Results and Problems in Cell Differentiation
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Editor

Cell Cycle in Development

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Preface

The purpose of this volume is to give the readers a current vision of the whole panorama of cell cycle-related events implicated in animal development. To assure the highest scientific level of this review the very best experts in their respective fields were invited to participate in this adventure. All chapters were peer-reviewed, and special thanks are due to Takeo Kishimoto (Tokyo), Rafal Piprek (Krakow), Malathesha Ganachari (Houston), and Orna Halevy (Jerusalem) whose help was particularly important.

The understanding of the specific mechanisms underlying the modifications of the cell cycle necessary to support development has a key role in comprehension of developmental processes. Specific adaptations of the cell cycle begin in unfertilized oocyte and occur throughout the whole embryonic and postnatal development. Even during senescence the cell cycle is modified in different tissues in a specific manner. Errors in these modifications may provoke pathologies. The cell cycle modification and adaptation to different developmental challenges are the leitmotif of this volume.

The initial chapters of the volume are devoted to general, *trans*-species questions: what was the beginning of the cell cycle, how it evolves today in respect to embryo development, how is it linked to the mechanical forces acting on each cell during the development, how the control of cell cycle progression is exerted at different developmental stages, and what are the links between the cell-sizing, cell cycle, and embryo development?

Subsequent chapters will guide readers through the problems at the intersection between the cell cycle and embryo development in several chosen species: *C. elegans*, ascidians, amphibians (*Xenopus laevis*), and mammals (mouse). Readers will find chapters devoted to selected problems, such as oocyte-to-embryo-transition, DNA replication, and meiotic regulation of Spindle Assembly Checkpoint, as well as chapters devoted to selected proteins or their families, such as Greatwall kinase, RanGTP, Cdk/cyclin complexes, or cyclins A.

A group of four chapters covers the cell cycle control in murine and human embryonic and adult stem cells. These ex vivo observations may greatly improve

our understanding of the mechanisms acting in situ during development. A final group of chapters deals with the neural cells. This part of the volume delivers an overview how the cell cycle is regulated and modified during brain development and senescence. The brain development is a very instructive example of what probably happens in other organs and tissues.

The very last chapter closing the volume deals with the cell cycle pathologies in adult brain and especially in Alzheimer's disease. It was written by the group of Mark A. Smith from the Case Western Reserve University in Cleveland, USA. Mark passed away in a tragic car accident shortly after writing this chapter. He was 45 years old and left behind his wife and two young sons. Mark's passing at the very top of his scientific career is much regretted. As this last chapter of the book was one of the last articles written by Mark, I would like to dedicate the whole volume to his memory.

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Chapter 1
Experimental Systems to Explore Life Origin: Perspectives for Understanding Primitive Mechanisms of Cell Division

Katarzyna Adamala and Pier Luigi Luisi

Abstract Compartmentalization is a necessary element for the development of any cell cycle and the origin of speciation. Changes in shape and size of compartments might have been the first manifestation of development of so-called cell cycles. Cell growth and division, processes guided by biological reactions in modern cells, might have originated as purely physicochemical processes. Modern cells use enzymes to initiate and control all stages of cell cycle. Protocells, in the absence of advanced enzymatic machinery, might have needed to rely on physical properties of the membrane. As the division processes could not have been controlled by the cell's metabolism, the first protocells probably did not undergo regular cell cycles as we know it in cells of today. More likely, the division of protocells was triggered either by some inorganic catalyzing factor, such as porous surface, or protocells divided when the encapsulated contents reached some critical concentration.

1.1 Studies of the Origin of Cellular Life

There is no commonly accepted definition of life. Most scientists working on the problem agree that life can be defined by the set of functions and features that must be possessed by the system to be called alive. Yet, the specificity of these functions remains undefined (Luisi 1998). Since there is no indisputable definition of life, it is also hard to define the event of the origin of life. For the purpose of this work, it will be assumed that the origin of life was the process during which the chemical reactions spontaneously arranged into a homeostatic system, and the newly formed living cells started undergoing spontaneous cell cycle of growth and division. As a

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beginning of cellular life, we understand a compartmentalized system capable of self-maintenance owing to a self-regeneration process from the inside.

Life originated on Earth at least 3.6 billion years ago. The oldest known traces of fully developed life are dated to approximately 3.465 billion years (Schopf 1994), and some evidences show a possibility of biochemical cycles existing as early as 3.8 billion years ago (Schidlowski 2001). The time between the origin of Earth's crust and primordial ocean 4 billion years ago (Morbidelli 2000) and the first known traces of life (date back to 3.8 billion years) is the time when all processes of the origin of life must have occurred. This leaves approximately 200–500 million years for the chemical evolution processes.

Various possible environments are considered as possible site of the prebiotic evolution and the origin of life. Prebiotic Earth provided many different sites for possible prebiotic chemistry reactions, including open water of the ocean, lagoons, surfaces of various minerals, thin layers of organic compounds, gaseous phase of the atmosphere, or submarine hydrothermal vents. Different prebiotic processes proposed in the literature are placed in different conditions. Nevertheless, the origin of life on Earth might not have been a singular accident; only one protocell lineage succeeded and survived, proliferating into all known forms of life. There is no reason to assume that our cells' metabolism represents the only possible type of metabolic process; yet, all the evidence suggests that all known life comes from a single ancestor.

The above mentioned ancestral cell, or population of cells, must have already some sort of functioning cell cycle, consisting of growth and division of the membrane and cell contents, driven by metabolic processes and genetically encoded. The exact nature of processes that have led to the development of the cell cycle is a subject of intensive studies. It is not impossible that the origin-of-life processes are still occurring, although it is much more difficult on the oxidized environment and on the planet absolutely possessed by one type of biological organisms; it is practically unimaginable to expect any other form of metabolism to grow enough to successfully compete with "our type" of life. Therefore, no effective biogenesis processes are observed today (Delaye and Lazcano 2005).

The chemical reaction system undergoing cycles of growth and division, selection and evolution, must have originated as a result of a long series of simpler, more primitive processes. These processes, chemical reactions leading to organic molecules, not based on any biological catalysts, are the subject of interest in prebiotic chemistry. To understand the mechanism of origin of modern cell cycle, simple models, the so-called protocells, have been studied (Luisi et al. 1999).

1.2 Protocell Membrane

To study the origin of elements of the cell cycle, particularly growth and division of protocell membrane, model protocell vesicles are commonly used (Chakrabarti et al. 1994; Walde et al. 1994; Segre et al. 2001). The self-assembled bilayer

membranes, semipermeable to small organic molecules and able to encapsulate bigger, polar compounds, are a good model of a prebiotic protocells.

Several authors, including the group of D. Deamer, proposed that at the earliest stage of the prebiotic bilayer membrane formation, membranes consisted of simple, long chain carboxylic acids (Fig. 1.1). The open question about the nature of the membrane in Last Universal Common Ancestor (LUCA) leaves many possible routes to the origin of lipid membranes during the earliest stages of protobiological evolution. In modern cells, apart from compartmentation, membranes perform several other functions, including energy transduction and transport of organic and inorganic compounds, and they are the docking site of many enzymes. Presumably, the very first role of the membranes was simple encapsulation – isolation of the reaction cycles (i.e., genetic materials or enzymatic peptides) from the environment. This could be done by the simplest amphiphiles, possibly available under the prebiotic conditions: medium-sized (up to C10) chain carboxylic acids (Fig. 1.2).

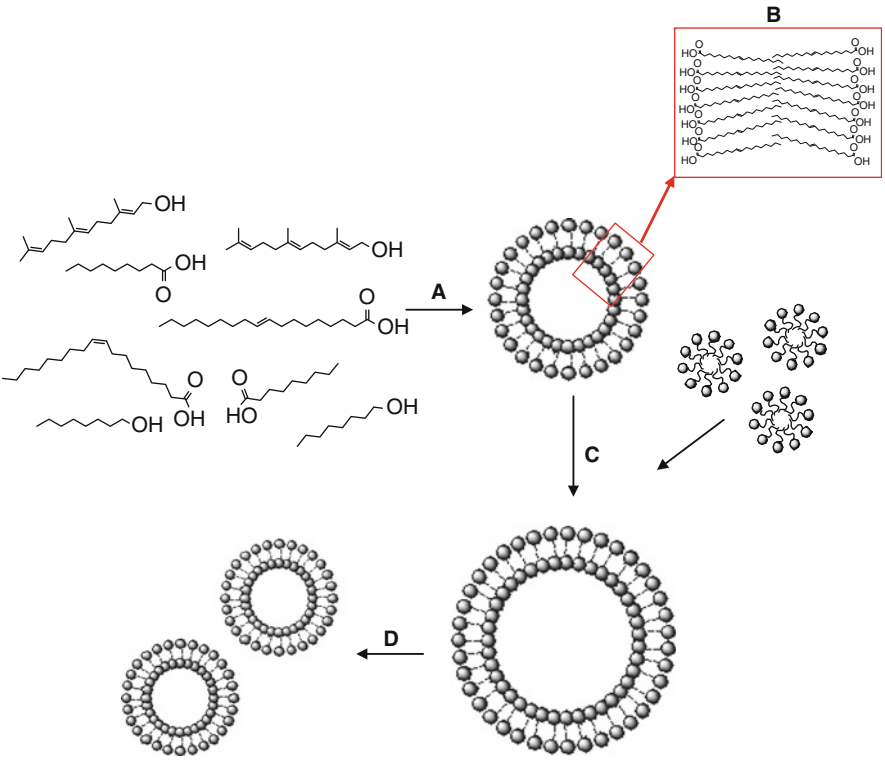

Fig. 1.1 Vesicles. (**a**) Vesicles are spontaneously forming from the amphiphilic monomers; (**b**) bilayer membrane of the vesicle, with polar, hydrophilic headgroups directed outside, and aliphatic, hydrophobic chains inside; (**c**) vesicles can grow upon addition of micelles; (**d**) vesicles can be forced to divide into daughter vesicles

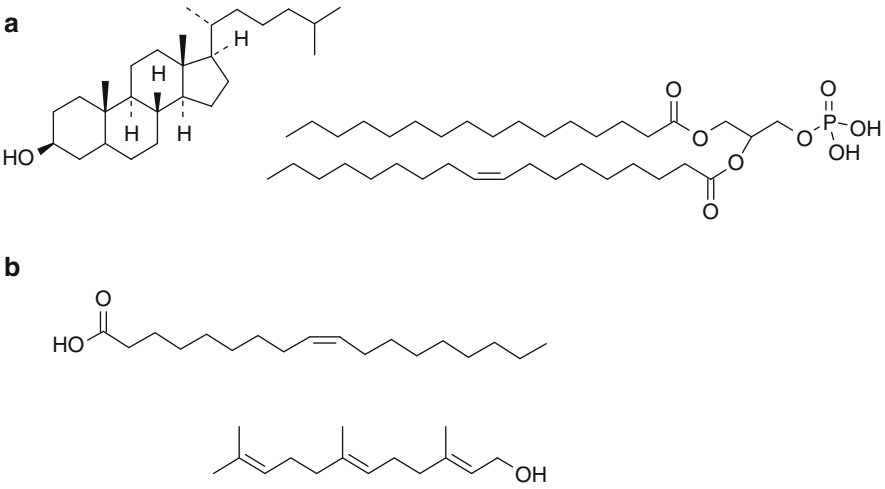

Fig. 1.2 Amphiphilic compounds building the membranes. (**a**) Modern cell's membrane building block; (**b**) possible prebiotic amphiphiles

The main building blocks of modern cells' membranes are phospholipids and sterols. Phospholipid glycerol esters and sterols are too complex to be synthesized under abiotic conditions. However, all these compounds can be derived from simplest building block – sterols from isoprene units and lipid derivatives from simple unsaturated carboxylic acids. The simple lipids might have been synthesized under prebiotic Earth conditions (Yuen et al. 1981; Allen and Ponnamperuma 1967), including environment of the underwater hydrothermal vents (McCollom et al. 1999). Simple amphiphiles were also detected in carbonaceous chondrite meteorites (Yuen and Kvenvolden 1973; Deamer 1985).

Compounds based on these simplest units could have formed the first membranes encapsulating biochemical cycles of the protocell. In a water solution, with the pH close to the polar headgroup p*K*a, the simplest amphiphiles spontaneously self-organize into bipolar membrane sheets that close into spherical vesicles (Apel et al. 2002).

Vesicles are commonly accepted as an approximation of the compartments of the earliest protocells (Walde 2006). Vesicle-like bilayer membranes were even observed in amphiphiles organic material from Murchison carbonaceous chondrite (Deamer 1985; Deamer and Pashley 1989), making its availability on prebiotic Earth more probable.

Vesicle structures can grow (Chen and Szostak 2004), divide (Hanczyc et al. 2003), and selectively take up compounds from the environment (Chen et al. 2004). Therefore, investigating properties of the different vesicle systems can give insight into possible routes to the origin of protobiological compartmentalization.

1.3 Models for Studying Protocell Growth and Division

Protocell vesicles can undergo cycles of growth and division based on simple physical properties of the bilayer membrane. Unlike modern cell cycle, the protocell size and shape changes are caused by external factors, such as addition of amphiphiles or pressure applied to the membrane, and not the internal metabolic processes (Oberholzer and Luisi 2002).

1.3.1 Growth of Vesicles

Simple prebiotic vesicles can grow upon addition of micelles, but the growth is triggered by addition of lipids from the external source, not as a result of reactions occurring inside protocells. The process of growth of simple fatty acid vesicles upon addition of micelles was first described by P.L. Luisi and coworkers (Fig. 1.3). Addition of fatty acid micelles in alkaline solution to buffered solution of vesicles causes vesicles to grow. Fatty acid micelles are stable only under highly alkaline

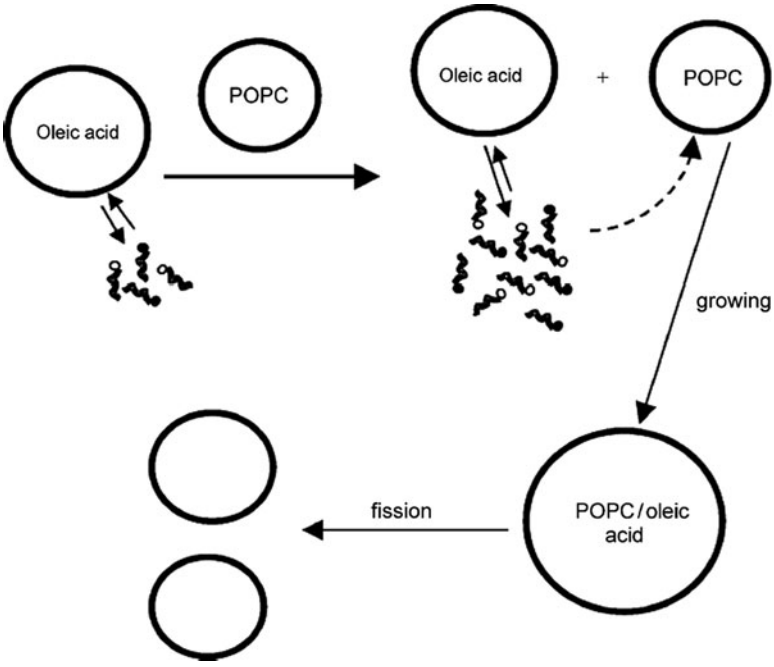

Fig. 1.3 Schematic representation of protocell vesicles competetive growth and division (from Cheng and Luisi 2003)

pH; when micelle solution is added to solution of vesicles, at pH slightly alkaline (i.e., pH 8 for oleic acid vesicles), micelles become thermodynamically unstable and either lipids from added micelles are taken up by the existing vesicles or de novo vesicles are formed. (Luisi et al. 2004; Berclaz et al. 2001; Blochliger et al. 1998; Rasi et al. 2003).

Addition of micelles is a plausible prebiotic model for vesicle growth. It is possible that lipids, such as simple fatty acids, were synthesized in one place on prebiotic Earth, and then transported to other place with lower pH, where they organized into vesicles or fuelled preexisting protocell vesicle population. This must have been caused by the arrival of lipids from external source, and not by processes of the protocells' internal metabolism. Thus, we can model the process of growth, necessary for the origin of cell cycle of growth and division.

Protocell vesicles can undergo competitive growth: when two populations of protocell vesicles are mixed, one made of simple oleic acids and the other made of phospholipids, the phospholipid vesicles grow on account of the oleic acid vesicles. (Cheng and Luisi 2003). This is also a good example of possible origin of competition on the protocell level.

Another process of competitive growth of simple prebiotic vesicles was described by J.W. Szostak and coworkers (Chen et al. 2004). One population of simple fatty acid vesicles can grow, on the expense of another population of vesicles made of similar amphiphiles, if there is a difference in osmotic pressure between those vesicles. Furthermore, the concentration gradient necessary for the competitive growth can be achieved with nucleotides and RNA molecules. That opens up the possibility of coupling two of the essential elements of cell cycle: growth of protocell vesicles in connection with the presence of genetic material.

Myelin-like giant multilamellar vesicles can divide in response to changes of osmotic pressure (Takakura and Sugawara 2004). This is not a particular prebiotically plausible example, since compounds used to build those vesicles are not simple lipids that can synthesize abiotically in aqueous environment. However, it is an interesting example of physical mechanism driving vesicle's membrane shape and size change.

1.3.2 Protocell Vesicle's Division

The first laboratory evidence of possible controlled division of protocell vesicles came from Luisi and colleagues (Berclaz et al. 2001); upon addition of oleic acid micelles to phospholipid vesicles, the diameter of the vesicles increased; at the end of the experiment, there were more vesicles present in the pool. The electron-dense protein ferritin was used as a marker of internal size of the vesicles. After the micelle addition, the vesicles were found not to contain any ferritin, or containing significantly less. This suggests the formation of new protocell vesicles during the process by the division of the grown original vesicles. This simple vesicle's division

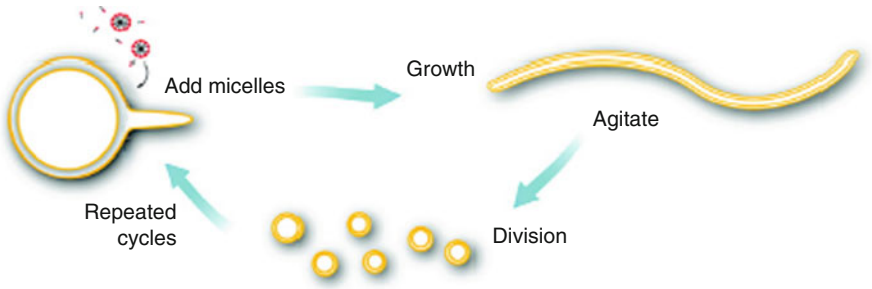

Fig. 1.4 The proposed mechanism of prebiotically plausible division of protocell vesicles (from Zhu and Szostak 2009)

is a proof of principle demonstration that protocell vesicles can be divided in a purely physical process, without any use of cell metabolism.

One of the simplest methods of protocell vesicle division is extrusion through porous cellulose membrane. This allows precise control of the size of daughter protocell vesicles, but a significant part of the contents encapsulated within vesicles is lost during the extrusion process. (Hanczyc et al. 2003) The extrusion is most commonly used during protocell "replication" experiments.

Upon addition of fatty acid micelles to previously formed large multilateral vesicles made of the same amphiphiles, vesicles develop thread-like structures, after gentle agitation, that separate and form new generation of protocell vesicles (Fig. 1.4). This process conserves the encapsulated contents of vesicles (Zhu and Szostak 2009) couples model protocell growth (by addition of micelles) and division.

If the acyltransferase enzyme is delivered inside the giant phospholipid vesicles, the 1-palmitoyl-sn-glycerol-3-phosphate is synthesized in the vesicles, and the change in membrane composition causes shrinkage of the parent vesicle. Also, small daughter vesicles are formed on the inner surface of the original giant vesicle (Wick and Luisi 1996). The inner protocell metabolism can be therefore coupled with the changes of the membrane shape and size and with the production of next generation of vesicles.

1.4 Conclusions

The cell cycle of modern cells, driven by complex networks of metabolic processes, must have originated in much simpler form in the protocell populations. The studies of protocell model systems can give insights into the origin and the underlying mechanisms of the modern cell cycle. Also, knowing the processes that have led to the origin of cellular life can help in the future in modifying cell cycle in different organisms and may be even designing entirely artificial cells.

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Chapter 2
Evolution of Bet-Hedging Mechanisms in Cell Cycle and Embryo Development Stimulated by Weak Linkage of Stochastic Processes

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Abstract Our current understanding of the origin and evolution of the cell cycle is largely filled with gaps and unresolved questions. Numerous similarities between the processes comprising the cell cycle in distant organisms from the Pro- and Eukaryota kingdoms provide some clues about the course that evolution has taken. Contemporary Prokaryotes and Eukaryotes regulate their cell cycles in a quite similar way, using a master oscillator that regulates cell division. Despite this striking similarity, they use entirely different molecules for this purpose. The necessity to keep the master oscillator intact for the survival of every cell/organism allows evolutionary changes in only the secondary mechanisms and processes of the cell cycle. This is especially clear in oocytes and embryos, which have a direct impact on the reproductive success of an adult organism. Here, we present examples of cues driving such mild evolutionary changes of certain aspects of cell cycle progression in oocytes and early embryos. We suggest that weak linkages between core processes that rely on randomness (stochasticity) have led to the evolution of strategies increasing fitness similar to bet-hedging, a stochastic-based survival strategy of risk minimization widely implemented by populations of bacteria, yeast, arthropods, and birds. Stochastic diversification of phenotypes by isogenic cells increases their fitness in unpredictable environments and improves their

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survival rate upon exposure to stress, a trait beneficial in evading antibiotic treatment by bacteria or withstanding chemotherapy by cancer cells. The evolution of bet-hedging has been observed experimentally for bacteria and attributed to specific molecular mechanisms involved in this strategy. In this chapter, we set out to answer whether similar strategies could have evolved at the level of oocytes and embryos. We indicate possible evolutionary cues capable of realizing bet-hedging-like mechanisms.

2.1 Introduction

The cell cycle regulation in Eukaryotes is based on cyclins that govern the major cell cycle transitions (I-phase/M-phase/I-phase). These cyclins function via a relatively simple accumulation/degradation mechanism that activates/inactivates the major cell cycle regulator CDK1 (or cyclin-dependent kinase 1) (Masui and Markert 1971). The oscillatory character of CDK/cyclin complex could appear early in evolution (Gopinathan et al. 2011). Yet, studies of prokaryotic cells demonstrate that many cyclins and CDKs are dispensable in efficient cell cycle control. No cyclin-like oscillating proteins have been found in bacteria, but strictly regulated proteolysis of a very different master cell cycle regulator called CtrA was identified to be a key event in the cell cycle regulation of *Caulobacter crescentus* (reviewed by Bowers et al. 2008; Jenal 2009). Thus, the major cell cycle oscillator is fully functional in this remarkable bacterium undergoing an asymmetric division, even though its resemblance to the eukaryotic cyclin-dependent kinase is tenuous. The differences between the prokaryotic CtrA-dependent regulation and the eukaryotic cyclin-dependent regulation are too important to allow constructing solid hypotheses regarding their possible relationship. However, the basic concept of accumulation/degradation is evidently perpetuated in both microbes and higher organisms. Likewise, some secondary processes, including inhibitory mechanisms that determine the site of cytokinesis in the cell division plane and are transmitted from the cell extremities, seem to act in surprisingly similar ways, despite the involvement of distinct molecular machinery (Moseley et al. 2009). Thus, a common mechanism to dictate the cell cycle clock via a master oscillator, which determines the robustness of the core cell-cycle control machinery, survived and was strongly conserved. Any modification in such a robust core mechanism must have catastrophic implications for the cell/organism and thus must be very difficult to pass on to the progeny.

A master oscillator controls cell division of both Prokaryotes and Eukaryotes; therefore, we shall refer to these two groups as Oscillokaryotes. The oscillatory character of cell cycle control was conserved because no cell could survive the abolition of a master oscillator. This leads to a hypothesis that, once an oscillatory mechanism became established, the principle of its action never changed. The CtrA master regulator in the *C. crescentus* precursor and CDK1/cyclin B in pre-Eukaryotes are both prime examples of such conserved mechanisms. The evolvability of the

cell cycle must therefore imply milder cues than changes in the main engine of cell division. As a result, only secondary events could be modified by means of extremely discrete mechanisms, such as weak linkage. Below, we discuss examples of how such associations could play a role.

2.2 Efficiency of a "Mild" Evolution

Kirschner and Gerhart (2005) noted that "most evolutionary change in the metazoa since the Cambrian has come not from changes of the core processes themselves or from new processes, but from regulatory changes affecting the deployment of the core processes". In this context, *regulation* encompasses a wide range of cellular mechanisms capable of altering the temporal dynamics and localization of gene expression as well as the activity and interaction potential of proteins involved in core processes. All of these changes make it possible for core processes to engage in new tasks by linking them *weakly* in new ways, thereby increasing an organism's flexibility and fitness in new environments. A number of cellular processes have been recognized to be capable of creating such a *weak linkage*. For instance, eukaryotic transcriptional regulation, a highly conserved process, allows for the generation of a staggering amount of phenotypic variation by linking existing simple units of transcription factors into elaborate regulatory circuits. An individual unit retains its simplicity while the increase in complexity occurs in the network or in the linkage between the units. For multicellular organisms, embryonic development is the most critical for triggering anatomical variations. Variability in the concentrations of chemical species due to stochastic events at the molecular level inherent to all biochemical processes is arguably another factor enhancing the propensity for creating weak links between core processes. Stochasticity alone can even become an underlying principle on which some core processes, such as bet-hedging or bacterial persistence, rely (Davidson and Surette 2008). Stochastic effects in the cell cycle and embryo development and their role in creating weak links and increasing fitness is the topic of this chapter. We discuss stochastic phenomena in embryonic development, which could give higher organisms a way to increase both their phenotypic plasticity and survival rate when faced with an unpredictable and randomly changing environment. We draw parallels between our experimental results and a mechanism of stochastic bet-hedging, or the minimization of risk – a survival strategy utilized at many levels of developmental organization, from bacteria through yeast to arthropods and birds (King and Masel 2007). The intuition behind the principle of bet-hedging is also familiar to humans; it is often expressed by a common proverb that it is safer not to keep all of your eggs in the same basket. Our emphasis is on the ability of seemingly unrelated processes to be coordinated into functional groups, largely stimulated by the mere existence of stochastic molecular mechanisms.

2.3 Bacterial Strategies for Survival: Stochasticity-Induced Population Heterogeneity

Organisms employ numerous strategies to survive in their environment. Spontaneous genetic mutations accompanied by the selection process lead to the fixation of new, more "successful" genotypes. However, the time scale of these changes is too long to adjust to changes in nutrient abundance taking place on an hourly basis, for instance. A multitude of sensory networks capable of inducing and regulating gene expression have evolved to cope with changing conditions. Even for bacterial sensing, which largely relies on relatively simple two-component networks, the high number of such networks (typically a few dozen per cell) and the fact that they operate in parallel with numerous cross-talks have led some to postulate a rudimentary form of intelligence embedded in the sensory network (Hellingwerf 2005). Whether their complexity is sufficient to exhibit neural network characteristics, such as memory or learning, is an intriguing concept that awaits further theoretical and experimental evidence.

While the ability to "intelligently" process environmental inputs by bacterial sensory machinery remains a hypothesis, the ability to reflect temporal patterns of extracellular conditions in the structure of regulatory networks has been demonstrated experimentally (Mitchell et al. 2009). Two model organisms used in this study, *Escherichia coli* and *Saccharomyces cerevisiae*, have evolved to be capable of activating parts of its machinery in anticipation of the sequence of stimuli. However, random and unpredictable fluctuations in environmental conditions pose a greater challenge for organisms. Likewise, rapid but infrequent cues (on a cellular generation timescale) that induce irreversible lethal changes may require a different type of response system. One solution is to maintain a multitude of regulatory mechanisms continuously prepared to face a vast array of environmental challenges. Even then, however, a cell fully equipped with sensory networks activating the appropriate genes may respond to a change only if integration of the extracellular signal lasts long enough to average out noise inherent in the sensing procedure. If the time required for gathering sufficient information about the environment exceeds the generation time of a single cell, the cell is never capable of reacting properly, which compromises the fitness of the population as a whole. Diversification of phenotypes may be a useful strategy to overcome this limitation.

The fitness advantage of a phenotypically heterogeneous population exposed to fluctuating conditions depends on the relative time scale of intracellular processes and changes in environmental cues (Kussell and Leibler 2005). Cells switching their phenotypes with the same frequency as changes in the environment have a greater survival rate as opposed to populations that remain out of synchrony with their surroundings (Acar et al. 2008). This observation hints at a possible choice of strategies aiming for adaptation (Perkins and Swain 2009). In environments with rapid, irregular or extreme changes, the cost of the maintenance of elaborate sensory networks may exceed benefits of a higher survival rate. Additionally, the

response of the network to a rapid change may be unsatisfactorily slow, thus promoting a simpler solution. An isogenic population could, for instance, increase its fitness by generating subpopulations via stochastic modulation of gene expression; each subpopulation performs suboptimally in the "average" environment but is able to survive critical changes – a strategy known as bet-hedging (for a comprehensive review, see Davidson and Surette 2008).

Stochasticity in cellular processes originates from thermal fluctuations, small number of molecules and macromolecular crowding. All of these factors may easily alter the rates of chemical reactions and local concentrations of reactants. This in turn affects the availability of certain genes for transcription (by means of random chromatin rearrangements, for instance) and affects the whole process of gene expression at various levels. In a number of experiments, isogenic populations starting at the same initial conditions have been propagated into cells with entirely different molecular makeup (Elowitz et al. 2002; Blake et al. 2006; Spencer et al. 2009) and proliferated into phenotypically distinct cellular entities with diversified biological functions (Balaban et al. 2004; Feinerman et al. 2008; Chang et al. 2008; Choi et al. 2008), a convincing demonstration of how random molecular events may affect the macroscopic observable – the phenotype. In the recent decade, researchers have been able to measure distribution of protein concentrations in a number of settings, taken as snapshots over the entire cell population, as well as the temporal changes in a fluctuating protein (Elowitz et al. 2002).

Bimodality in the gene product level as depicted in Fig. 2.1 is one of the elementary mechanisms behind the induction of population diversity in a population of isogenic cells. Two peaks of the steady-state product distribution have been traditionally attributed to bistability in the dynamic system resulting from some form of feedback regulation, or so-called "feedback-based multistability". A thorough review concerning this type of mechanism can be found in Smits et al. (2006). Here, we discuss a different case in which two distinct levels of a protein across the population emerge solely due to stochastic gene regulation (Kepler and Elston 2001; To and Maheshri 2010). The emergence of a stochastic bet-hedging strategy in a bacterial population has been recently observed in an elegant experiment by Beaumont et al. (2009). After 15 rounds of subjecting cells to two opposing environments, each favoring a different phenotype, a new genotype evolved capable of stochastic switching between the two conditions. Stochastic gene expression has been also shown to confer a fitness advantage in a population of yeast cells exposed to antibiotic stress (Blake et al. 2006). Due to bursts in production of the protein conferring resistance to an antibiotic, at least part of the population is in a position to respond rapidly. This translates to an overall increase in the persistence of the whole population. Survival upon antibiotic treatment in general has been attributed to population heterogeneity stemming from stochastic switching between two phenotypes with distinct survival rates, a phenomenon known as bacterial persistence (Balaban et al. 2004; Kussell et al. 2005; Bishop et al. 2007). Similarly, Sorger and colleagues (Spencer et al. 2009) have demonstrated how cancer cells escape drug treatment thanks to stochastically induced cell-to-cell variability. Such studies should help answer a long-standing question of why seemingly identical

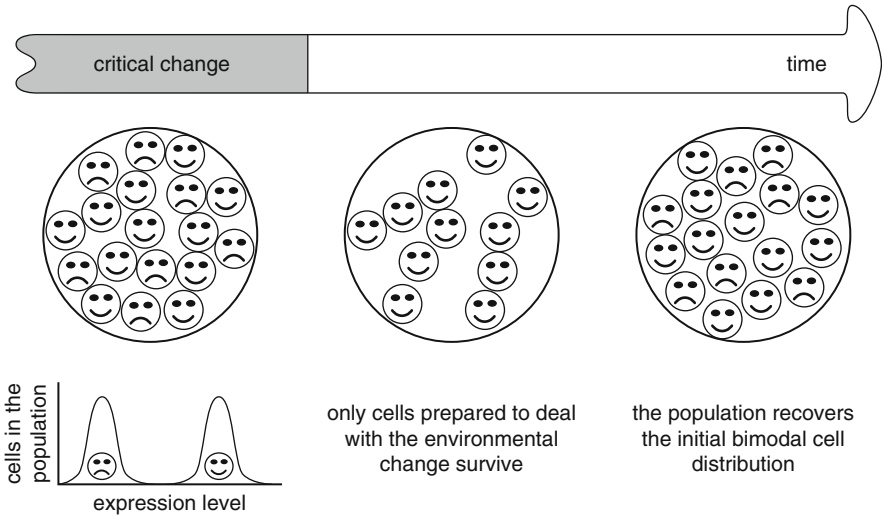

Fig. 2.1 Population heterogeneity increases survival rate. In the simplest case, the abundance of the protein dealing with environmental challenge (e.g., neutralizing a toxin) has a bimodal distribution due to stochastic gene expression or feedback regulation (so-called feedback-based multistability). An example of the latter mechanism could be positive feedback in a sporulation network of *Bacillus subtilis*. More generally, bimodality could refer to meta-states in which expression profiles of many genes undergo significant changes. Bimodality results in a stable, phenotypically heterogeneous population. Only individuals "prepared" for the upcoming change in the environment survive. The initial heterogeneity is recovered after few generations

cells respond differently to the same drug. In a remarkable experiment by Cohen et al. (2008) the levels and locations of approximately 1,000 endogenous proteins were tracked in individual cells after administering a chemotherapy drug. The presence of the drug evoked a higher variability in protein levels, and in some cases, the protein concentrations exhibited a bimodal distribution. The low and high levels in protein concentration corresponded to the survival or death of a cell. Thus, the presence of the environmental stressor induced a stochastic strategy, which allowed part of the cancer cell population to escape their deadly fate. However, phenotypic diversity in a clonal population is rarely a result of fluctuations in the expression of a single gene. Cellular states with distinct functionalities (phenotypes) usually correspond to transcription profiles differing in the expression of many genes. Slowly decaying fluctuations may promote reversible transitions between such meta-states, implying various cell fates, as has been demonstrated for mammalian progenitor cells (Chang et al. 2008).

The role of stochasticity has been also suggested for early events in embryo development, the patterning of the mouse blastocyst (Dietrich and Hiiragi 2008). A step preceding determination of cell fates and their subsequent sorting within the inner cell mass has been argued to rely on molecular fluctuations. At the eight-cell stage, a stochastic process induces variability across blastomeres in the concentration of Nanog – a protein required to maintain cellular pluripotency. This variability

in the molecular signature of blastomeres prescribes their further allocation in the inner cell mass. Further experiments using this system verified that the distribution of Nanog has a bimodal distribution (analogous to the one shown in Fig. 2.1) due to slow fluctuations in gene expression (Kalmar et al. 2009).

Diversification of the microbial population benefits its survival rate when faced with environmental changes, but it also allows these simple organisms to solve complex tasks. In one such system, a population of *Salmonella* bacteria split stochastically into two subpopulations. One of the phenotypes facilitates infection in the gut lumen by triggering the inflammation. By doing so, it contributes to the "public good" of the whole bacterial invasion; however, these bacteria are killed by the host's immune response (Ackermann et al. 2008). This self-destructive cooperation elegantly illustrates the population benefit of phenotypic noise and its role in bacterial pathogenesis. It is also an interesting contribution to recent experimental studies on the evolution of cooperation by engineering simple bacterial ecologies (Santorelli et al. 2008; Gore et al. 2009; Khare et al. 2009).

Microbial bet-hedging strategies typically involve stochastic switching between contrasting phenotypes in a genetically identical population of cells. As mentioned earlier, the fitness of organisms that employ this strategy increases if the rate of stochastic switching correlates with the frequency of environmental changes (Kussell and Leibler 2005; Acar et al. 2008). Consequently, this strategy is successful mainly for large bacterial populations; due to slow switching rates, small ensembles are less likely to exhibit an entire array of phenotypic variability necessary to survive various environmental stresses. Higher organisms, in contrast, cope with this limitation by generating an increased diversity among progeny in every generation when exposed to stress. By doing so, metazoans can induce a substantial variability in a group as small as two daughter cells. This mechanism, typical of metazoans, has been recently observed experimentally for the first time in microbes. When exposed to stress, the bacterium *Sinorhizobium meliloti* expresses a diversification strategy by dividing into two daughter cells with phenotypes differing in their capacity to store carbon (Ratcliff and Denison 2010). We shall discuss examples of bet-hedging strategies of this kind in the section below.

2.4 Reproductive Success in the Lake: Bet-Hedging in *Daphnia*

Fitness-increasing stochastic bet-hedging strategies are not exclusive to cellular populations. Numerous examples indicate that the strategy has evolved on individual or population level in primitive invertebrates and in evolutionarily advanced vertebrates, such as birds. Differences lie only in the complexity of strategy regulation (both at the biochemical and physiological levels), but the strategies themselves remain fundamentally unchanged.

Reproduction of both animals and plants can be often described in terms of this strategy. For example, it is known that eggs of parasitic nematodes from the species *Nematodirus battus* hatch in a bimodal temporal distribution. Some of the eggs

hatch in autumn, and some hatch only after winter cooling in spring. The autumn and spring hatches take place randomly in the whole population. This solution increases the chance of infecting the host, ruminants in the case of *N. battus*, and clearly increases the reproductive success of the parasite (van Dijk and Morgan 2010). Similarly, numerous plant species exhibit random germination of seeds deposited in the soil, an adaptation to changing and unpredictable environmental conditions (e.g., sudden drought) that also increases the persistence of plant populations in particular localities (Evans and Dennehy 2005). Trade-offs between the size of eggs or seeds and their number characterize the reproduction of both animals and plants. The parental organism possessing limited resources must somehow "decide" whether it should invest in the number or quality of the offspring. Most often, the nutrient supply of eggs or seeds and their number is random, strongly suggesting that a bet-hedging strategy is involved (Olofsson et al. 2009).

Currently, additional data has become available on stochastic phenomena underlying some aspects of animal behavior. Recent discoveries indicate that the flight direction during autumn migration in passerine birds is chosen randomly. Young birds from one nest fly to the south. However, each of them flies independently in slightly different directions. The weather during bird migration as well as variation in the strength and direction of winds are crucial for successful migration. Young birds have limited energy resources, and they only reach their destination when winds are favorable. If all the individuals were flying together in exactly the same route under poor weather conditions, none of them would survive. Randomly choosing the flight direction increases the probability that more young birds will reach the wintering grounds and survive the period of migration (Reilly and Reilly 2009). Thus, bet-hedging may apply to both fixed characteristic such as egg/seed volume and behavioral aspects, as like in the case of migratory birds.

Crustaceans from the genus *Daphnia* are ideal model organisms to experimentally examine bet-hedging strategies in animal behavior. They are easy to culture in laboratory, have a short generation time, and most importantly, they reproduce parthenogenetically in favorable conditions, as is typical in bacterial populations. In the case of *Daphnia*, parthenogenesis is characterized by no DNA recombination; the female is genetically identical to its offspring. Thus it is possible to culture genetically identical *Daphnia* populations (Peters and De Bernardi 1987). These clones may simplify the study of bet-hedging strategies by negating the influence of genetic variation.

In its natural environment in lake pelagial, *Daphnia* face several environmental threats, including inadequate food and the presence of cyanobacterial toxins and predators. *Daphnia* respond to the presence of visually hunting predators (e.g., planktivorous cyprinid fish) by migrating to the deep, dark, and cool layers of the lake (hypolimnion) (Zaret and Suffern 1976). Due to low temperatures and the scarcity of food, *Daphnia* bear the costs of safety in this refuge. These costs include prolonged periods of embryonic and juvenile development leading to prolonged time gaps between successive reproductive episodes, as well as a decrease in brood size. However, *Daphnia* may perform diel vertical migration (DVM), returning to the warm and food-rich surface layers of the lake at night. DVM is a pattern of daily

migration undertaken by some organisms living in the large water reservoir, such as ocean, sea, and lake. Especially fish and crustaceans move up to the epipelagial at night and return to hypopelagial during the day (Lampert 1989; Loose and Dawidowicz 1994).

To test *Daphnia* behavior, "plankton organs" are used. These devices simulate the thermal conditions of lake pelagial (Loose and Dawidowicz 1994). Plankton organs consist of glass tubes put vertically in aquarium filled with water. A heater close to the water surface and radiator at the bottom help to maintain summer stratification typical for dimictic lakes. Plankton organs allow for testing the influence of varied environmental conditions on *Daphnia* as different media may be pumped through each glass pipe. *Daphnia* individuals may freely migrate in the water column limited only by the walls of glass pipes.

A trade-off between the costs mentioned in the previous paragraph and the benefits of vertical migration causes variation in *Daphnia* clones regarding alternative behavioral strategies. As field observations and laboratory experiments using plankton organs have shown, some *Daphnia* clones showed the migratory strategy while others stayed close to the surface for 24 h (Stich and Lampert 1984; Pijanowska et al. 2007).

Some *Daphnia* clones do not stay at a particular depth but rather disperse in the entire available water column during the day. There is a high degree of noise in the plasticity of this behavior. Statistical analyses indicate that the distribution of individuals in the water column is indeed random. Here again, the natural conditions in lake pelagial may change rapidly and in an unpredictable way. During substantial and rapid environmental change, the "classic" phenotypic plasticity may by insufficient, and *Daphnia* may be surprised by the sudden appearance of planktivorous fish. A random distribution of individuals increases the probability that a higher number survive if a predator attacks.

The stochastic behavior of *Daphnia* is probably an extremely important adaptation to a changeable and variable environment, as is the random timing for growth of seeds or the random selection of the direction of flight in birds. The scientific description of the stochasticity of events and effects observed in different organisms is gaining more and more attention, and this focus enables a better understanding of ecological phenomena and their relationship to reproductive success.

2.5 Structural Role of Specific RNAs in Oocytes: Hazard Makes a New Function

The ability to perceive stress signals and rapidly respond to a changing environment is a prerequisite for the survival and evolution of any organism. For many decades, the firmly established consensus has been that responses to environmental changes occur via appropriate sensory, regulatory, chaperon, and structural proteins. However, following the discovery of catalytic RNAs in the early 1980s, more and more

studies on prokaryotes and eukaryotes have shown that RNA is able to directly recognize various environmental or intracellular cues and in a protein-independent manner, regulate cellular stress responses via conformational changes and the formation of appropriate secondary structures (Altuvia and Wagner 2000; Bevilacqua and Russell 2008; Helmann 2007; Romby and Wagner 2008; Waters and Storz 2009).

RNA molecules are extremely dynamic and can switch rapidly between different conformations. Numerous studies on bacteria, fungi, plants, and humans have shown that metal ions (magnesium, iron, manganese) and free metabolites can directly bind to and induce conformational changes in the regulatory RNAs known as riboswitches (Altuvia and Wagner 2000; Waters and Storz 2009). In addition, riboswitches, which are the regions of 5′ end of the RNAs they regulate, and various sRNAs and protein-binding RNAs are also quorum and temperature sentinels (Altuvia and Wagner 2000; Bevilacqua and Russell 2008; Helmann 2007; Ray et al. 2009; Romby and Wagner 2008; Waters and Storz 2009). Interestingly, many of these regulatory RNAs belong to a general category of "localized RNAs", which are restricted to specific subcellular compartments and/or structures, such as Cajal bodies and the P bodies of somatic cells and embryos (reviewed in Waters and Storz 2009).

The localization of RNA within a cell or an embryo is crucial for proper cellular function and development (Kloc et al. 2002). Several years ago, our laboratory discovered a novel, structural function of localized RNAs in *Xenopus* oocytes and embryos. We found that several RNAs localized at the vegetal cortex of *Xenopus* oocytes, including the noncoding Xlsirts RNA and the coding VegT and Fatvg mRNAs, play a structural, protein-independent role in maintaining the integrity of the cytokeratin and actin network (Chan et al. 2007; Kloc et al. 2005; Kloc 2008, 2009). Using molecular beacons, we also showed that these RNAs are integrated in the cytoskeleton of vegetal cortex of the oocyte (Fig. 2.2a; Kloc et al. 2005, 2007; Kloc 2008, 2009). The removal of these RNAs from the oocytes, using an antisense deoxyoligonucleotide approach, causes hyperpolymerization of actin and the collapse and fragmentation of the cytokeratin network in the oocyte vegetal cortex (Kloc et al. 2005, 2007; Kloc 2008, 2009). This in turn causes the aggregation of germinal granules (germ cell fate determinants for the developing embryo that are anchored to the cortex by cytokeratin filaments) and their displacement from the vegetal cortex (Kloc et al. 2005, 2007; Kloc 2008, 2009). Embryos resulting from such oocytes (fertilized eggs) showed drastic reduction in germ cell number (Kloc et al. 2005; Chan et al. 2007). Interestingly, the collapse of the cytokeratin network also occurs naturally during oocyte maturation (Kloc et al. 2005). After natural activation of the oocyte by sperm or artificial prick activation, the cytokeratin network reconstitutes (Kloc et al. 2005). Because there is no destruction of the structural RNA in this case, it suggests that a rather reversible change in RNA folding takes place during oocyte maturation/activation, resulting in the dispersion/reconstitution of the cytokeratin network. This in turn suggests that the change in RNA folding may be a fast-acting mechanism. Other laboratories found more structural RNAs to be associated with the mitotic spindle and asters in *Xenopus*

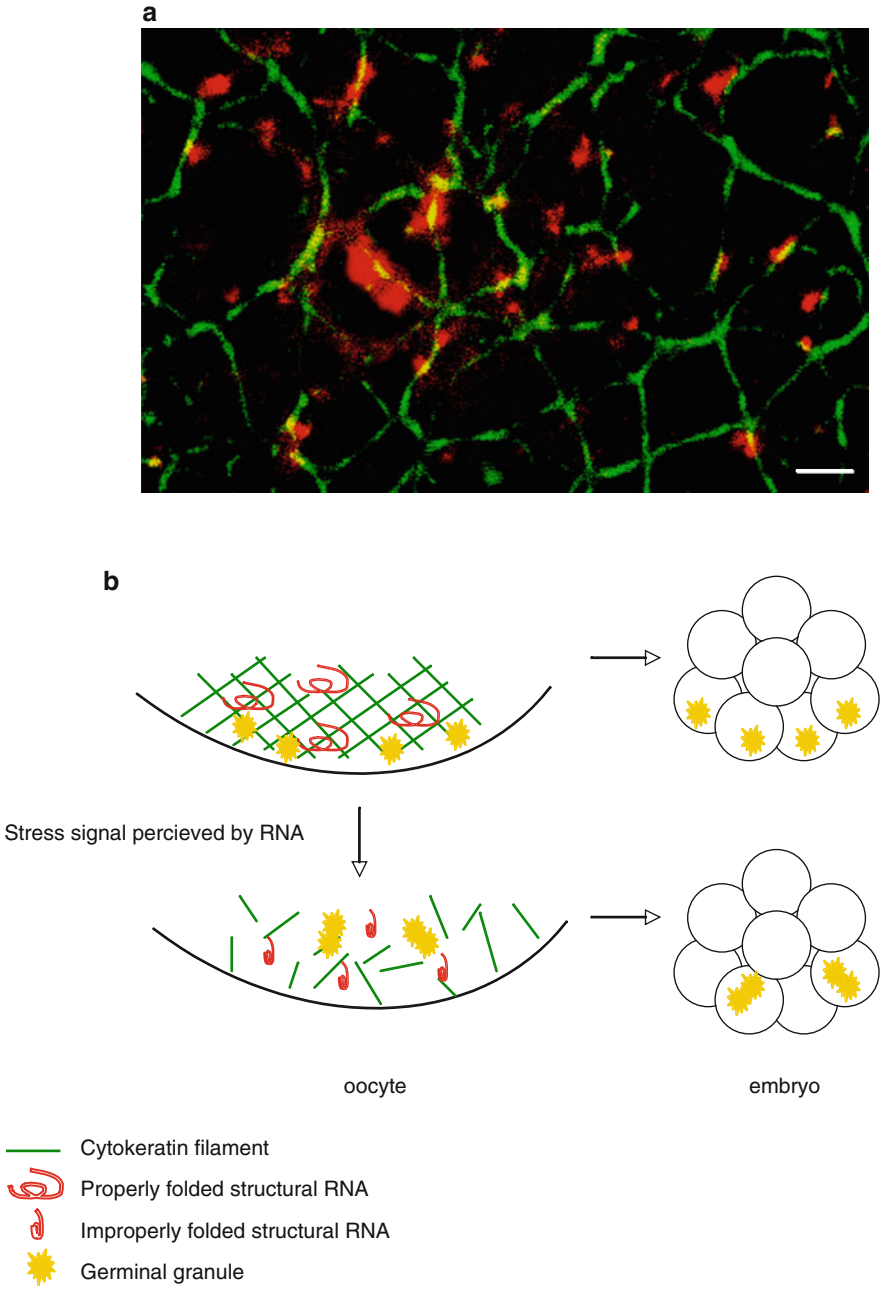

Fig. 2.2 (**a**) Fragment of the vegetal cortex of a *Xenopus* stage VI oocyte. Structural RNA (VegT mRNA, *red*, hybridized with Texas *red*-labeled molecular beacons) is associated with the network of cytokeratin filaments (*green*, stained with FITC-conjugated anti-cytokeratin antibody). With the permission of Elsevier, the picture included is from Kloc (2008). Bar is equal 5 μm. (**b**) Hypothetical

egg extracts and in human HeLa cells. These RNAs control the dynamics of microtubules and play a direct, translation-independent role in the assembly of the mitotic spindle apparatus, and the removal of these RNAs affects spindle formation (Blower et al. 2005). Another example of unforeseen function of mRNA comes from recent studies on *Drosophila*, which suggest that oskar mRNA may function structurally as a scaffold for the assembly of cytoplasmic complexes essential for the oocyte development (Lécuyer et al. 2007). All these findings demonstrate a novel structural role of coding and noncoding RNAs in the organization of the cellular cytoskeleton and architecture.

As we described above, the vegetal cortex of *Xenopus* oocytes houses germinal granules, which are determinants of germ cell fate and are incorporated exclusively into germ cell precursors during development. Numerous studies showed that mechanical or chemical interference with the integrity of germinal granules has deleterious effect on the number of germ cells in the embryo and fecundity of the adult (Kloc et al. 2004). Because the oocyte cortex is the most external part of the oocyte, it has to be prepared to respond to stress signals from a constantly changing environment. It is reasonable to assume, then, that the cortex should contain mechanism(s) for the protection of germinal granules' integrity. It is plausible that the structural RNA present in *Xenopus* oocyte cortex could act as a stress sensor and allow disintegration of the cytoskeleton by rapidly changing its conformation. This would result in the release of germinal granules from their cytokeratin anchor as well as their aggregation and displacement toward the "safer" oocyte interior (Fig. 2.2b). Although such a mechanism would allow for "protection" of germinal granules from the harmful influence of the external environment, the aggregation of germinal granules would result in their abnormal segregation during cleavage and the formation of an embryo with a decreased number of germ cells (Fig. 2.2b). Thus, this type of protective mechanism, although not perfect, would secure at least some degree of fecundity.

It seems unlikely that *Xenopus* would use a "conservative" bet-hedging strategy (Olofsson et al. 2009) and rely exclusively on one (RNA-based) oocyte stress response pathway. The more likely scenario would be that the evolution of a "diversified" bet-hedging strategy in *Xenopus* resulted in increased fitness in variable and unpredicted environments (Olofsson et al. 2009). A "diversified" bet-hedging strategy uses two oocyte stress response pathways: one RNA-based and

Fig. 2.2 (Continued) model showing theoretical RNA-based response to stress in *Xenopus* oocyte. *Left hand panels* represent fragments of the oocyte vegetal cortex with cytokeratin network organized through the interaction with structural RNA. Cytokeratin filaments anchor germinal granules, which are the determinant of germ cell fate. The stress signal, sensed directly by RNA, leads to conformational change in RNA. This in turn results in the disruption of cytokeratin network, the release and aggregation of germinal granules and their displacement to the "safer" oocyte interior. *Right-hand panels* represent cleaving embryos with primordial germ cells (blastomeres), which acquired germinal granules. The aggregation of germinal granules in response to stress will result in their abnormal segregation during cleavage and the formation of an embryo with a decreased number of germ cells

one protein-based. Each of these two pathways would be used in different circumstances, or one of them would be a back-up strategy. Several questions arise regarding this system: which pathway is the original, more ancient pathway, which later evolved and why, and how they are used?

There are at least three possibilities. First, the protein-based response could be an ancestral strategy and the RNA-based response could be derived, which increased the sensitivity of responses or was a redundant, back up strategy. Second, the RNA-based response could be ancestral, and a more derived and sophisticated protein-based strategy evolved later. The RNA-based strategy may be the remnant of the "RNA world", if, as predicted by the RNA-world hypothesis, RNA, with its information/catalytic properties, predated DNA/protein based life. Third, RNA- and protein-based strategies may have evolved in parallel with the complementary pathways that differed in dynamics (rapidity) and the quality of the response. Because the RNA molecule acts both as a sentinel and an executor, it has enormous potential for diversity of sequence, structure, and function. Thus, the RNA-based response is probably much faster than the complicated, protein-based response required to turn on the activity of various proteins, each performing a different function. Thus, whenever an extremely rapid response is required, the RNA-based response, which relies on existing localized RNA, would be the preferred pathway. Most probably, RNA-based and protein-based pathways act as complementary strategies, and the various combinations and degree of involvement of each pathway modulates the ultimate stress response.

2.6 Usefulness of Unnecessary Processes for Cell Cycle Evolution: Omen Versus Omre

Evolution requires certain tools triggering genotypical and phenotypical changes that assure selection of individuals characterized by better survival and reproductive fitness. Because modifications of core mechanisms seem too difficult, unnecessary cellular processes linked to the control of the cell cycle may play potentially important roles in the evolution of the cell cycle. They offer the advantage of not modifying the most stable core mechanisms. An apparent disadvantage could be their low impact on the processes of cell cycle regulation. Paradoxically, this low impact may be advantageous from an evolutionary point of view.

Estrogen-regulated Protein 45 (EP45 also known as Seryp or pNiXa) is involved in yolk digestion and embryo nutrition in *Xenopus laevis*, but it has no specific role in meiotic resumption in oocytes responding properly to physiological doses of progesterone (Marteil et al. 2010). However, EP45 efficiently enhances meiotic resumption when expressed in oocytes poorly responding to low doses of progesterone (Marteil et al. 2010). Thus, EP45 overexpression ameliorates the quality of oocytes suffering from low reactivity to progestins (Marteil et al. 2009). Under fluctuating conditions provoked, for instance, by changes in the environment,

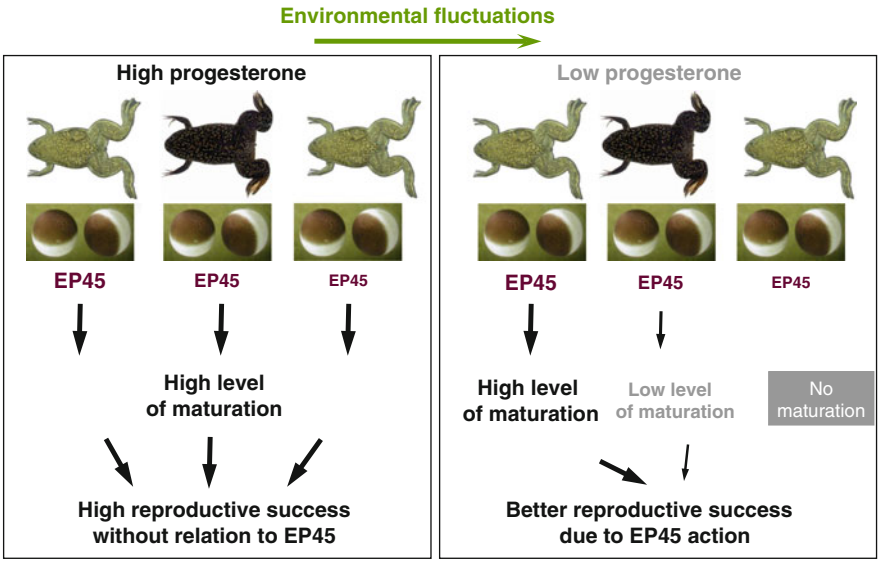

Fig. 2.3 Hypothetical role of EP45 in reproductive fitness of females in changing environmental conditions

females may exhibit low progestin levels causing a drastic fall in the number of oocytes entering meiotic maturation and thus reducing the female reproductive fitness. In these novel conditions, the so far unnecessary EP45-dependent process gains its importance in oocytes accumulating by chance the highest levels of EP45 or disposing of the highest activity dependent on EP45 (Fig. 2.3). As such, EP45 becomes an unexpected survival factor for the oocytes in this novel situation and may have an important impact on the reproductive fitness of a female, a population or even a species. Thus, a pathway physiologically unnecessary for cell cycle control and most likely not directly related to oocyte maturation initiation in normally high progestin conditions may have a great value for the survival and reproductive fitness of *X. laevis* upon decreased progestin levels in females. In addition, EP45-dependent enhancement of meiotic resumption seems to be an extremely efficient tool for evolution because it is seemingly ready-to-use (Marteil et al. 2010).

ERK2 MAP kinase in *X. laevis* oocytes and ERK 1/2 MAP kinases in mouse oocytes may belong to the same family of oocyte maturation enhancers, if we consider their capacity to enter the meiotic M-phase as a criterion for the quality of gametes. ERK2 is not necessary for the meiotic M-phase entry in *X. laevis*; however, it enhances the process (Gross et al. 2000). In the mouse oocytes, ERK 1/2 are naturally activated following GVBD (Verlhac et al. 1994). Major problems in maturation of both *Xenopus* and mouse oocyte without ERK MAP kinases activity appear only following MI (Gross et al. 2000; Verlhac et al. 1996). However, it is not known how small differences in these activities, comparable to differences

in EP45, may influence the efficiency of oocyte maturation and female fertility. One can only guess that the reproductive success of females with such a defect should be low. Contrary to EP45, we do not know what environmental cues, if any, could lower MAP kinases activity in maturing oocytes. Curiously, one of the potential pathways stimulated by serpins (EP45 belongs to this group of proteases inhibitors as well) in *X. laevis* oocytes may be ERK2 (Marteil et al. 2010).

The EP45 pathway is not unique in its "unnecessary" character. Similar accessory cellular processes may be present in oocytes and transmitted to the progeny due to their neutral character or their usefulness only in cell cycle unrelated processes, such as the regulation of yolk plate digestion in the case of EP45 (Jorgensen et al. 2009). UBE2S, identified as an anaphase-promoting complex/cyclosome (APC/C) auxiliary protein, was shown to be dispensable for the physiological mitotic exit, but crucial for spindle assembly checkpoint (SAC) slippage (Garnett et al. 2009). Thus, both M-phase entry (via EP45) and M-phase exit (via UBE2S) are accompanied by "unnecessary" cell cycle related processes that deliver fantastic tools for evolution; such processes constitute a reservoir of emergency solutions at the cellular level. This resembles, to some extent, the silent point mutations at the genomic level. However, the unique advantage of these processes for evolution comes from the possibility of their immediate application upon changes in the environment, as opposed to silent point mutations in DNA, which increase diversity and only facilitate amino-acids replacements in proteins. The potential mode of action of such phenomena in evolution resembles the strategy of bet-hedging.

Oocyte maturation-enhancing (Omen) activity suggested for EP45 (Marteil et al. 2010) may be equilibrated by a repressive activity (oocyte maturation repressor or Omre; D'Inca et al. 2010). The equilibrium may again resemble the robust mode of cell cycle control via phosphorylation/dephosphorylation, methylation/demethylation, and similar reversible protein modifications. Thus, Omen and Omre are perfect candidates for easy-to-use tools for evolution at the cell cycle level.

2.7 Bet-Hedging and Weak Linkage in the Cell Cycle and Development: Conclusions

The architecture of cellular processes involves numerous mechanisms conserved over the course of the evolution across a multitude of species and genera. The cell cycle, with its elaborate checkpointing and time synchronization mechanisms, is one of them. Protein and mRNA localization in the cell is another example. Given the low frequency of establishing such core processes in evolutionary history (i.e., those involved in generating the phenotype), their reuse becomes an economical alternative for the organism to introduce novelty in its anatomy and physiology. Combining relatively few core elements by way of weak links is arguably the way evolution has solved two seemingly contradictory requirements: robustness and adaptability (Kirschner and Gerhart 2005).

In this chapter, we argued that two initially unrelated processes (the EP45 protein essential for yolk digestion and embryo nutrition and meiotic resumption) become subject to such weak linkage. Under the stressful condition of low progestin levels, meiotic maturation is disrupted and reproductive fitness is significantly reduced. However, meiotic resumption remains intact in those cells that happen to contain high levels or activity of EP45. Regardless of the nature of the stimulating effect of the EP45 protein on meiosis, the level of EP45 is not a mere response to the environmental change; it is independent of the progestin level. The explanation we advocate here is that the regulation of the EP45 pathway occurs independently from the stage of meiosis and the copy number distribution of EP45 between embryos is purely random. Similarly, interactions between localized mRNA and the cytoskeletal network of oocytes may also rely on such a relationship. mRNAs may have acquired their role of structural regulation based on their stochastic distribution in relation to their future function.

The "weak linkage" suggested for these two examples reveals itself under stress conditions. Without a feedback response, only a high abundance of EP45 enables cells to complete their meiotic cycle. An analogous link may exist between the localization of specific mRNAs and their capacity to develop structural functions in oocytes. Such "function hijacking" may increase the survival rate at a very low-cost. The distribution of EP45 between cellular ensembles is already present in the cell and no additional regulatory mechanisms are required to advance at least part of the cells to maturation. This behavior is akin to a bet-hedging strategy employed by a variety of species, ranging from microbial populations to arthropods and even human societies (Miller and Page 2007), to evade stressful conditions.

Whether the magnitude of fluctuations in the level of EP45 protein or randomness in the distribution of structural mRNAs in the cellular population is indeed subject to selective forces awaits further theoretical and experimental verification. Stochastic processes, even though widely present at the cellular level and capable of affecting the rates of chemical reactions and steady-state protein distributions, are frequently filtered out by a number of regulatory processes (e.g., cascade architecture of signaling networks (Thattai and van Oudenaarden 2002), large time scale separation between up- and downstream processes (Bruggeman et al. 2009), feedback loops (Acar et al. 2005). Subject to those constraints, molecular noise is therefore incapable of affecting cellular physiology or the phenotype. On the other hand, stochastic protein synthesis, or gene expression taking place in bursts (McAdams and Arkin 1997; Shahrezaei and Swain 2008; Dobrzyński and Bruggeman 2009), is a potent mechanism of generating phenotypic heterogeneity in a population of isogenic cells. Such noise-induced variability in the properties of individual cells may confer a fitness advantage on the population level in the face of antibiotic exposure (Blake et al. 2006), chemotherapy treatment (Cohen et al. 2008; Spencer et al. 2009; Sharma et al. 2010) or general changes in extracellular conditions (Acar et al. 2008). Embryo development and cell cycle transitions arguably employ similar mechanisms to increase the reproductive success in the face of unexpected environmental changes. The EP45 fluctuations and random concentration of structural mRNA across oocytes could underlie such evolutionary adaptations. However,

further experiments are required to reveal to what degree noise in cellular processes is regulated, which properties of the noise spectrum are modulated and what parts of the regulatory machinery actively control the noise.

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Chapter 3
Mechanics and Regulation of Cell Shape During the Cell Cycle

Andrew G. Clark and Ewa Paluch

Abstract Many cell types undergo dramatic changes in shape throughout the cell cycle. For individual cells, a tight control of cell shape is crucial during cell division, but also in interphase, for example during cell migration. Moreover, cell cycle-related cell shape changes have been shown to be important for tissue morphogenesis in a number of developmental contexts. Cell shape is the physical result of cellular mechanical properties and of the forces exerted on the cell. An understanding of the causes and repercussions of cell shape changes thus requires knowledge of both the molecular regulation of cellular mechanics and how specific changes in cell mechanics in turn effect global shape changes. In this chapter, we provide an overview of the current knowledge on the control of cell morphology, both in terms of general cell mechanics and specifically during the cell cycle.

3.1 Introduction

The shape of a cell is defined by its mechanical properties and its interactions with the environment (Thompson 1917), and thus the study of cellular mechanics is a prerequisite for the understanding of cell shape control. Cell mechanics can be approached at two levels: (1) by development of physical descriptions of the cell and (2) by experimental studies that combine biological and biophysical methods. While the first approach is critical for understanding which mechanical properties are important for the control of cell shape and how these properties interact to give rise to global cell morphology (Ingber 1993; Bereiter-Hahn 2005; Hoffman and Crocker 2009), the second is essential for understanding how molecular pathways

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control cellular mechanical properties and, as a result, govern cell shape (Janmey and McCulloch 2007; Lecuit and Lenne 2007; Montell 2008; Paluch and Heisenberg 2009). Such experimental studies rely heavily on the development of tools and methods for measuring cellular mechanical properties and the physical forces generated by living cells.

In this chapter, we discuss cell shape changes that occur throughout the cell cycle. We focus on the mechanical properties that have been shown to be involved in cell shape control and discuss how these properties are regulated, particularly by key biochemical pathways that drive the cell cycle. We first introduce several basic mechanical concepts that are useful for the study of cell shape. The subsequent section describes the geometrical changes in cell volume and surface area that occur during the cell cycle. Sections 3.4 and 3.5 focus on the mechanical control of shape changes during mitosis and interphase, respectively. Section 3.6 discusses specific aspects of cell mechanics within tissues. Finally, the last section summarizes the current knowledge on how cellular mechanics are controlled by cell cycle-related biochemical pathways.

3.2 Physical Descriptions of the Cell

This section briefly introduces the most common physical descriptions of the cell, as well as the basic mechanical concepts that will be used throughout this chapter. Mechanics is the branch of physics that deals with the movements and deformations of physical bodies. Classical mechanics is often subdivided into two branches: kinematics and dynamics. While kinematics describes the motion of objects without considering the causes of this motion, in dynamics, laws of mechanics (e.g., Newton's laws) are used to predict the motions and deformations caused by the forces exerted on a system. To use a dynamics approach to study the deformation or movement of a cell, one must know not only the forces exerted on the cell, but also the physical properties of the cell (such as its elasticity and viscosity), which determine how it will respond to such forces. A major focus of biomechanics is to design experiments to determine these forces and properties (Bereiter-Hahn et al. 1987). However, it is not always possible to directly measure the mechanical properties of a cell and the forces to which it is subjected, particularly for cells in tissues (Davidson et al. 2009). In the absence of such direct measurements, kinematic approaches, which focus on analyzing the motion and geometrical changes of cells, can provide important insights into cell mechanics (see Sects. 3.2.5 and 3.3, Blanchard et al. 2009).

Mechanical models for cell shape changes, particularly during cell division, and also in the larger context of embryonic development, have been a focus of interest since the late nineteenth century (Roux 1894; Thompson 1917; Rappaport 1996). In the simplest models, the cell is considered as a liquid bag surrounded by a homogenous shell under tension (see Sect. 3.2.2). Successive levels of complexity can then be added by taking into account the physical properties of cytoplasmic

structures and spatial variations in the physical parameters describing the cell. The addition of substrate adhesions and interactions with neighboring cells is essential for understanding cell shape in the context of a tissue (Box 3.1).

Box 3.1 Major Physical Properties of the Cell

One of the first attempts to experimentally determine cell surface mechanical properties was with a device called the "Cell Elastimeter," which uses a pressure differential to aspirate single cells inside of a glass capillary (Mitchison and Swann 1954). This technique, along with other cell aspiration and deformation methods, has been used to measure a number of cell surface properties important for the study of cell mechanics (Hochmuth 2000; Lomakina et al. 2004; Krieg et al. 2008b).

Cortical Tension

The most basic cortical structure is present under the membrane of red blood cells. In these cells, a very thin (5–10 nm) network of spectrin and short actin filaments is tightly attached under the plasma membrane. Interfacial tension of red blood cells was one the first experimentally measured and is approximately 15 pN/μm (Rand and Burton 1964). In contrast to red blood cells, most cell types have a tensile actomyosin cortex under the plasma membrane with a thickness ranging between several hundred nanometers and several microns depending on cell type (Hiramoto 1957; Hanakam et al. 1996). The magnitude of the resulting cortical tension also varies between cell types. For example, neutrophils have a relatively low tension of approximately 35 pN/μm (Evans and Yeung 1989), which is comparable to the cortical tension of early embryonic cells isolated from zebrafish embryos (Krieg et al. 2008a). In contrast, fibroblasts typically display tensions of 500–1,000 pN/μm (Thoumine et al. 1999; Tinevez et al. 2009), while in *Dictyostelium discoideum* cells, cortical tension can reach several thousand pN/μm (Pasternak et al. 1989; Dai et al. 1999; Schwarz et al. 2000).

Relationships Between Tension and Other Physical Properties of the Cortex

In the literature, tension is sometimes confused with elasticity (also often called stiffness or rigidity). However, tension and elasticity are two distinct physical properties. Whereas elasticity is a measure of deformability for a solid-like material, tension is related to the sum of all forces within a material. In the context of the cortex, tension is the local stress in the cortical network (i.e., the forces per unit area in the network) integrated over the thickness of the cortex. In cells, surface tension is the combined result of the physical properties of the membrane and of the underlying cortical network. Provided that the cortex is tightly coupled to the membrane, the total surface tension is

(continued)

the sum of the in-plane tension of the plasma membrane, γ, and of cortical tension, T_{cortex}:

$$T_{\text{total}} = \gamma + T_{\text{cortex}}.$$

In most cells with an actomyosin cortex, plasma membrane tension is negligible and surface tension is dominated by cortical tension (Krieg et al. 2008a; Tinevez et al. 2009). For a steady-state cortex in the absence of elastic deformations or flows, cortical tension is primarily determined by the active tension (T_{active}), which is the result of active processes such as myosin motors pulling on the actin network. Active tension is equal to $\zeta\Delta\mu{\cdot}h$, where h is the thickness of the cortex and $\zeta\Delta\mu$ is the active stress exerted in the cortical network (ζ is a coefficient relating the energy provided by ATP hydrolysis, $\Delta\mu$, to the active stress; ζ depends both on the activity and on the concentration of myosin motors; Kruse et al. 2004). In the presence of deformations or flows, additional terms that depend on viscoelastic properties of the cortex must be taken into account. For example, for purely elastic deformations:

$$T_{\text{cortex}} = T_{\text{active}} + T_{\text{elastic}} = \zeta\Delta\mu \cdot h + Eh\frac{(A - A_0)}{A_0}$$

where E is the elastic modulus, A is the surface area, and A_0 is the equilibrium surface area of the cortex (Tinevez et al. 2009). In this particular case, cortical tension depends on cortex elasticity, though this is not true for all deformations.

Membrane-to-Cortex Attachment

In order to translate cortical movements into movements of the plasma membrane, the cortex and the membrane must be attached. Membrane-to-cortex attachment is achieved by specific proteins that bind both to actin filaments and to transmembrane proteins or lipids. The best-studied examples are the ezrin/radixin/moesin (ERM) family proteins, though other molecules such as myosins-1 and filamin are also involved in membrane-to-cortex attachment (Popowicz et al. 2006; Fehon et al. 2010). Membrane-to-cortex attachment can be measured in isolated cells by pulling membrane tubes from the cell: the force necessary to pull and maintain a membrane tether directly depends on the energy of membrane-to-cortex attachment (Sheetz 2001; Brochard-Wyart et al. 2006; Nambiar et al. 2009; Diz-Muñoz et al. 2010).

Cell–Substrate and Cell–Cell Adhesion

Physical descriptions of isolated cells in suspension have provided important insights into cellular mechanics. However, cells both in tissue and in

(continued)

culture physically interact with their environment, be it a culture dish, a matrix or neighboring cells in a tissue. Including the effect of substrate adhesion into mechanical models of cells is not trivial and is still being actively investigated. In most models, a continuum adhesion term is added to liquid core/viscoelastic contractile shell-type descriptions of the cell (Chu et al. 2005; Krieg et al. 2008b; Borghi and Nelson 2009). Such purely physical descriptions, which do not include complex biochemical regulation of cell–substrate adhesion, successfully account for the dynamics of early spreading of a variety of cell types over solid substrates (Cuvelier et al. 2007).

While cortical tension favors a spherical cellular shape, cell–substrate and cell–cell adhesions provide counteracting forces that tend to favor a more flattened, spread shape. The balance between these opposing forces determines the areas of cell–cell or cell–substrate interfaces and defines the overall shape of an adherent cell (Cuvelier et al. 2007; Montell 2008). Predicting the shape of a single cell on a flat substrate or of a doublet of adhering cells is already a theoretical challenge, and modeling the shape of cells within tissues or interacting with three-dimensional substrates is considerably more complex and has only been approached in a few specific situations (Lecuit and Lenne 2007).

Notably, even the most complex physical models of cellular objects are only valid within a set of predefined initial conditions and constraints, and these models only account for observations in a given framework. A model that accurately accounts for a set of observations using a certain experimental setup may have no value in another experimental situation. Given the complexity of the cell, it is not clear that physical modeling will ever provide a complete mechanical description of the cell, or even if such a complete description is possible. The primary goal of physical models is not necessarily to completely describe cellular mechanics, but also to provide tools for experiments. A physical description is necessary to interpret any quantitative mechanical experiment. For example, in measurements of cellular tension that rely on cell deformations induced by pipette aspiration or by laser ablation, a physical model is necessary to deduce the tension from the observed deformation (Evans and Yeung 1989; Hochmuth 2000; Rauzi et al. 2008). Models also help to determine which physical properties are likely to affect a given cell shape change and aid in designing experiments to test the effect of these properties on cell morphology. Multidisciplinary approaches that couple experimental manipulation and modeling are currently attracting a growing interest in the field of cell morphogenesis (Farhadifar et al. 2007; Lecuit and Lenne 2007; Solon et al. 2009). These include studies of cell mechanics and shape changes during mitosis (Théry et al. 2007; Stewart et al. 2011).

3.2.1 *Physical Descriptions of the Cell: A Multicomponent Complex Object*

Cells are made of "soft" structural components. As such, their behavior can differ considerably from that of rigid, engineered structures typically modeled in the framework of conventional mechanics. Physical properties of cells are usually approached in the context of soft matter physics, which deals with materials such as liquids, colloids, and polymer gels (Boal 2002). A typical soft-matter-type behavior of biological materials is viscoelasticity. When subjected to a force, a viscoelastic material displays liquid-like (viscous) or solid-like (elastic) behaviors, depending on the timescale of the perturbation. Although the time dependency of the response to stress differs among various types of viscoelastic materials, many biological materials respond to an applied force in a solid-like, elastic manner on short timescales and in a liquid-like, viscous manner on long timescales.

In the past few decades, soft-matter physicists have developed theoretical tools for the study of many biological materials, including viscoelastic cytoskeletal networks and fluid lipid bilayers (reviewed in Lipowsky and Sackmann 1995; Boal 2002; Janmey and McCulloch 2007). However, the cell is a complex object, composed of many constituents with very different physical properties that vary in space and time, and thus it cannot be fully described by a simple viscoelastic or fluid model (Bereiter-Hahn et al. 1987; Hochmuth 2000). Moreover, cytoskeletal networks can display active behaviors, such as treadmilling or contraction, where ATP consumption is converted into mechanical work. The presence of active processes makes passive mechanical models incomplete. Recently developed "active gel" models, which integrate such active behaviors with more classical viscoelastic descriptions of cytoskeletal meshworks, provide a more accurate description of cellular networks (Kruse et al. 2004, 2005).

One challenge in modeling cell mechanics resides in choosing the level of detail for a given model. Cellular mechanical properties are the consequence of interactions at the molecular level. However, an accurate model of how molecular interactions give rise to global mechanical properties would require detailed knowledge of all the molecular players involved and the characteristics (e.g., time constants) of their interactions. Thus, only the behavior of relatively simple cytoskeletal structures can be predicted from molecular data using simulations. Such molecular approaches have proven successful in modeling in vitro actin networks (Carlsson 2003; Dayel et al. 2009; Rafelski et al. 2009) and microtubule bundles and spindles (Janson et al. 2007; Dinarina et al. 2009). However, in most cellular processes, the data necessary to develop microscopic models are simply not available. Moreover, molecular models cannot always account for mesoscopic properties like pressure and friction. In contrast to molecular descriptions, coarse-grained models use phenomenological parameters that integrate the influence of microscopic processes without requiring a detailed knowledge of how they occur. For example, in coarse-grained models of cytoskeletal networks, viscosity is described by a generic parameter that globally accounts for all sources of viscosity in the network

(turnover of cross-linkers, filament turnover, etc.) without requiring molecular details of the viscosity-generating mechanisms (Kruse et al. 2005; Mayer et al. 2010). Coarse-grained models are frequently used to describe mechanical properties at cellular length scales. They are able to describe processes that are too complex to model at the microscopic level or where emergent mesoscopic properties, rather than molecular details, are central. However, they do not necessarily provide a physical understanding of how molecules influence a given physical property. Therefore, coupling coarse-grained and molecular approaches is necessary to achieve a thorough understanding of cell shape changes.

From experimental and theoretical studies, a number of core mechanical properties have emerged as major determinants of cell shape. These properties, which include cortical tension, membrane-to-cortex attachment and cell–substrate/cell–cell adhesion, are discussed in detail in Box 3.1.

3.2.2 Simplest Models: A Liquid in a Shell

Because most cells assume a spherical shape when put in suspension, a behavior characteristic of liquids, early models describe suspended cells as "liquid drops." In liquids, surface tension, which determines the energy cost of an interface between the liquid and the surrounding medium, favors the shape with the minimal surface for a given volume (i.e., a sphere). Though very simplistic, liquid drop models have, for example, proven successful in predicting the shape of suspended cells aspirated into a pipette (Evans and Yeung 1989; Yeung and Evans 1989; Dai et al. 1999; Hochmuth 2000).

Cellular surface tension results from a combination of the tensions of the plasma membrane and the cytoskeletal structure present immediately under the membrane, the cell cortex (Box 3.1, Fig. 3.1a). In most cells, a cortical network of cross-linked actin filaments supports the plasma membrane (Bray and White 1988), and this meshwork is under tension as a result of myosin motor activity. Actomyosin tension is different from the passive plasma membrane tension in that it is actively generated by myosins pulling on actin filaments. Its mechanical effects are nevertheless similar to that of a passive surface tension (Dai et al. 1999; Hochmuth 2000; Bereiter-Hahn 2005; Tinevez et al. 2009). Provided that the cortex is tightly attached to the membrane, the total cellular surface tension is the sum of the tensions of the plasma membrane and of the cortex. However, if the coupling of the membrane to the cortex is loose, such that membrane and cortex can move separately from one another, membrane and cortex tensions are not simply additive. Regardless, the tension of the plasma membrane itself is usually negligible compared to the tension of the underlying cortex (Dai et al. 1999; Tinevez et al. 2009).

In liquid drops, surface tension results in a hydrostatic pressure inside of the drop, given by the law of Laplace. Similarly, in cells, the contractile cortex generates pressure in the cytoplasm, which is related to cortical tension by the law of Laplace (Fig. 3.1b). This has implications for the regulation of cell volume as increasing cortical tension could, in principle, cause the cell to contract and drive

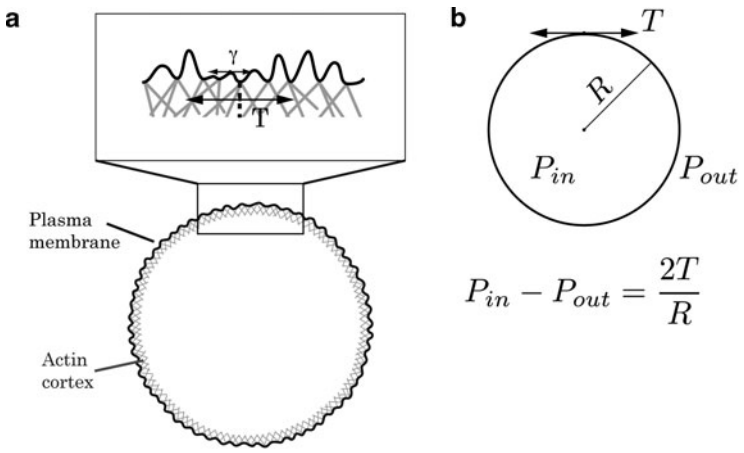


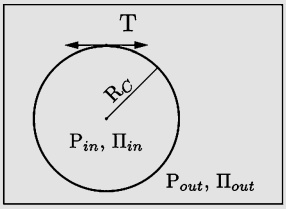
Fig. 3.1 Surface tension, intracellular pressure and implications for the regulation of the cell volume. (**a**) In most cells, a cortical network of cross-linked actin filaments lies under the plasma membrane. The surface tension of the cell is a combination of the tension of the plasma membrane, γ, and the cortical tension, T. If the cortex is tightly attached to the membrane, the total surface tension is simply $\gamma + T$. In most cells, γ is orders of magnitude smaller than T, and γ can thus be neglected. (**b**) Cortical tension generates a hydrostatic pressure in the cytoplasm. The law of Laplace relates the difference between intracellular pressure, P_{in}, and external pressure, P_{out}, to the cell radius, R, and the cell surface tension, T

water out of the cell, thereby reducing cell volume. At steady state, when the cell volume does not vary, the hydrostatic pressure has to be exactly balanced by the osmotic intracellular pressure (Box 3.2; Bereiter-Hahn 2005). However, even the highest measured cortical tensions (several thousand pN/μm for *Dictyostelium* cells, see Box 3.1) result in a hydrostatic pressure differential across the cell membrane of around 1,000 Pa (given by the law of Laplace applied to a typical *Dictyostelium* cell with a tension of 2,500 pN/μm and a radius of 5 μm). In contrast, the osmotic pressures of physiological media are typically around 5×10^5 Pa. Therefore, even a complete disassembly of the contractile actin cortex, reducing the hydrostatic pressure differential from 1,000 to 10 Pa, would result in a volume change of only a few percent (Tinevez et al. 2009; Stewart et al. 2011; see Box 3.2 for details). Cell volume is therefore primarily dictated by osmotic pressure, which is regulated by ion fluxes, while the main function of cortical tension is rather to provide physical resistance to external forces exerted on the cell surface and to drive cellular deformations.

3.2.3 The Structure of the Cytoplasm

Describing the cytoplasm as a viscous fluid can be sufficient for slow deformations. However, in the case of fast shape changes, elastic, solid-like properties of cytoplasmic structures may play an important role in resisting deformations. Indeed, the

Box 3.2 Osmotic Pressure, Cortical Tension and Cell Volume Control



Hydrostatic Pressure:

$$P_{in}\text{-}P_{out}=\frac{2T}{R_C}$$

Osmotic Pressure:

$$\Pi_{in}-\Pi_{out}=\frac{n_{osm}R\Theta}{V_c}-\Pi_{out}$$

(Eq. 1) Δ Hydrostatic Pressure = Δ Osmotic Pressure

(Eq. 2) $$P_{in}\text{-}P_{out}=\Pi_{in}\text{-}\Pi_{out}$$

(Eq. 3) $$\frac{2T}{R_C}=\frac{n_{osm}R\Theta}{V_c}-\Pi_{out}$$

(Eq. 4) $$V_c=\frac{n_{osm}R\Theta}{\Pi_{out}+\frac{2T}{R_C}}$$

$\sim 10^5$ Pa $\sim 10^2 - 10^3$ Pa

(Eq. 5) $$V_c\approx\frac{n_{osm}R\Theta}{\Pi_{out}}$$

For a spherical cell at steady state, cell volume is fixed, and no net flow of water through the cell membrane occurs. While the difference in hydrostatic pressure across the membrane is given by the law of Laplace, the osmotic pressure difference is the difference between intracellular osmotic pressure, which depends on the number of moles of intracellular osmolites, n_{osm}, and the extracellular osmotic pressure, which is approximately 10^5 Pa for physiological media [see (Eq. 3), where T is the cortex tension, R_{C} is the cell radius, R is the gas constant, θ is the temperature, and V_{C} is the cell volume]. At steady state, intracellular hydrostatic pressure is exactly balanced by osmotic pressure [(Eq. 1); Bereiter-Hahn 2005; Salbreux 2008; Tinevez et al. 2009]. This means that the hydrostatic pressure differential across the cell membrane is equal to the differential in osmotic pressure (Eq. 2) and (Eq. 3). For cells in culture and in vivo, the absolute value of the extracellular osmotic pressure is much higher than the typical pressure difference across the plasma membrane (Eq. 4). As a consequence, even a dramatic change in cortical tension will be balanced by a change in osmotic pressure resulting from a very small change in cell volume; the contribution of cortical tension can therefore be neglected, and cell volume can be reasonably approximated using osmotic pressure alone ((Eq. 5); see also Salbreux 2008). For example, if cortical tension is reduced from 2,500 to 10 pN/μm (corresponding to the change in tension resulting from cortex disassembly in cells with high surface tension), the

(continued)

hydrostatic pressure difference between the inside and the outside of the cell drops by about 1,000 Pa. This drop represents only 1% of the exterior osmotic pressure. Such a change in pressure would thus be mechanically balanced by an expansion of the cell volume by ~1%. This calculation implies that the cortex is *not* primarily responsible for preventing cell expansion due to the osmotic pressure difference (Tinevez et al. 2009). This is likely the reason why cells treated with the actin depolymerizing drug Cytochalasin D, for example, which reduces cortical tension by 90%, only display a small increase in volume and do not immediately explode (Stewart et al. 2011).

nucleus, intracellular cytoskeletal networks (including intermediate filaments) and membrane organelles display elastic properties, at least on short timescales (Brown et al. 2001; Caille et al. 2002; Ingber 2003; Liu and Wang 2004; Mitchison et al. 2008).

A more realistic model of the cell body treats the cytoplasm as a sponge-like poro-elastic material, where fluid cytosol fills an intracellular viscoelastic network (Mitchison et al. 2008). Such poro-elastic models have been successfully used to quantitatively describe cellular deformations driven by flows of cytosol and changes in intracellular pressure (Charras et al. 2008; Tinevez et al. 2009). The presence of a cytoplasmic network has important implications for intracellular hydrostatic pressure. Indeed, a porous cytoplasm hinders the motion of the fluid cytosol, and thus slows down the equilibration of hydrostatic pressure throughout the cell (Charras et al. 2005). The timescale of pressure equilibration is directly related to the effective meshsize of the cytoplasmic network; if the meshsize is small (in the 30 nm range), pressure gradients across the cell could persist on timescales of seconds, and may thus influence cell motility (Mitchison et al. 2008). Experiments in cultured filamin-deficient M2 cells suggest the presence of such gradients (Charras et al. 2005), while experiments in other cell types argue for a faster equilibration of cytoplasmic pressure (Tinevez et al. 2009; Maugis et al. 2010). It is possible that cytoplasmic mesh sizes are within a range where small changes could drastically affect the timescale of pressure equilibration, and thus the structure of the cytoplasm must be tightly regulated. Interestingly, a massive disassembly of intermediate filament networks is observed in mitosis, controlled by $p34^{Cdc2}$ (Chou et al. 1990), suggesting that the meshsize of the cytoplasmic network increases and that intracellular pressure equilibration may occur faster in this phase of the cell cycle.

3.2.4 Spatial Variations: Cells Are Not Spheres

The models described above mostly consider the cell as a spherical object. To account for cell polarization and local cell deformations, one must take into account

spatial inhomogeneities. There are a number of examples where local variations in cellular physical parameters lead to cell deformations:

- Local changes in dynamics or spatial polarity of cytoskeletal networks underlying the membrane can be sufficient to explain a variety of complex cell shapes. For example, the shapes of keratocytes gliding over flat surfaces can be successfully described by a two-component model where a treadmilling actin network pushes against an inextensible membrane bag (Keren et al. 2008).
- Local changes in the physical properties of the cortex itself can account for shape changes. For example, physical models of cytokinesis can account for the formation of the cleavage furrow by introducing a gradient in contractility between the poles and the equator (White and Borisy 1983).
- Local shape changes can result from inhomogeneities in membrane-to-cortex attachment. For example, at places where the cortex is locally decoupled from the membrane, intracellular pressure can drive the formation of membrane protrusions called blebs, which are involved in cell migration, cytokinesis, cell spreading, and apoptosis (Sheetz et al. 2006; Charras and Paluch 2008; Fackler and Grosse 2008).

3.2.5 Mechanics of Cells in Tissues

Beyond the level of the individual cell, tissue morphogenesis also relies on mechanical processes (Thompson 1917). Tissue mechanics arises from the physical properties of the cells that compose the tissue. Deformations of individual cells are coupled by cell–cell or cell–matrix interactions and therefore give rise to global tissue movements. Although there is no general understanding of how these individual properties impact tissue morphogenesis, studies in developing embryos point to cell–cell and cell–substrate adhesion and actomyosin contractility as core physical properties for many of the shape changes observed during embryonic development (Lecuit and Lenne 2007; Montell 2008). For example, the vertex model, which only considers cell elasticity, cell–cell adhesion and actomyosin contractility at apical junctions, can account for various packing geometries observed in the *Drosophila* wing epithelium as stable and stationary network configurations (Farhadifar et al. 2007; Staple et al. 2010). Moreover, if perturbations in the network due to cell proliferation are introduced in the model, the resulting cell rearrangements are similar to those observed during *Drosophila* development. Thus, using the vertex model, the epithelial packing geometries observed during development can be successfully accounted for by the combined action of cell mechanics and cell proliferation in the tissue. Further refinements of the vertex model, coupling cell polarity to mechanical properties, account for global tissue polarity during morphogenesis (Aigouy et al. 2010). A similar approach has been used to investigate the mechanics of germ-band elongation during *Drosophila* development (Rauzi et al. 2008). Coupling a vertex-type model to quantification of cell deformations and laser ablation-based force distribution mapping within cells

has identified the tensile properties of individual cells, as opposed to external forces, as the major contributor to the remodeling of the elongating germ-band. Similar models that couple contractility to cell–cell adhesions have been used to describe cell patterning in the *Drosophila* retina (Käfer et al. 2007; Hilgenfeldt et al. 2008) and to account for size determination during the growth of the *Drosophila* wing disk (Hufnagel et al. 2007).

There are a number of challenges associated with experimental studies of cell mechanics in tissues. One major problem in investigating which mechanical properties are important during tissue morphogenesis is that it is difficult to specifically modulate a single physical property without affecting others (Lecuit and Lenne 2007). Indeed, many molecular pathways are likely to affect several mechanical properties simultaneously. For example, proteins controlling actin turnover may influence the viscosity of the cortex as well as cortical thickness and tension (Tinevez et al. 2009; Mayer et al. 2010). Moreover, feedback loops between mechanical properties and molecular pathways provide an additional level of coupling between specific mechanical properties. For example, physical stress enhances the recruitment of adhesion proteins to focal adhesions and thus enhances adhesion strength (Riveline et al. 2001). This suggests that a feedback loop between cortical tension, which tends to reduce the area of cell–cell contact, and cell–cell adhesion, which tends to increase this area, could contribute to the regulation of cell shape in tissues (Rauzi et al. 2008; Paluch and Heisenberg 2009). A second problem is the lack of tools to directly measure the mechanical properties of individual cells in a tissue. While many methods exist to quantify properties of isolated cells, such as cell–cell or cell–substrate adhesion, cell elasticity, and cortical tension (reviewed in Paluch and Heisenberg 2009), most of these methods rely on direct manipulation of cells, which is difficult in tissues. The method most commonly used at present to assess mechanical properties of cells within tissues is laser ablation, where the relaxation of a cell–cell boundary upon disruption with a laser gives an indirect measurement of cortical tension or cellular elasticity (Hutson et al. 2003; Landsberg et al. 2009; Rauzi et al. 2008).

Ideally, biomechanics provides an understanding of cellular shape from "first principles," which means that cellular movements and deformations are analytically derived from the forces causing these movements. Such an approach can, however, be experimentally challenging, given the difficulties associated with directly measuring the physical properties of cells and the forces they generate. When methods to directly measure cellular mechanical properties are not available, quantification of cell movements and deformations can itself provide information about the forces generated in single cells as well as in tissues. Quantitative data on the shapes and positions of cells can be used to solve the so-called *inverse problem*, where mechanical parameters and force distributions are extracted from the observed cellular deformations. Such kinematic approaches, which focus on describing the motion of objects, have been used to map strain rates during dorsal closure (Blanchard et al. 2009; Gorfinkiel et al. 2009) and germ-band extension (Butler et al. 2009) in *Drosophila*. The distribution of forces within the tissue can be extrapolated from strain rate maps and these deformation maps can then be

quantitatively compared for different experimental conditions, providing insight into the molecular pathways involved in force generation (Butler et al. 2009; Brodland et al. 2010). In such kinematic approaches, changes in cell geometry must be precisely quantified in order to extract information about the physical properties of cells and the forces acting upon them. The following section provides an overview of the changes in cell shape occurring during the cell cycle.

3.3 Regulation of Cell Volume and Surface Area During the Cell Cycle

Cells undergo considerable geometrical changes during each division cycle, particularly during mitosis. At the onset of M-phase, cells both in vivo and in vitro transform from various adhesive shapes to being nearly spherical and virtually detached from the substrate in the process commonly known as "cell rounding," or "rounding up" (Strangeways 1922; Sanger and Sanger 1980; Cramer and Mitchison 1997). Concomitant with this drastic shape change, cells undergo significant changes in surface area and volume during the course of M-phase as well as in the final stage of M-phase, cytokinesis, before re-spreading as two daughter cells. Precisely monitoring these geometrical changes can provide information about the underlying forces that drive cell deformations.

3.3.1 The Geometry of Cell Rounding

The surface area of a sphere is the minimal area possible for a given volume. Therefore, when a cell transforms from a flat shape to a sphere during rounding, it must undergo either a reduction in surface area (assuming volume is constant) or an increase in volume (assuming surface area is constant) (Fig. 3.2a; Théry and Bornens 2008). In other words, the surface area-to-volume ratio must decrease as a cell rounds up, and this can be achieved by changing either one or both of these geometrical parameters. In reality, both surface area and volume appear to decrease during cell rounding, but because the reduction in surface area is greater, the surface area-to-volume ratio decreases (Fig. 3.2b; Erickson and Trinkaus 1976; Boucrot and Kirchhausen 2008).

3.3.2 Changes in Cell Volume

Cell volume gradually increases during interphase to between two and three times the volume observed in early interphase (Graham et al. 1973; Knutton et al. 1975; Habela and Sontheimer 2007). During M-phase, it has been observed that cell

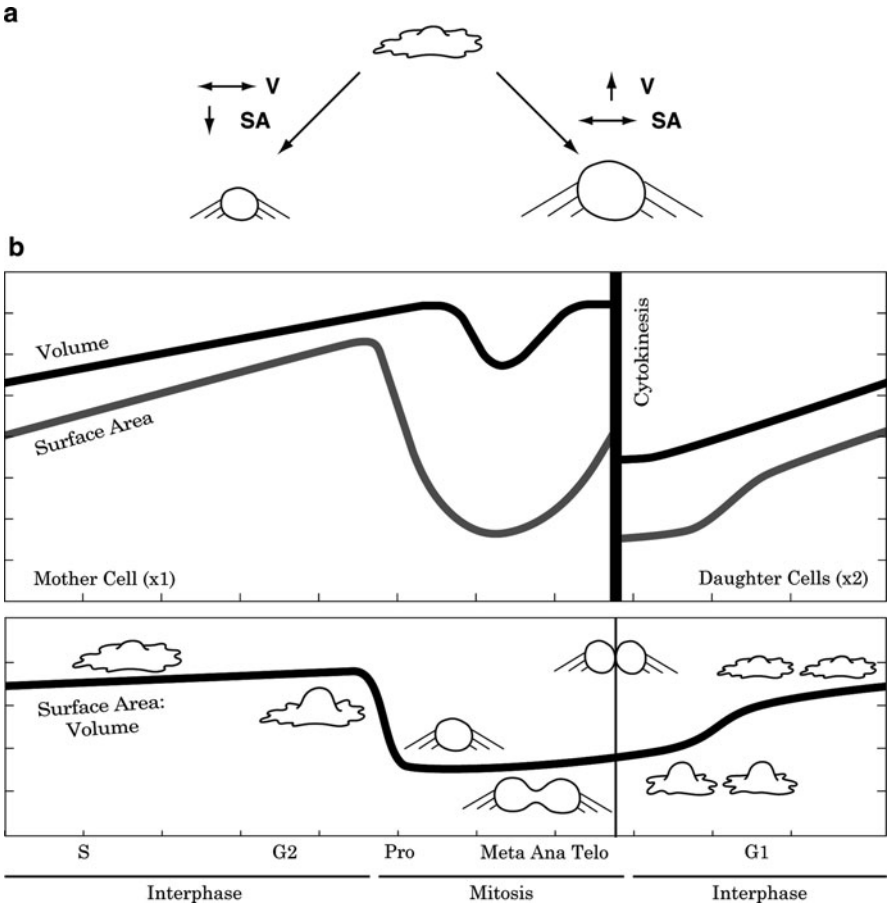


Fig. 3.2 Cell volume and surface changes during the cell cycle. (**a**) Cell rounding requires a decrease in the cell surface area-to-volume ratio. This can be achieved by decreasing the cell surface area (SA) and/or by increasing cell volume (*V*). (**b**) Evolution of cell volume and surface area (*upper panel*) and surface-to-volume ratio (*lower panel*) during the cell cycle. Note: after cytokinesis, the volume and surface area of one of the daughter cells are plotted, which explains the jump in volume and surface values in the upper panel at cytokinesis

volume is reduced by approximately 30% from prophase to metaphase, and subsequently returns to the prophase volume just prior to cytokinesis. Following cytokinesis, each daughter cell then has a volume that is equal to half of the mother's at the beginning of cytokinesis (Fig. 3.2b; Boucrot and Kirchhausen 2008). Notably, as volume measurements generally rely on three-dimensional reconstructions of cell shape, they are subject to significant errors in accuracy (Tzur et al. 2009), and the extent of mitosis-related volume changes is still a matter of debate. Changes in cell volume could in principle be caused by changes in cortical tension, and the resulting hydrostatic pressure, or in osmotic pressure. However, given the respective magnitudes of extracellular osmotic pressure and of the pressure differential

across the plasma membrane, such significant volume changes are more likely controlled by pathways that modify the cell's osmotic potential rather than by the cytoskeleton (see Sect. 3.2.2 and Box 3.2; Salbreux 2008; Tinevez et al. 2009; Stewart et al. 2011).

3.3.3 *Changes in Surface Area*

Scanning electron micrographs of cultured mammalian fibroblasts show that the plasma membrane is not a blanket of lipids pulled tightly over the cell, but rather forms a continuous series of folds and ridges overlying the cell (Fig. 3.3a; Knutton et al. 1975; Erickson and Trinkaus 1976). Therefore, one must distinguish between the surface area that includes these folds, the plasma membrane surface area (PMSA), and the apparent cell surface area (CSA), which excludes these folds (Fig. 3.3b). Unlike PMSA, CSA can be measured with a light microscope, which does not resolve membrane folds or very small protrusions. PMSA is controlled by the balance of exocytosis and endocytosis (Steinman et al. 1983), wherein lipids are removed from the PM by endocytosis, sorted in endosomes and re-inserted into the PM by exocytosis. Additionally, new lipids can be synthesized

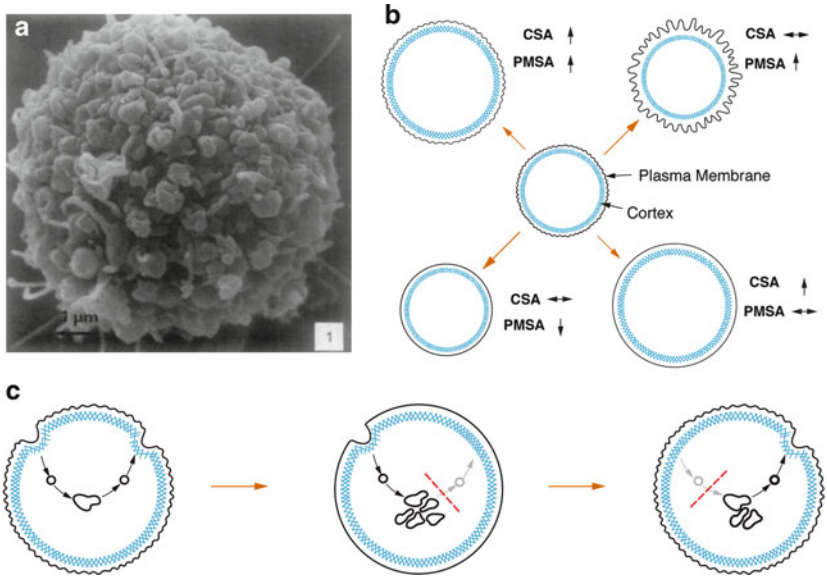


Fig. 3.3 Cell surface versus plasma membrane area. (**a**) Electron micrograph of a BHK21 cell detached from the substrate by trypsinization. The plasma membrane displays numerous folds and ridges, which indicates that PMSA is higher than CSA. Picture adapted from (Erickson and Trinkaus 1976). (**b**) Plasma membrane surface area (PMSA) and cell surface area (CSA) can vary independently from one another. (**c**) PMSA is controlled by membrane trafficking. Blocking exocytosis decreases PMSA, whereas blocking endocytosis increases PMSA

de novo in the Golgi apparatus and then trafficked to the PM (Fig. 3.3c). Electron micrographs indicate that the PMSA-to-CSA ratio, which describes the excess of plasma membrane, increases from approximately 1.5 to 3 between G_1 and G_2 phases (Erickson and Trinkaus 1976). This suggests that CSA and PMSA are regulated independently of one another. Indeed, while PMSA is necessarily regulated by membrane trafficking, CSA modification does not require changes in membrane trafficking, but rather results from cytoskeleton-driven shape changes (Fig. 3.3b, c).

3.3.3.1 Changes in Cell Surface Area

Cell surface area (CSA) doubles from early interphase to early M-phase, which is accompanied by a comparable increase in cell volume. The subsequent cell rounding upon M-phase entry results in a twofold decrease in surface area (Erickson and Trinkaus 1976). Because cell shape is consistently spherical throughout the first half of M-phase, and volume has been shown to decrease (Boucrot and Kirchhausen 2008), surface area also decreases during this period. Assuming that cell volume does not change during late anaphase, CSA increases as cytokinesis progresses because the cell becomes less spherical. Following cytokinesis, the two new daughter cells re-spread and surface area increases to the original interphase value (Fig. 3.2b).

3.3.3.2 Changes in Plasma Membrane Surface Area

Plasma membrane surface area (PMSA), which is controlled by membrane recycling, varies throughout the cell cycle, though the magnitude of this change is unclear. Estimations of PMSA based on scanning electron micrographs of detached cells indicate that PMSA increases 1.5-fold from early to late interphase (Knutton et al. 1975). During the transition from interphase to metaphase, measurements based on fluorescence intensity of membrane markers indicate a significant decrease in PMSA, though the observed change ranges from two- to sixfold depending on the cell type (Boucrot and Kirchhausen 2007). This loss of PM occurs simultaneously with a decrease in endosomal trafficking and/or exocytosis, which, consistently, would result in a net loss of plasma membrane lipids as endosomes accumulate in the cytoplasm (Berlin et al. 1978; Berlin and Oliver 1980; Warren et al. 1984; Schweitzer et al. 2005; Boucrot and Kirchhausen 2007). Just prior to cytokinesis, PMSA suddenly returns to its interphase value (Boucrot and Kirchhausen 2007). The increase in PMSA during late M-phase is likely the result of exocytosis of endosomes accumulated earlier in M-phase, as exocytosis has been observed to resume during this period, and Golgi-derived vesicles (which contain newly synthesized lipids) are not required for the PMSA increase (Warren et al. 1984; Schweitzer et al. 2005; Boucrot and Kirchhausen 2007). Following cytokinesis, the PM is split between the two daughter cells, which then re-spread as they enter the next round of interphase.

Cyclin Dependent Kinase (Cdk)-Cyclin complexes, the master regulators of cell cycle progression, have been implicated in the regulation of membrane trafficking throughout the cell cycle, though their exact role is still unclear. Cdk1 (the homolog of yeast *cdc2*, also known as $p34^{cdc2}$ in other systems), the mitotic Cdk that controls M-phase entry, blocks membrane fusion in vitro (Tuomikoski et al. 1989). This suggests that Cdk1 could be responsible for the observed decrease in exocytosis rates in early M-phase, when Cdk1 activity is highest. However, Cdk1 has also been shown to reduce endocytosis rates, as treatment with mitotic cell extract or purified cyclinB-$p34^{cdc2}$ decreases invagination of endocytic pits (Pypaert et al. 1991). Such a decrease in endocytosis rates would, in principle, counteract the effects of the observed decrease in exocytosis. These apparently conflicting data indicate that regulation of membrane trafficking by Cdk1 must be precisely tuned.

In addition to global PMSA regulation, local deposition of new membrane at the cleavage furrow is required in many systems for proper cytokinesis. This coincides temporally with the observed increase in PMSA just prior to cytokinesis (Boucrot and Kirchhausen 2007). In the final stages of cytokinesis, membrane vesicles endocytosed from the poles and Golgi-derived vesicles are inserted into the PM at the midbody and contribute to final abscission (Schweitzer et al. 2005; Goss and Toomre 2008). In early divisions of *Xenopus* embryos, new membrane is inserted specifically in the cleavage furrow throughout cytokinesis, and likely fulfills a requirement for a local increase in PMSA in these very large cells (Bluemink and de Laat 1973). Membrane insertion has also been implicated in cytokinesis and cellularization in *Drosophila*, *Caenorhabditis elegans*, and echinoderms (Burgess et al. 1997; Skop et al. 2001; Shuster and Burgess 2002). Following cytokinesis in some cultured cells, PMSA continues to increase during re-spreading. This continued growth in PMSA may directly result from increased membrane tension during spreading, which can both block endocytosis and stimulate exocytosis (Raucher and Sheetz 1999; Gauthier et al. 2009).

Kinematic approaches in cell mechanics, in which cell movements and changes in surface area and volume are precisely quantified, can provide clues about the forces responsible for such changes. For example, during cell rounding, the fact that cells become more spherical (i.e., their surface area-to-volume ratio decreases) suggests that there could be an increase in cortical tension during rounding (see also Sect. 3.2.2). Such changes in cell shape observed throughout the cell cycle can be further studied using a dynamics-style approach, where the forces required to produce certain cell shapes and the proteins responsible for generating these forces are considered.

3.4 Shape Changes During M-Phase: Cell Rounding and Cytokinesis

Cells undergo two major shape changes during M-phase, cell rounding, which occurs at M-phase entry, and cytokinesis, the final step of mitosis. Cortical tension and cell–substrate or cell–cell adhesion both play major roles in determining cell

shape during M-phase. In cultured cells, for example, cell rounding involves an increase in tension and a decrease in cell adhesion that causes a transformation from a spread morphology to a sphere (see also Sect. 3.3, Fig. 3.2). In cytokinesis, a spatial gradient of active tension effects a shape change that results in the physical division of one cell into two. These physical properties are controlled directly by the actomyosin cytoskeleton and adhesion proteins.

3.4.1 Cell Rounding

Entry into M-phase is characterized morphologically by cell rounding, which results from major changes in a cell's physical properties between interphase and M-phase. For example, the elastic modulus of the cell surface increases approximately threefold between interphase and M-phase (Matzke et al. 2001; Maddox and Burridge 2003; Kunda et al. 2008). Rounding is observed both in cultured cells and in vivo. However, most mechanistic studies of cell rounding have been performed using cultured cells. Therefore, this section will deal only with rounding in cultured cells, though most of the described shape changes and underlying mechanics are likely to be similar during mitotic rounding in tissues. For most cultured cell lines, interphase cells have a spread morphology and stable cell surface adhesions. The actin cytoskeleton is organized into flat, ruffled actin-filled protrusions at the periphery and stable, contractile actin bundles, called stress fibers, in the cell body (Cramer and Mitchison 1997). In addition, interphase cells have an actin cortex on their "top" side that supports the plasma membrane (Morone et al. 2006; Estecha et al. 2009), though this structure does not seem to be as well-developed as in M-phase. In sharp contrast, cells in M-phase have a nearly spherical shape with a uniform, well-developed actin cortex at their periphery as well as retraction fibers, thin, actin-filled tethers that connect the cells to the underlying substrate (Bradley et al. 1980; Cramer and Mitchison 1997).

3.4.1.1 Surface Tension and Adhesion: Opposing Forces During Cell Rounding

Cell rounding is thought to result from two simultaneous processes: (1) disassembly of cell surface adhesions and (2) reorganization of the actin cytoskeleton into an active, stress-generating cortex (Bradley et al. 1980; Cramer and Mitchison 1997). While cell surface adhesions cause the cell to increase its contact area with the substrate, and hence promote a flattened morphology, increased surface tension will cause the cell to become more spherical (Lecuit and Lenne 2007). A number of observations suggest that both a reduction in cell–substrate adhesion and an increase in tension contribute to cell rounding.

During rounding in cultured cells, stable focal adhesions are disassembled (Yamakita et al. 1999), which suggests that cell–substrate adhesion is important for retaining a flat morphology. Indeed, cells can be forced to round up by treatments that cause disassembly of focal adhesions. For example, when flattened cells are treated with proteases like trypsin, which disrupts cell–substrate adhesions, they round up (Britch and Allen 1980). Interphase cells can also be forced to round up by treatment with functional antibodies against integrin β1, a protein that links actin filaments to focal adhesion proteins and whose phosphorylation is important for mitotic rounding (Suzuki and Takahashi 2003). In the case of M-phase rounding, not all focal adhesions are disassembled, as evidenced by the presence of retraction fibers, which stay attached to the substrate (Mitchison 1992). Also, the forces generated by cell rounding are higher for M-phase rounding than for trypsin-mediated rounding (Stewart et al. 2011), indicating that focal adhesion disassembly is not the only mechanics change during M-phase rounding.

In addition to disassembly of focal adhesions, the presence of a contractile actin cortex is required for cell rounding in mitosis. Addition of the actin depolymerizing drug Cytochalasin D, which causes disassembly of the cortex, inhibits mitotic cell rounding (Cramer and Mitchison 1997). Myosin-2 activity has been shown to be required for cell rounding during M-phase, both in human and *Drosophila* cultured cells, and is thought to generate the cortical contractility required for cell rounding (Maddox and Burridge 2003; Kunda et al. 2008). The presence of a well developed, contractile actomyosin cortex is likely the reason for the observed increase in elastic modulus from interphase to mitosis (Matzke et al. 2001), and is directly responsible for higher forces during mitotic rounding compared to trypsin-mediated rounding (Stewart et al. 2011).

3.4.1.2 Moesin in Cell Rounding

In addition to myosins, another family of actin-binding proteins, the Ezrin–Radixin–Moesin (ERM) family, which link the cortex to the plasma membrane (see Box 3.1), has also been implicated in cell rounding. Moesin, the single ERM family member present in *Drosophila*, is required for cell rounding in *Drosophila* S2 cells (Carreno et al. 2008; Kunda et al. 2008). Moesin, which is activated by the mitotic kinase Slik, is five times more active in M-phase (Carreno et al. 2008). Strikingly, when myosin-2 is knocked down, overexpression of a phosphomimetic version of moesin is sufficient to induce rounding in S2 cells. In these myosin-2 knockdown cells, the activation of moesin during M-phase may contribute to cell retraction by causing actin filaments to preferentially align parallel to the plasma membrane, which could increase cortex tension (Carreno et al. 2008; Kunda et al. 2008). Moesin may also play a signaling role during this process, as ERM proteins can activate RhoA (Bretscher et al. 2002), an upstream activator of actin polymerization and actomyosin contractility (Kimura et al. 1996; Jaffe and Hall 2005). Indeed, RhoA is required for proper cell rounding (Maddox and Burridge 2003), and moesin knockdown causes mislocalization of

RhoA during M-phase in *Drosophila* cultured cells (Carreno et al. 2008). These data suggest that at least part of moesin's role in mitotic rounding is mediated by RhoA signaling.

3.4.2 Cytokinesis

As the initially spherical metaphase cell enters anaphase, it elongates and progressively forms a cleavage furrow at the cell equator. The cleavage furrow then ingresses until only a narrow bridge, the midbody, remains between the two daughter cells. Finally, sometimes hours after the completion of cytokinesis, midbody abscission occurs, leading to complete separation of the daughter cells (Rappaport 1996).

The mechanics of cytokinesis have been the subject of investigation for more than 100 years (Flemming 1895), and a multitude of physical models of cytokinesis have been proposed (reviewed in Rappaport 1996). In most metazoan cells, cytokinesis is the result of myosin-driven contraction (Glotzer 2001). In metaphase, the actomyosin cortex is uniformly distributed under the plasma membrane. Upon anaphase entry, actin and myosin progressively accumulate at the cell equator by a combination of local assembly of new filaments and cortical flows of existing filaments from the poles towards the equator (Cao and Wang 1990a, b; Fishkind et al. 1991; DeBiasio et al. 1996). This results in a higher actomyosin tension at the equator, which leads to furrow ingression (White and Borisy 1983; Matzke et al. 2001). Although most cells require myosin-2 for cytokinesis, adherent *Dictyostelium* cells are able to divide in the absence of myosin-2 (Neujahr et al. 1997). Daughter cell separation in these cells appears to be achieved without the formation of a contractile ring. Instead, the two daughters form lamellapodia and seem to tear themselves apart by crawling away from one another (Nagasaki et al. 2009; King et al. 2010).

The position of the cleavage furrow is crucial for accurate segregation of genetic material between the two daughter cells, and thus, furrow positioning must be precisely coupled to chromosomes separation (Rappaport 1996). The mitotic spindle, which segregates the chromosomes, also directly controls the formation of spatial gradients in cortical contractility and directs furrow formation (Rappaport 1961; Werner and Glotzer 2008). There are two major theories regarding how the spindle achieves this (1) astral spindle microtubules locally reduce contractility at the poles of the cell or (2) the spindle midzone and/or equatorial microtubules increase contractility at the equator (reviewed in Rappaport 1996; Glotzer 2004; Burgess and Chang 2005). These mechanisms are not mutually exclusive, and both appear to be used to various extents in different cell types (Wang 2001; Bringmann and Hyman 2005).

Early studies of force generation during cleavage furrow ingression often modeled the contractile ring as a sarcomere-like structure where parallel bundles of

actin filaments are contracted by myosin motors in a muscle-like manner (Rappaport 1996; Pollard 2010). Although in some species, such as fission yeast, the equatorial ring appears to indeed be formed by parallel bundles of filaments (Kamasaki et al. 2007; Pollard and Wu 2010), in animal cells, the ring is rather an accumulation of actomyosin cortex with varying levels of alignment of the actin filaments (Wang and Taylor 1979; Maupin and Pollard 1986; Mabuchi et al. 1988; Schroeder 1990; Fishkind and Wang 1993). Moreover, the cortex itself is far from being a stable structure, as actin filaments in the cortex constantly turn over; it has both been shown experimentally and predicted theoretically that this dynamic behavior is essential for proper ingression of the cleavage furrow (Mukhina et al. 2007; Zumdieck et al. 2007; Salbreux et al. 2009; Pollard 2010).

Finally, although mechanical studies of cytokinesis have mostly focused on the cleavage furrow, an actomyosin cortex remains present at the poles of the dividing cell throughout cytokinesis. Passive mechanical resistance of this polar cortex to deformation must be taken into account in mechanical models of cell division (Robinson and Spudich 2004; Reichl et al. 2005). Moreover, active contractile forces exerted by the polar cortex can lead to asymmetric division. During asymmetric division of *Drosophila* neuroblasts, for example, myosin accumulates at the cortex of the smaller of the two future daughter cells (Barros et al. 2003; Cabernard et al. 2010). A similarly polarized distribution of cortical myosin has been shown to be essential for asymmetric division in some neuroblasts of the *C. elegans* Q neuroblast lineage, resulting in the generation of two daughter cells of different sizes (Ou et al. 2010). In symmetric divisions, polar cortex contractility can perturb cytokinetic mechanics and lead to cell shape instabilities where unbalanced polar contractions propel cytoplasmic material between the two poles, destabilizing the position of the cleavage furrow (Sedzinski et al. 2011).

3.5 Mechanical Changes During Interphase

Most studies of cell mechanics focus on mitosis, likely because shape changes are most dramatic during this phase of the cell cycle. However, there is evidence that cells have different mechanical properties at different stages of interphase. For example, global cell viscosity has been shown to increase approximately 1.5-fold from G_1 to S-phase, and though viscosity is primarily governed by the actin cytoskeleton, microtubules are responsible for this G_1 to S-phase viscosity increase (Tsai et al. 1996). There are only few direct mechanical studies of physical changes during interphase. However, several indirect examples indicate that such changes occur. Cell cycle-dependent differences in cell migration and cell cycle-specific morphogenesis in neural stem cells are both indirect indications that cell mechanics differ between the different stages of interphase.

3.5.1 Differences in Cell Migration During Interphase

In a number of different cell lines, cell migration dynamics vary throughout interphase. In general, migration is enhanced during G_1 and early S-phase compared to late S-phase and G_2 (Ratner et al. 1988; Iwasaki et al. 1995; Walmod et al. 2004). However, the reasons for reduced migration efficiency vary across cell lines; for some cell types, reduced migration is a result of lower migration speed, while in others, it is a result of decreased migratory persistence (Walmod et al. 2004).

Variations in the strength of cell–substrate adhesion could cause cell cycle-dependent differences in cell migration. Indeed, various cultured mammalian cell lines have different optimal cell–substrate adhesiveness that maximizes migration speed (Palecek et al. 1997), and the observed increase in adhesion force as cells progress from G_1 to S and then to G_2/M-phase coincides with a gradual reduction in migration speed (Giet et al. 2002). These examples imply that cell cycle-mediated changes in adhesion are likely to play an important role in the observed differences in cell migration. In addition to variations in cell–substrate adhesion, it is likely that changes in other mechanical properties, such as contractility, also contribute to differences in cell migration at different stages of interphase. Indeed, a theoretical model combining effects of cell–substrate adhesion and contractility suggests that the interplay of these two properties determine cell migration speed (DiMilla et al. 1991).

3.5.2 Interkinetic Nuclear Migration and Cell Cycle-Specific Morphogenesis in Neural Stem Cells

Another indirect example of changes in mechanical properties of cells during interphase is interkinetic nuclear migration in developing neuroepithelia of vertebrates. In these tissues, neurons have an elongate shape with a single bulge formed by the nucleus and are attached to the basal and apical membranes at the extremities of the tissue. For most neural stem cells, M-phase onset is characterized by a rapid movement of the nucleus toward the apical surface (Gotz and Huttner 2005; Norden et al. 2009). Following division, nuclei migrate away from the apical surface and back toward the basal lamina. Although these movements have been observed in a number of different vertebrate systems, the underlying mechanisms are unclear; actomyosin contractility, microtubule/dynein-based transport, and passive displacement have all been implicated to varying degrees (Schenk et al. 2009; Norden et al. 2009; Tsai et al. 2010). Most neurons within the neuroepithelium indeed undergo mitosis at the apical surface, though a subset of these neurons, the basal progenitors, division occurs more basally, and this eventually gives rise to a subventricular zone rich in basal progenitors (Haubensak et al. 2004). During G_1 phase, basal progenitors retract their apical processes and transform from an elongate shape, spanning the entire ventricular zone and neuronal layer, to a short and wide morphology residing in the basal or subventricular zone (Miyata et al.

2004). The nucleus therefore cannot translocate to the apical surface during mitosis, and as a result, these cells divide on the basal side of the tissue. This shape change may be the result of a decrease in adhesion and/or an increase in cortical tension. Notably, in this example, this interphase-specific change may be responsible for part of the overall structure of the vertebrate nervous system.

3.6 Cell Cycle-Related Shape Changes in Tissues

Although much of the work related to cell mechanics during the cell cycle has been done in culture and in embryos up to blastula stage, cell shape changes are also an essential part of tissue morphogenesis. Changes in the mechanical properties of the individual cells that make up a tissue affect the general organization of the tissue, as in the example of interkinetic nuclear migration in brain development (Sect. 3.5.2). Because cells within a tissue are connected by cell–cell adhesions, when a single cell in a tissue rounds up or undergoes cytokinesis, for example, it will push and pull on neighboring cells. This coupling of single cell mechanics and cell–cell adhesion gives rise to tissue-scale morphogenesis and has been directly related to cell cycle progression in the cases of mitotic cell rounding and division plane orientation.

3.6.1 Cell Rounding in Tissues

Cell rounding at mitosis entry has been observed in cultured cells as well as in vivo, both during early embryonic development and in early epithelia. Cells in early amphibian embryos, for example, undergo periodic changes from being disk-shaped to being spherical, and the timing of these periodic changes corresponds with the timing of the cell cycle (Selman and Waddington 1955; Hara et al. 1980). The extent of cell shape change is not as great in vivo as in culture, as cells in tissues are never as flat as cells in culture, which is largely a consequence of extensive cell–cell contacts of cells within tissues. In tissues, cells do not undergo a disassembly of cell–cell contacts at mitosis, at least in the case of confluent epithelial cells. This includes the retention of desmosomes, tight junctions, and intermediate junctions (E-cadherin-mediated junctions; Baker and Garrod 1993). Indeed, maintenance of cell– cell adhesions is vital for preservation of a tissue, as a loss of such adhesions (especially if the cell cycle were synchronized between cells, as in the case of early development) would cause the tissue to simply fall apart. This suggests that rounding in tissues may be more dependent on the contraction of a well-developed actomyosin cortex rather than on disassembly of adhesions. In contrast to cell–cell adhesions, cell–ECM adhesions, specifically those mediated by integrins, may be partially disassembled in a cell cycle-dependent manner (B Baum, personal communication). An additional consequence of extensive cell–cell contacts is that cells

in tissues stay much more tightly packed than cells in culture, and as a result, retraction fibers (see Sect. 3.4) are typically not observed.

3.6.2 Division Plane Orientation

Orientation of the division plane can play a significant role in determining the shape of a tissue or epithelium. A coordinated bias of division plane orientation can have substantial effects on the shape of a tissue, regardless of whether or not total cell volume increases (Fig. 3.4). Division axis orientation can be determined by a number of mechanisms, including anisotropies in cell shape or stresses upon cells, or by polarity proteins.

3.6.2.1 Effects of Division Plane Orientation on Tissue Organization

Oriented cell divisions are known to play a role in early embryonic development, specifically during gastrulation. Polarized divisions have been implicated as early as during formation of the primitive streak, an elongated group of cells that specifies the site of gastrulation in higher amniotes (Wei and Mikawa 2000). Although treatment with cell division inhibitors does not disrupt initial development of the primitive streak, such treatment does inhibit extension of the streak once it is formed, though it is not entirely clear if oriented divisions or simply cell divisions in general are required for this process (Cui et al. 2005). The preferential orientation of cell divisions during gastrulation has been observed in a number of different organisms (Hertzler and Clark 1992; Concha and Adams 1998; Gong et al. 2004; Wang et al. 2008). In zebrafish gastrulae, for example, oriented cell division has

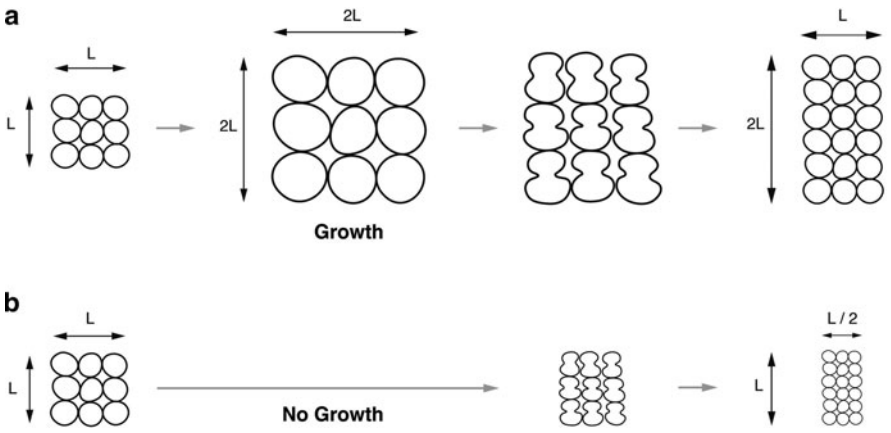


Fig. 3.4 Effect of a coordinated bias in division plane orientation on the shape of a tissue. A coordinated bias towards one axis in division plane orientation leads to tissue elongation both in the presence (panel **a**) and in the absence (panel **b**) of tissue growth

been shown to contribute significantly to the required tissue extension (Gong et al. 2004). Despite this, it has been shown that some organisms, including sea urchins, do not require cell divisions for proper gastrulation (Stephens et al. 1986). The difference in the requirement of oriented cell division in sea urchins and other organisms may be due to the vast difference in the geometries of gastrulation between model systems.

Biased division plane orientation is also important later in development, during tissue morphogenesis. Division plane bias during neural development, for example, has been proposed to contribute to the elongated geometry of the early neural tissue in a number of different model systems (Schoenwolf and Alvarez 1989; Sausedo et al. 1997; Geldmacher-Voss et al. 2003). It has also been proposed that oriented cell divisions promote cell intercalation, which in turn drive further oriented cell divisions based on the resultant cell shapes and pulling forces. This leads to a positive feedback between these two morphogenetic processes that allows for formation of the characteristically long, thin neural tissue (Kimmel et al. 1994). A physical model of this type of positive feedback suggests that coupling of oriented cell divisions and cell intercalation movements is sufficient to drive anisotropic tissue growth in development of the *Drosophila* imaginal wing disk (Bittig et al. 2009), which agrees with experimental evidence showing that oriented cell divisions are important in determining the final morphologies of organs developing from imaginal disks (Baena-López et al. 2005). Division plane orientation is also important in model tissues in culture. For example, Madin–Darby canine kidney cells are capable of forming lumens in culture, and this process has been shown to depend on oriented cell divisions (Qin et al. 2010; Rodriguez-Fraticelli et al. 2010).

3.6.2.2 Mechanisms of Division Plane Orientation

Division plane orientation can be regulated through a number of physical and molecular mechanisms. From a geometrical standpoint, cells tend to divide in a plane perpendicular to their long axis. This is the result of orientation of the mitotic spindle parallel to the long axis of the cell during metaphase. Such geometry-dependent spindle orientation can occur even when the length of the long and short axes differs only by a few percent and has been observed in a number of systems, including cultured mammalian cells, mouse zygotes, *Xenopus* embryos, fission yeast, and plant cells (Rappaport 1960; O'Connell and Wang 2000; Smith 2001; Gray et al. 2004; Strauss et al. 2006; Vogel et al. 2007). In addition, it has been shown in several of these systems that if the long axis is changed, the mitotic spindle will reorient such that it aligns parallel to the new long axis, and thus the division plane will be perpendicular to it (O'Connell and Wang 2000; Gray et al. 2004). Despite the prevalence of this phenomenon, the mechanism by which it occurs is poorly understood.

Geometry-dependent division plane orientation has been shown in some cases to be overridden by internal signaling cues, as in the first cleavage of *C. elegans* embryos. Compression of *C. elegans* zygotes to change the long axis does not

change the position of the division plane. In these cells, spindle plane orientation is rather governed by activity of the Par family proteins, which are important for polarity in a number of biological contexts including asymmetric cell division (Cheng et al. 1995). It should be noted, however, that geometry dependence of spindle plane orientation is "restored" in embryos with compromised Par function (Tsou et al. 2003).

Spindle plane orientation, and therefore division plane orientation, is dependent on an intact actin cortex (Gray et al. 2004; Théry et al. 2007; Carreno et al. 2008; Kaji et al. 2008; Kunda et al. 2008) as well as on astral spindle microtubules and dynein (O'Connell and Wang 2000; Théry et al. 2005). In cultured cells, mitotic spindle orientation can be directed by anisotropic stresses. These stresses can either be caused by pulling from unequally distributed retraction fibers or can be applied externally (Fink et al. 2011). Anisotropic stresses promote polarized reinforcement of the cortex, which is thought to recruit dynein, and this in turn pulls the spindle poles toward this region (Fink et al. 2011; Théry and Bornens 2006). This mechanism ensures that cells preferentially divide in a plane perpendicular to the force field (Fink et al. 2011). In cultured *Drosophila* cells, spindle orientation also requires moesin activity (Carreno et al. 2008); in these cells, activated Moesin accumulates at the bases of retraction fibers (Kunda et al. 2008), and may play some role in local cortex reinforcement or dynein recruitment. It is possible that such mechanisms may also lead to biased division plane orientation in tissues, though division plane orientation has previously been shown in tissues to be primarily controlled by polarity signaling pathways, namely by the Planar Cell Polarity (PCP) pathway and the Par polarity proteins (Geldmacher-Voss et al. 2003; Gong et al. 2004; Siegrist and Doe 2006). In addition, oriented divisions in tissues are dependent on adherens junctions (Lu et al. 2001; Geldmacher-Voss et al. 2003), though it is not clear whether junctions play a signaling or mechanical role in this process.

Finally, in addition to cortical cues, intracellular actin networks can also contribute to spindle positioning. In mouse oocytes, spindle repositioning to the cortex, which precedes polar body extrusion, depends on a cytoplasmic actin network nucleated by Formin 2 (Azoury et al. 2008; Schuh and Ellenberg 2008). Myosin 2 is enriched at the spindle poles and repositions the spindle to the cortex by physically pulling on the intracellular actin network (Schuh and Ellenberg 2008). Dynamic, Arp2/3 nucleated cytoplasmic actin structures have also been observed during symmetric divisions of cultured cells (Mitsushima et al. 2010) where they may also influence the orientation of the spindle (Fink et al. 2011).

3.7 Linking Cell Cycle Biochemistry and Cell Mechanics

In order to understand how cells control their shape during the cell cycle, one must understand not only which mechanical properties drive specific shape changes, but also how the proteins regulating these properties are controlled. Like other cell

cycle-regulated processes, mechanical changes are controlled by Cdk–Cyclin complexes. These complexes, and in particular the mitotic Cdk–Cyclin complex CyclinB-Cdk1, were first postulated to affect cell mechanics based on their influence on cell shape during surface contraction waves in amphibian embryos and during mitotic cell rounding. Although it is not clear exactly how it affects cell mechanics, CyclinB-Cdk1 signals via a number of downstream pathways to eventually affect the actomyosin cytoskeleton and cell adhesions. These signaling pathways, most of which involve other protein kinases, modulate the regulation of actin-binding proteins and adhesion-associated proteins; such proteins can in turn affect properties like actin dynamics, myosin activity or turnover of adhesions, which govern physical properties like tension or cell–substrate or cell–cell adhesion. As it is clear that CyclinB–Cdk1 does not act on the actin cytoskeleton or adhesions via a single pathway, it is likely that the combined effects of many pathways allow Cdk–Cyclin complexes to precisely control cell shape during the cell cycle.

3.7.1 CyclinB–Cdk1 and Surface Contraction Waves

One of the first known examples where Cdk–Cyclin complexes were shown to influence cell mechanics is in surface contraction waves in early amphibian embryos. During early development, amphibian embryos undergo a series of surface contractions that are regulated cyclically by CyclinB-Cdk1. Before each cleavage in early axolotl, newt, and *Xenopus* embryos, a wave of surface relaxation, followed closely by a wave of surface contraction, moves from the animal pole to the vegetal pole (Hara 1971; Sawai and Yoneda 1974; Hara et al. 1980). Such surface contraction waves can be observed simply by tracking pigment displacements at the surface of these embryos. These waves are driven by transient waves of CyclinB-Cdk1 activity (Rankin and Kirschner 1997; Perez-Mongiovi et al. 1998) and still occur in cells lacking nuclei or that do not actually cleave (Sawai 1979; Hara et al. 1980). Although it is clear that the changes in surface properties associated with *Xenopus* surface contraction waves are organized by CyclinB-Cdk1 signaling, the mechanism of propagation of these activity waves and how exactly these biochemical signals affect cortical contractility is not understood.

3.7.2 CyclinB-Cdk1 in Cell Rounding

The CyclinB-Cdk1 complex also controls cell rounding in cultured cells. In addition to a high temporal correlation between CyclinB-Cdk1 activation and mitotic rounding (Gavet and Pines 2010), perturbation of CyclinB-Cdk1 activity has been shown to affect rounding. Injection of purified CyclinB-Cdk1 causes disassembly of stress fibers and ectopic cell rounding in interphase mouse fibroblasts, similar to

the changes seen during mitotic rounding (Lamb et al. 1990). Consistently, injection of functional antibodies against Cdk1 prevents entry into mitosis and results in a failure to round up, but does not disrupt maintenance of a round morphology in mitosis (Riabowol et al. 1989). Though it is clear from these data that CyclinB-Cdk1 affects cell rounding (and thus the underlying cell mechanics), it is not understood whether these effects are direct, or whether they are mediated by intermediate pathways.

3.7.3 The LIMK1-Cofilin Pathway in Cell Rounding

One biochemical pathway that has been shown to directly affect cell rounding is the LIM Domain Kinase 1 (LIMK1)-Cofilin pathway. Though no direct relationship between CyclinB-Cdk1 and this pathway has yet been documented, LIMK1 is a cell cycle-regulated kinase that affects M-phase cell mechanics by deactivating the actin severing protein cofilin. Cofilin regulates actin dynamics through its ability to sever actin filaments and is inactive when phosphorylated (Moon and Drubin 1995; Bamburg et al. 1999; Pollard and Borisy 2003; Hotulainen et al. 2005; Pfaendtner et al. 2010). As cofilin knockdown is sufficient to cause a twofold increase in surface tension in cultured cells (Tinevez et al. 2009), its inactivation during mitosis is likely to contribute to increasing surface tension. LIMK1 can induce stabilization of actin filaments in a cofilin phosphorylation-dependent manner (Arber et al. 1998; Yang et al. 1998), and this actin stabilizing effect is likely the cause of increased surface tension, presumably through an increase in cortex thickness (see Box 3.1 for the relationship between cortex thickness and tension). Cofilin can also affect cell rounding and morphology. Cells expressing nonphosphorylatable cofilin are still able to round up, but often have morphological irregularities (Kaji et al. 2008). In addition, the actin interacting protein 1 (AIP1), which decreases cofilin's ability to sever actin filaments, is required for mitotic cell rounding (Fujibuchi et al. 2005).

3.7.4 Other Pathways Involved in Cell Rounding

A number of other biochemical pathways have been implicated in control of cell rounding, though none has been as firmly linked to mitotic rounding as the CyclinB-Cdk1 and LIMK1-Cofilin pathways. Nonetheless, these other pathways display high activity during M-phase and are known to affect properties such as actomyosin contractility and substrate adhesion, both of which are important for cell rounding. In addition, several of these pathways have links to the CyclinB-Cdk1 pathway, which indicate that CyclinB-Cdk1 may regulate a number of separate pathways during M-phase to effect changes in cell mechanics and thereby promote mitotic cell rounding.

3.7.4.1 The PAK Pathway

The p21-activated kinases 1 and 2 (PAK-1, PAK-2) are required for proper mitosis and are regulated in a cell cycle-dependent fashion. These kinases affect cell mechanics via their downstream effects on actin and myosin. PAK-1 autophosphorylation, which has been shown in vitro to indicate PAK-1 activity, is highest during M-phase, and PAK-1 depletion delays mitotic entry (Maroto et al. 2008). In addition, PAKs promote lamellipodia retraction, focal adhesion disassembly and increased contractility, and this suggests that they may be involved in the mechanical changes leading to cell rounding (Kiosses et al. 1999; Zeng et al. 2000; Szczepanowska et al. 2006). More specifically, PAKs-1 and -2 have been shown in a number of cell types to phosphorylate Myosin Regulatory Light Chain (MRLC) on S19, which increases myosin-2 motor activity (Chew et al. 1998; Sells et al. 1999; Zeng et al. 2000; Szczepanowska et al. 2006; Coniglio et al. 2008). PAKs are also involved in myosin-2 regulation through phosphorylation of caldesmon (Foster et al. 2000; Eppinga et al. 2006; Van Eyk et al. 1998). Dephoshporylated caldesmon inhibits myosin-2 activity, and its phosphorylation relieves this inhibition (Yamashiro and Matsumura 1991; Foster et al. 2000). Therefore, mitosis-specific phosphorylation of caldesmon by PAKs is likely to increase cortical tension during rounding by promoting myosin-2 activity. Consistent with this, if caldesmon phosphorylation is blocked by expression of a nonphoshporylatable dominant negative, cell rounding and entry into mitosis are delayed, and cells fail to sufficiently disassemble stress fibers (Yamashiro et al. 2001). High PAK activity during M-phase, therefore, is likely to promote an increase in actomyosin contractility, via its effect on myosin II. In addition to its regulation by PAKs, caldesmon can also be phosphorylated directly by CyclinB-Cdk1 (Yamashiro et al. 1990).

PAK1 also interacts with the actin cross-linking protein filamin (Vadlamudi et al. 2002). Filamin-deficient cells in interphase display lower migration speeds, increased cell blebbing and a twofold increase in global cellular elastic modulus (Cunningham et al. 1992). Filamin and PAK have a coordinated activation, wherein PAK1 phosphorylates filamin, and phosphorylated filamin can in turn bind PAK1, thereby allowing for autophosphorylation and activation of PAK1 (Vadlamudi et al. 2002). The filamin branch of the PAK pathway is thus a secondary mechanism by which high PAK activity during M-phase can affect actomyosin activity and dynamics to control cell rounding.

3.7.4.2 Nonreceptor Tyrosine Kinases

The Src family of nonreceptor tyrosine kinases has been implicated in cell rounding, both by promoting disassembly of adhesions and by modification of the actin cytoskeleton. Injection of mouse fibroblasts with functional antibodies against the three Src kinases, c-Src, Fyn, and Yes, results in arrest prior to mitosis without rounding and without nuclear envelope breakdown (Roche

et al. 1995). The Src family member pp60$^{c\text{-}Src}$ is highly phosphorylated during M-phase and its activity increases up to sevenfold from interphase to mitosis (Chackalaparampil and Shalloway 1988). pp60$^{c\text{-}Src}$ has been implicated in both disassembly of cell adhesions and reorganization of actin (Henderson and Rohrschneider 1987; Warren and Nelson 1987). In mouse oocytes, Fyn kinase is required for maturation and polar body extrusion; in this system, Fyn is enriched at the cortex near the meiotic spindle and is involved in signaling for contractile ring formation (Levi et al. 2010). Additionally, the Transmembrane and Associated with Src kinases (Trask) protein can induce cell rounding when overexpressed (Bhatt et al. 2005). Trask is a cell adhesion protein that, like Src kinases (David-Pfeuty and Nouvian-Dooghe 1990), undergoes a dramatic membrane-to-cytoplasm redistribution upon entry into mitosis (Bhatt et al. 2005), indicating again that Src kinases may also be involved in disassembly of adhesions during cell rounding.

3.7.4.3 pEG3/Kin1/PAR-1/MARK Kinase Family

Another kinase family that is implicated in M-phase specific shape changes is the pEG3/KIN1/PAR-1/MARK family (*Xenopus*/*S. pombe*/*Drosophila*, *C. elegans*/mammalian homologs, respectively). Members of this family localize to the actomyosin cortex specifically during M-phase in an actin-dependent fashion (Chartrain et al. 2006) and are most highly phosphorylated (and therefore most active) during mitosis (Blot et al. 2002). Disruption of these proteins causes aberrant morphologies in cultured mammalian cells by interfering with microtubule dynamics (Drewes et al. 1997); it is not clear if this change in microtubule dynamics directly affects cell shape or plays a signaling role. This family of proteins is therefore potentially important in regulating M-phase-specific mechanical changes given its high mitotic activity and effects on cell morphology in interphase.

3.7.4.4 Rap1 GTPase

Rap1 is a small GTPase that has been shown in a number of systems to promote integrin- and cadherin-mediated adhesion via a number of downstream effectors (Bos 2005). Rap1 activity is significantly reduced during M-phase, correlating temporally with a loss of cell–substrate adhesion, and expression of constitutively active Rap1 prevents focal adhesion and stress fiber disassembly at mitosis onset, leading to defects in cell rounding. Furthermore, cells expressing dominant negative Rap1 round up ectopically and fail to re-spread following division (Dao et al. 2009). Although the Rap1 inactivating protein Rap1GAP can be phosphorylated by Cdk1, both in vitro and in cultured cells (Rubinfeld et al. 1992; Janoueix-Lerosey et al. 1994), it is not clear whether this phosphorylation contributes to Rap1 inactivation during M-phase.

3.7.4.5 The TCTP Chaperone

In addition to protein kinases, translationally controlled tumor-associated protein (TCTP) has also been implicated in control of cell rounding. Knockdown of TCTP, which is important in calcium regulation (Arcuri et al. 2005) and is a potential chaperone protein (Thaw et al. 2001), causes rounding defects. During M-phase, knockdown cells often retain long protrusions, indicative of a failure to sufficiently de-adhere from the substrate (Bazile et al. 2009). Surprisingly, TCTP knockdown actually decreases cell–substrate adhesion on several different substrates in other cell types (Ma et al. 2009). This well-conserved and highly expressed protein is known to associate with actin and microtubules and could be involved in local folding of proteins important for regulating the cytoskeleton or adhesions during mitosis, given its potential as a chaperone (Bazile et al. 2009).

3.7.5 The Role of Mechanosensing in Cell Cycle Regulation

In addition to modification of cell mechanics by cell cycle-mediated signaling, there are many indications that forces experienced by the cell may in turn affect cell cycle progression. For example, pharmacological treatments leading to increased cell spreading in cultured cells also cause an increase in DNA synthesis and cell proliferation (Folkman and Moscona 1978), and if cultured cells are subjected to physical stresses, their proliferation rate increases (Nelson et al. 2005). Furthermore, the Rho/ROCK/Diaphanous pathway, an upstream signaling pathway for actin assembly and contractility, is necessary for entry into S-phase (Seasholtz et al. 1999; Iwamoto et al. 2000; Zhao and Rivkees 2003), suggesting that contractility itself could potentially affect cell cycle progression.

Beyond its influence on cell cycle progression, mechanotransduction is also used to fine-tune cell shape changes. *Dictyostelium* cells, for example, demonstrate an M-phase-specific response to mechanical perturbation, wherein an externally induced deformation promotes local recruitment of myosin-2 and the actin-binding protein cortexillin I, which counteracts the deformation and helps to stabilize cell shape (Effler et al. 2006; Ren et al. 2009). Such mechanical feedback systems allow cells to more precisely control their shape and can also contribute to the maintenance of correct cell shape in the presence of external forces.

3.8 Conclusion

Progression through the cell cycle involves a number of shape changes that rely on the precise modification of a cell's physical properties. As these events are mechanical in nature, a firm theoretical understanding of the cell as a multicomponent complex

object is necessary to understand how key parameters such as tension, viscoelasticity, and cell adhesion give rise to the diverse shapes observed in different phases of the cell cycle. Experimentally, understanding cell cycle biomechanics can be approached using kinematics, wherein cell movements and deformations are precisely quantified, providing information on the forces leading to these movements, or using dynamics, where forces and physical properties are measured directly and then used to predict cell behavior. These forces and physical properties are mediated on the molecular level by a number of proteins, including actin, actin-binding proteins, and cell adhesion proteins, whose activity is controlled by Cdk cyclins and other upstream signaling pathways. The study of cell cycle-mediated shape change therefore requires a combination of general cell mechanics and the use of modern biophysical and cell biological methods. Such interdisciplinary approaches have helped cell cycle biomechanics to reemerge as a major field in cell and developmental biology.

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Chapter 4
The Spindle Assembly Checkpoint: Clock or Domino?

María de Medina-Redondo and Patrick Meraldi

Abstract In each cell division, the newly duplicated chromosomes must be evenly distributed between the sister cells. Errors in this process during meiosis or mitosis are equally fatal: improper segregation of the chromosome 21 during human meiosis leads to Down syndrome (Conley, Aneuploidy: etiology and mechanisms, pp 35–89, 1985), whereas in somatic cells, aneuploidy has been linked to carcinogenesis, by unbalancing the ratio of oncogenes and tumor suppressors (Holland and Cleveland, Nat Rev Mol Cell Biol 10(7):478–487, 2009; Yuen et al., Curr Opin Cell Biol 17(6):576–582, 2005). Eukaryotic cells have developed a mechanism, known as the spindle assembly checkpoint, to detect erroneous attachment of chromosomes to the mitotic/meiotic spindle and delay the cell cycle to give enough time to resolve these defects. Research in the last 20 years, has demonstrated that the spindle assembly checkpoint is not only a pure checkpoint pathway, but plays a constitutive role in every cell cycle. Here, we review our current knowledge of how the spindle assembly checkpoint is integrated into the cell cycle machinery, and discuss some of the questions that have to be addressed in the future.

4.1 Introduction

Cell cycle progression is organized in such a way, that the initiation of a particular event is dependent on the completion of an earlier event. The cell cycle has been therefore described as being regulated by clocks and dominoes, which constitute the cell-cycle control system (Hartwell and Weinert 1989; Murray and Kirschner 1989). The former ones act as timers, which provide a fixed amount of time for the completion of each event. The latter ones constitute checkpoints (also known as surveillance mechanisms) that sense whether some malfunction prevents the successful completion of the processes, and delay the progression to the next phase.

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Checkpoint systems consist of three types of units: a sensor detecting possible defects and triggering a signal, a signal-transduction cascade, and an effector that delays cell-cycle progression (Hartwell and Weinert 1989). Checkpoint mechanisms tend to sense negative intracellular signals that arrest the cell cycle, rather than counting all the positive signals, since the last arising positive signal in a sea of positive signals is harder to detect than the loss of the last negative signal. Under ideal conditions, prototypical checkpoints are not essential for normal cell-cycle progression.

An interesting example is the spindle assembly checkpoint (also known as spindle checkpoint or kinetochore attachment checkpoint), which ensures the fidelity of chromosome segregation during cell division in eukaryotes. To produce two genetically identical daughter cells, the replicated chromosomes have to be evenly segregated to each daughter cell. Accurate chromosome segregation depends on the proper attachment of chromosomes to spindle microtubules emanating from opposite spindle poles and their positioning in the middle of the spindle. Microtubule attachment is carried out by kinetochores, multiprotein complexes assembled on centromeric DNA that contain up to hundred different proteins (Cheeseman and Desai 2008; Musacchio and Salmon 2007; Santaguida and Musacchio 2009). The spindle assembly checkpoint senses the interactions between chromosomes and microtubules and delays anaphase onset until all chromosomes are attached in a bipolar manner. It is mediated by a set of evolutionarily conserved proteins that functions as a signal transduction system and includes Mad1, Mad2, Mad3/BubR1, Bub1, Bub3, and Mps1 (Hoyt et al. 1991; Li and Murray 1991; Weiss and Winey 1996).

The Bub and Mad proteins were first identified in the budding yeast *Saccharomyces cerevisiae* (Hoyt et al. 1991; Li and Murray 1991). The groups of Andrew Murray and Andrew Hoyt aimed to investigate the feedback control that prevents cells with incompletely assembled spindles from exiting mitosis. As by definition checkpoints are not necessary for cell cycle progression under normal conditions, screens were designed to find nonessential mutations, which override a mitotic arrest resulting from a perturbed spindle. Eight different mutants were described and named *bub* (for "budding uninhibited by benzimidazole") or *mad* (after "mitotic arrest defective"). Genetic analysis showed that they belonged to five different complementation groups that were termed *BUB1*, *BUB3*, *MAD1*, *MAD2*, and *MAD3*. In agreement with the checkpoint definition, budding yeast Bub and Mad genes are not essential (Hoyt et al. 1991; Li and Murray 1991). Here, we review the mechanisms governing the spindle assembly checkpoint, and explore to which extend it is a real "checkpoint". While we briefly discuss the molecular mechanisms controlling the spindle checkpoint (for more extensive reviews on this point we recommend Ciliberto and Shah 2009; Musacchio and Salmon 2007), our emphasis will lie on the general significance of this checkpoint, and the way it is integrated into the cell cycle machinery of embryonic and somatic cells of different model systems.

4.2 Brief Description of the Spindle Assembly Checkpoint

In a classical study of 1995, the group of Conly Rieder showed that the spindle assembly checkpoint is a kinetochore-microtubule attachment checkpoint, as it demonstrated that the presence of a single unattached kinetochore is sufficient to generate a "wait-anaphase" signal (Rieder et al. 1995). To segregate chromosomes sister kinetochores bind spindle microtubules (K-fibers) emanating from the two poles to ensure the proper distribution between the two daughter cells. Since kinetochore-microtubule attachment is a stochastic process, defective attachments can arise. These include attachments of both sister kinetochores to microtubules coming from the same pole (syntelic attachment), attachment of one sister kinetochore to microtubules from both spindles poles (merotelic attachment), lack of attachment to one sister kinetochore (monotelic attachment), and absence of kinetochore-microtubule attachments. With the exception of merotelic attachments, all these erroneous configurations activate the spindle checkpoint, either due to lack of microtubule occupancy at one or both sister kinetochores, or due to lack of tension between improperly attached sister kinetochores. It is still controversial whether lack of tension directly activates the checkpoint, or whether it leads to unattached kinetochores as a consequence of Aurora B activity, which detects and disrupts tension-free kinetochore-microtubule attachments (Nezi and Musacchio 2009). Merotelic attachments, in contrast, are corrected as cells progress through mitosis, including during chromosome segregation in anaphase (Cimini et al. 2001).

At the molecular level unattached kinetochores accumulate the conserved spindle assembly checkpoint proteins Mad1, Mad2, Mad3 (BubR1 in metazoans), Bub1, Bub3, and Mps1 (Chen et al. 1996; Hoffman et al. 2001). Although the proteins that act as landing pads for these proteins at kinetochores have been partially identified, the molecular mechanisms governing the specific accumulation of these proteins at kinetochores are still unclear. The Bub and Mad proteins and Mps1 function as a signaling cascade at kinetochores, with the protein kinases Mps1, Bub1, and BubR1 at the top of the cascade (Kops 2009; Musacchio and Salmon 2007). These proteins recruit through an unknown mechanism Mad1 to kinetochores. Mad1 then itself recruits Mad2, leading to the "activation" of the checkpoint cascade (Chen et al. 1998). Specifically, Mad1 forms a dimer with two Mad2 binding sites that can each bind one Mad2 dimer (De Antoni et al. 2005; Sironi et al. 2002). Interestingly, the two subunits within the Mad2 dimer are not equivalent, as each protein adopts a different conformation. One subunit binds tightly Mad1 in the so-called "closed" conformation, while the other subunit, in the so-called "open" conformation can bind to Cdc20, the target of the spindle checkpoint (De Antoni et al. 2005; Luo et al. 2000). This binding process releases open Mad2 from the Mad1:Mad2 complex and results in the closing of its conformation on Cdc20.

An active spindle assembly checkpoint delays the onset of anaphase by inhibiting the E3-ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C; Clute and Pines 1999). The APC/C controls anaphase onset by targeting Cyclin B

and securin for degradation by the 26S proteasome, resulting in the activation of separase, a protease that cleaves the cohesin complex between sister kinetochores (Peters 2006). The final consequence is the separation of sister chromatids and progression into anaphase. The spindle assembly checkpoint inhibits APC/C by targeting its activator Cdc20; however, the molecular mechanisms by which it inhibits APC/C^{Cdc20} are not fully elucidated. Originally it had been proposed that Mad2 alone acts as the effector of the spindle assembly checkpoint; however, its binding to Cdc20 is not sufficient to block APC/C activity at physiological concentrations (Fang 2002; Fang et al. 1998; Tang et al. 2001). A second model proposes that APC/C is inhibited by the Mitotic Checkpoint Complex (MCC), a complex that consists of Bub3, BubR1, Cdc20, and Mad2 (Fraschini et al. 2001; Sudakin et al. 2001). Biochemical experiments indicate that the inhibitory activity of the MCC is at least 2,000-fold greater that single recombinant Mad2. The model is further based on the idea that the signaling cascade at kinetochores catalyzes the formation of the MCC to inhibit APC/C. Interestingly the MCC is already present in the cytoplasm of G2 cells, before the onset of mitosis, in a state that is compatible with inhibition of the mitotic APC/C (Sudakin et al. 2001). This implies that cells already contain an APC/C-inhibitory activity at the onset of mitosis and that the activity of the spindle assembly checkpoint at kinetochores might be crucial to sustain the formation of MCC. Finally, a third attractive model has emerged recently, which proposes that the MCC is only a transient step in the activation of the spindle assembly checkpoint (Nilsson et al. 2008). In this model Mad2 bound to Cdc20 acts as a catalyst for the binding of Mad3/BubR1 to Cdc20 (Kulukian et al. 2009; Nilsson et al. 2008). Mad3/BubR1 not only acts a pseudosubstrate for Cdc20–APC/C, it can also target Cdc20 itself for ubiquitination by the APC/C, leading to the degradation of Cdc20 (Burton and Solomon 2007; Nilsson et al. 2008). In such a model APC/C activity is restrained by the degradation of its Cdc20 activator. Consistent with such a model, it was found that the mitotic progression of cells expressing a nondegradable Cdc20 could not be blocked by the spindle assembly checkpoint (Nilsson et al. 2008).

The increasing molecular complexity of the checkpoint signaling is also reflected at the level of its integration into cell cycle control. Indeed, experimental evidence in metazoans indicates that the checkpoint is much more tightly integrated into cell cycle progression than originally assumed. During the past 20 years we have learned many new aspects of this integration, but many questions remain unresolved.

4.3 A Checkpoint or Timing Mechanism?

The spindle assembly checkpoint was first described in budding yeast as a pure checkpoint that is nonessential for proper chromosome segregation or cell viability (Hoyt et al. 1991; Li and Murray 1991). However, when studied in other organisms it became rapidly clear that the spindle assembly checkpoint is essential in

mammalian systems. Knock-out of Mad2 in mice is embryonic lethal, and although it is possible to obtain Mad2$^{-/-}$ mouse embryo fibroblasts, this is only possible in cells that have at the same time lost the ability to induce p53-dependent apoptosis (Burds et al. 2005; Dobles et al. 2000). The decisive difference between murine and budding yeast cell division is that *S. cerevisiae* cells establish kinetochore-microtubule attachments immediately after centromere replication and undergo a closed mitosis with intranuclear microtubules (Westermann et al. 2007). In contrast, metazoans undergo an open mitosis, in which microtubules only reach kinetochores after nuclear envelope breakdown in prometaphase. The spindle checkpoint is therefore active in every single metazoan cell division, since at the onset of prometaphase cells will have only unattached kinetochores. In contrast, in budding yeast, it is estimated that the spindle assembly checkpoint is only active in about one division out of thousand, a rare event in case kinetochore attachments go wrong (Warren et al. 2002). This indicates that the spindle assembly checkpoint in metazoan cells is not a pure checkpoint mechanism, but rather an intrinsic part of the cell cycle machinery.

The view that the spindle assembly checkpoint is not just a checkpoint mechanism was further strengthened by the findings that microinjection of anti-Mad2 or anti-BubR1 antibodies in human cells, not only prevented a mitotic arrest in the presence of microtubule poison, such as nocodazole, but also led to a very premature anaphase onset when compared to mock-injected cells (Gorbsky et al. 1998). This indicated that the checkpoint proteins control the timing of cell division (Canman et al. 2002; Gorbsky et al. 1998; Shannon et al. 2002; Taylor and McKeon 1997). A more detailed study based on RNAi depletions of all the human Mad and Bub proteins revealed the existence of two different, separable phenotypes: while all the Mad and Bub proteins are necessary for delaying anaphase in the presence of unattached kinetochores, only Mad2 and BubR1 depletion also resulted in a significant acceleration of mitosis when compared to control-depleted cells (Meraldi et al. 2004). This suggested that Mad2 and BubR1, in addition to their checkpoint function, also act as mitotic "timers", while the other proteins, Bub1, Bub3, and Mad1, have a pure checkpoint role with regard to mitotic timing. Later studies further indicated that the remaining checkpoint kinase Mps1, participated as Mad2 and BubR1 in the timing mechanism (Tighe et al. 2008). Interestingly, the dual functions of Mad2 and BubR1 correlated with different protein pools, localized at kinetochores (checkpoint) or in the cytoplasm (timer), respectively (Meraldi et al. 2004).

Based on these results, a model was proposed in which APC/C activity is hold in check by a series of inhibitors as cells approach anaphase. First, APC/C is inhibited by Emi1 during G2 and prophase (Reimann et al. 2001a, b). In late prophase, Emi1 is phosphorylated by Plk1, leading to its recognition by the ubiquitin ligase SCF and its subsequent proteasome-dependent degradation (Guardavaccaro et al. 2003; Margottin-Goguet et al. 2003). At this stage, the spindle assembly checkpoint is not yet fully active, as kinetochores are still recruiting many of their components, which could lead to a premature activation of APC/C (Sudakin et al. 2001). However, to prevent such a premature activation of APC/C towards Cyclin B and securin a cytosolic pool of Mad2 and Mad3/BubR1 is present that inhibits

APC/C^{Cdc20} and thus functions as a mitotic timer, by inhibiting anaphase onset. As kinetochores rapidly recruit all their components, they will activate the spindle assembly checkpoint machinery in the absence of microtubule attachment, resulting in a sustained APC/C^{Cdc20} inhibition until all kinetochores are attached and the cell is ready to segregate their sister chromatids accurately (Meraldi et al. 2004). An alternative hypothesis is that such a timing mechanisms represent a very basic form to prevent premature anaphase onset, without direct necessary input from the kinetochores. Such a basic timing mechanism might be useful in the very early embryonic cell cycles, which often do not have a spindle assembly checkpoint, or only a very weak checkpoint. Indeed, *Caenorhabditis elegans* embryos have only a very weak spindle checkpoint, that can only delay anaphase onset by a few minutes (Essex et al. 2009; Kitagawa 2009; Kitagawa and Rose 1999), while *Xenopus* egg extracts do not have a functional spindle checkpoint at all, unless one increases massively the nucleus to cytoplasm ratio (Chen et al. 1996; Minshull et al. 1994). Nevertheless, these extracts contain all the spindle assembly checkpoint proteins, suggesting that they fulfill a function at this stage of embryonic development.

It is important to note that another nonspindle assembly checkpoint-related protein has been suggested as a putative mitotic timer: Cyclin A (Geley et al. 2001). Indeed, Cyclin A degradation is independent of the spindle checkpoint and required for anaphase onset (Geley et al. 2001). Cyclin A degradation by the APC/C already starts in prometaphase and is a relative long process, providing cells with enough time to attach all their chromosomes and form proper metaphase plates (den Elzen and Pines 2001; Geley et al. 2001). Thus it seems clear that time is such a crucial parameter for mitosis in many organisms, that cells might have developed different, independent pathways that cooperate to ensure each cell has enough time to attach and align chromosomes before proceeding to anaphase.

The existence of the timing function of Mad2 and BubR1 has been confirmed in other organisms, in particular to the fly *Drosophila melanogaster*. *Mad2*- or *BubR1*-null mutants have an accelerated mitosis, whereas the disruption of other spindle checkpoint genes does not lead to such short cell divisions (Buffin et al. 2007; Rahmani et al. 2009). However, despite a shortened mitosis and a lack of a spindle assembly checkpoint activity, mad2-null mutants in *Drosophila* are viable and fertile. This suggests that in the vast majority of the cells, kinetochores become bipolarly attached to microtubules and aligned on the metaphase plate before Cyclin B and securin are degraded to an extent that allows chromosome segregation and anaphase onset. Therefore, these cells do not need any mitotic timer or checkpoint to prolong metaphase to fulfill all these requirements before exiting mitosis (Buffin et al. 2007). Indeed, the duration of prometaphase (time to bind and align all kinetochores) is only 5 min in wild-type *Drosophila* cells, compared to the average 20 min or more in vertebrate cells, most likely due to the very low numbers of chromosomes ($n = 4$). However, in conditions in which spindle formation is perturbed, such as loss of centrosomes, the spindle assembly checkpoint becomes also essential in *D. melanogaster* (Buffin et al. 2007). This demonstrates that, despite the fact that the molecular mechanisms of the spindle assembly checkpoint are highly conserved, the biological and cell cycle context determines whether the

checkpoint plays an essential role, as in mammals, or a more subsidiary role, as in fly and fungi. Another important question is whether the timing mechanisms associated with Mad2 and BubR1 is specific for metazoans or whether it is also present in fungal systems. This is difficult to evaluate at the current stage, since this timing mechanism becomes only visible in mammalian cells when using nuclear envelope breakdown as a clear morphological starting point $t = 0$ at the onset of mitosis. In the absence of a dramatic cytoskeletal and nuclear reorganization in fungal systems it might be difficult to test for the importance of a timing mechanisms. Finally, it is presently unclear how this timing mechanism is shut off, and whether this follows similar molecular mechanisms as for the inactivation of the spindle assembly checkpoint itself (see Sect. 4.7).

4.4 Is It Only Checking Kinetochore-Microtubule Attachments?

According to their original definition, checkpoints arrest the cell cycle for the cell to repair errors before resuming cell cycle progression. Although the spindle assembly checkpoint clearly fulfills this requirement, it has also become evident that the spindle checkpoint machinery goes beyond that, as its components are also involved in regulation and repair of kinetochore-microtubule attachments. Indeed, all three spindle checkpoint kinases, Mps1, BubR1, and Bub1, regulate and sometimes correct defective kinetochore-microtubule attachments (Kops 2009; Kops et al. 2010; Musacchio and Salmon 2007). Importantly, Bub1 and BubR1 separation of function mutants have demonstrated that this ability to regulate kinetochore-microtubule attachments is independent of their capacity to control spindle checkpoint signaling (Elowe et al. 2010; Klebig et al. 2009; Malureanu et al. 2009; Warren et al. 2002). BubR1 controls the dynamics of kinetochore-bound microtubules, while Bub1 has been proposed to contribute to the transition of lateral kinetochore-microtubule attachments to end-on attachments during chromosome alignment (Gillett et al. 2004; Lampson and Kapoor 2005; Meraldi and Sorger 2005). Finally, Mps1 has been involved in the correction of defective kinetochore-microtubule attachments, possibly by regulating Aurora B at kinetochores (Jelluma et al. 2008; Maure et al. 2007). Such a role is very reminiscent of the function ascribed to the so-called DNA-damage checkpoint (Rouse and Jackson 2002; Zhou and Elledge 2000). DNA-damage checkpoint proteins not only arrest the cell cycle in the presence of DNA damage, such as double-strand breaks, but also act as landing pads for DNA repair proteins at the site of the DNA damage, thus acting in a dual role of checkpoint signaling and repair mechanisms (Martin et al. 1999; Mills et al. 1999). The situation is even more extreme for spindle checkpoint kinases, as the functional data suggest that they not only contribute to the repair of defective attachments but also play a constitutive role in every cell division. This is not only the case in systems with an open mitosis, which always go through a stage with 100% of unattached kinetochores, but also in fungi with closed mitosis that have

microtubules bound to kinetochores during the whole cell cycle: budding yeasts lacking *BUB1* show severe chromosome segregation defects, that occur at a much higher rate than in yeasts lacking *MAD2* (Warren et al. 2002). This indicates that Bub1 is not just repairing defective attachments, but really systematically contributes to the establishment of correct attachments to kinetochores.

The dual role in checkpoint signaling and attachment regulation also raises the question as to where to draw the boundary between "checkpoint" proteins and other components of the kinetochore machinery. Several other components of the kinetochores are essential for the spindle assembly checkpoint, such as the so-called KMN (*K*nl-1-*M*ind-*N*dc80) network, which is required for kinetochore-microtubule attachment and acts as landing pad for most classical spindle checkpoint proteins on kinetochores (Cheeseman et al. 2004, 2006; Kiyomitsu et al. 2007; Martin-Lluesma et al. 2002). Should such proteins be considered as a part of the checkpoint or not? Given their essential role for kinetochore-microtubule attachment in every organism, probably not, but this shows that when analyzing every aspect at the molecular level, it becomes more difficult to define "checkpoint" proteins. The most ambivalent example is the protein kinase Aurora B (Ipl1 in budding yeast or Ark1 in *Schizosaccharomyces pombe*). This protein kinase, which is located at the centromeres between the two sister-kinetochores, plays an essential role in the correction of erroneous kinetochore-microtubule attachment (Liu and Lampson 2009; Nezi and Musacchio 2009). Specifically, it phosphorylates the microtubule-binding KMN network at kinetochores in a proximity-dependent manner if kinetochores are not stretched apart through a bipolar attachment (Liu et al. 2009; Welburn et al. 2010). But it has also been implicated directly in the spindle checkpoint signaling, as it contributes to the loading of Mad2 to kinetochores in human cells and is essential for the spindle checkpoint in *S. pombe* (Johnson et al. 2004; Vanoosthuyse and Hardwick 2009). Given that Ipl1/Ark1 is essential in fungi, it is not considered a checkpoint protein. However, its molecular functions are so close to the other checkpoint kinases, that it becomes very difficult to rationalize this strict separation.

One critical tool to understand these dual functions in the future will be to use separation of function mutants to elucidate the relative contributions to each pathway of these proteins. Moreover, there is a strong need for the identification of substrates of the checkpoint kinases to obtain more precise molecular investigative tools such as phospho-deficient or phospho-mimetic mutants. While multiple Aurora B substrates at kinetochores have been identified, we only know very few targets of Bub1, BubR1, or Mps1 involved in checkpoint signaling or kinetochore-microtubule attachments.

4.5 The Meiotic Spindle Assembly Checkpoint

Errors in chromosome-microtubule attachments are not exclusive to mitosis, but occur also during meiosis. Therefore, it is not surprising that the spindle checkpoint also functions during meiotic divisions (Shonn et al. 2000). Absence of tension at

kinetochores can activate the spindle checkpoint in meiosis (Li and Nicklas 1995) and, similar to mitosis, the checkpoint is required to induce metaphase arrest in the presence of perturbed kinetochore-microtubule attachments. In addition, there is considerable evidence that, as in mitosis, the spindle checkpoint machinery is not only acting as a pure checkpoint, but rather plays a constitutive role that is important for the distinct features of equational division (mitosis, meiosis II) and reductional division (meiosis I). This is particularly evident in *S. cerevisiae*, as the spindle checkpoint machinery is nonessential for mitotic division in this organism, but essential for meiotic divisions (Shonn et al. 2000).

S. cerevisiae Mad2 and Bub1 are required for proper chromosome segregation in meiosis I (coincident with APC^{Cdc20} activity), although their absence does not compromise chromosome distribution in meiosis II (Bernard et al. 2001; Shonn et al. 2000). Bub1 in particular, has multiple roles, as it monitors kinetochore-microtubule attachments, contributes to the unification of sister chromatids, and prevents the removal of the cohesin Rec8 from centromeric regions (Bernard et al. 2001). At the molecular level, Bub1 regulates this latter function by controlling the centromeric localization of Sgo1 through the phosphorylation of Histone H2A (Kawashima et al. 2010). The recruitment of Sgo1 in turn protects Rec8 from degradation during meiosis by safeguarding it from the separase (Kitajima et al. 2004). This Bub1 function relies on its Cdc2-dependent phosphorylation during meiosis I (Yamaguchi et al. 2003). Budding yeast cells lacking Mad2 showed a nondisjunction phenotype of chromosomes in meiosis I, which can be reverted by delaying anaphase I. More generally, the spindle checkpoint also detects lack of tension in meiosis I and delays the degradation of the securin Pds1 and thus prevents meiotic progression (Shonn et al. 2000).

Similar results were also obtained in vertebrate systems, in particular in murine oocytes. Loss of Bub1 leads to a premature resolution of chiasmata, massive chromosome segregation errors due to a failure to biorient bivalent chromosomes and a premature anaphase onset (McGuinness et al. 2009). A similar role is also known for BubR1, since its loss generally disrupts the ability of the spindle assembly checkpoint to inhibit APC/C^{Cdc20} (Homer et al. 2009), but also leads to chromosome alignment defects in meiosis II (Baker 2004). The most surprising finding is that BubR1 depletion in mouse oocytes results in the override of the physiological prophase I arrest, leading to germinal vesicle breakdown, and a subsequent arrest before anaphase I onset. At first sight this is inconsistent with its spindle checkpoint function, according to which its depletion should accelerate anaphase I entry, indicating that BubR1 must have other functions apart from its spindle assembly checkpoint role. Consistently, the presence of BubR1 is necessary to stabilize the APC/C activator Cdh1, which is crucial to prevent premature entry into meiosis I (Homer et al. 2009). BubR1/Mad3 is also essential for prophase I in both yeast and fly meioses, suggesting that this additional meiotic role is conserved (Cheslock et al. 2005; Malmanche et al. 2007). Again, as for the mitotic cycle, it will be important to identify the meiotic targets of Bub1 and BubR1 and to compare them to their mitotic counterparts, to better understand how these kinases adapt to the specific context of meiosis.

4.6 Checkpoint Function at Kinetochores Versus Cytoplasm

The spindle assembly checkpoint is defined as a kinetochore-attachment checkpoint. This is based to the fact that the checkpoint remains active as long as cells have erroneous kinetochore-microtubule attachments, and the fact that disruption of the kinetochore structure, either through laser ablations, genetic means or RNAi depletion, abrogates checkpoint signaling (Meraldi et al. 2004; Rieder et al. 1995; Tavormina and Burke 1998). This had led, for good reasons, to a very kinetochore-centric view of the spindle assembly checkpoint. However, at the molecular level the checkpoint has to also function outside of kinetochores. Indeed, if one single unattached kinetochore results in APC/C^{Cdc20} inhibition, this kinetochore must emit a diffusible signal that can inhibit cellular APC/C^{Cdc20} activity. Elegant experiments with fused cells containing two neighbouring bipolar spindles indicate that the diffusion rate of this signal is limited, as an unattached kinetochore in one spindle could not inhibit anaphase progression in the neighbouring spindle, despite the presence of a common cytoplasm (Rieder et al. 1997). Overall, this leads to the question which part of the checkpoint requires the kinetochore as a catalyst and which part occurs or can occur in other parts of the cells.

The strongest evidence for a nonkinetochore function comes from the set of proteins that act as inhibitors of APC/C^{Cdc20}. These include the MCC, comprising Mad2, BubR1, Bub3, and Cdc20. This complex can already be detected during interphase in human and yeast cells (Sudakin et al. 2001), at a time where functional kinetochores are not yet assembled, indicating that it acts outside of kinetochores. The same is true if BubR1 is the effector that ultimately inhibits APC/C^{Cdc20}, as both in a reconstituted functional *Xenopus laevis* assay, human cell extracts or mouse embryo fibroblasts, BubR1 appears to act as a cytoplasmic APC/C inhibitor (Kulukian et al. 2009; Malureanu et al. 2009; Nilsson et al. 2008). These data are also consistent with functional data in human cells, which are based on the RNAi-mediated depletion of Mad2 and BubR1 or on the depletion of the landing pads of these proteins on kinetochores (Meraldi et al. 2004). While complete depletion of Mad2 or BubR1 abrogates the mitotic timing and checkpoint function of these proteins, disruption of their kinetochore localization only disrupts the checkpoint function, indicating that their timing function must reside outside of kinetochores.

Recent experiments both in fission yeast and human cells suggest that such a behavior is not only limited to the effector proteins, but can also be found for the more upstream kinase Bub1 (Klebig et al. 2009; Windecker et al. 2009). A first study based on a structure-function study of human Bub1 mutants in an Bub1 RNAi background, indicates that Bub1 does not need to be located at the kinetochores to control chromosome congression and spindle assembly checkpoint even though there is a minor impairment of each function (Klebig et al. 2009). The same is true in *S. pombe*, where *bub3* mutants, which fail to localize Bub1 to kinetochores, are still able to mount a Bub1-dependent checkpoint response and to control Shugoshin localization at centrosomes through Bub1 (Windecker et al. 2009). This behavior is not restricted to Bub1, since BubR1 does not need to localize to kinetochores to

control chromosome alignment (Klebig et al. 2009). These data suggest that Bub1 and BubR1 are not mere scaffold proteins at the kinetochores and that their binding to kinetochores only increases the efficiency by which they control kinetochore-microtubule attachment and spindle checkpoint signaling. Although these two kinases are likely to act mostly at kinetochores in wild-type conditions, these results suggest that a more thorough investigation of the nonkinetochore bound pathways of the spindle assembly checkpoint machinery might reveal more surprising results.

4.7 How Is the Spindle Assembly Checkpoint Turned Off?

Once all chromosomes are bipolarly attached to microtubules emanating from both spindle poles through their sister kinetochores, the spindle assembly checkpoint is satisfied and must be inactivated to allow anaphase onset. However, spindle assembly checkpoint inactivation is clearly the least understood aspect of this pathway.

A first question is whether checkpoint inactivation is an active, dominant process, in which a molecular entity is turned on, once the checkpoint is satisfied, or whether it has a more passive nature, i.e. if the checkpoint signal is not maintained, it will fade away. Experiments with fused cells containing two mitotic spindle in a shared cytoplasm, found that spindle assembly checkpoint satisfaction in one spindle could induce anaphase onset in the other spindle, even if it still had unattached kinetochores (Rieder et al. 1997). This suggested that once the spindle checkpoint is satisfied, cells emit a dominant diffusible signal that will suppress the inhibitory signal of the spindle assembly checkpoint. However, the molecular nature of this suppression is not clearly defined.

A first system that was found to stop the checkpoint signal at kinetochores is the dynein/dynactin system. The spindle assembly checkpoint proteins Mad1, Mad2, and BubR1, are removed from kinetochores upon microtubule attachment and transported along microtubules to the spindle pole (Howell et al. 2000, 2001), a transition that is supposed to silence the signal emanating from kinetochores. This spindle checkpoint protein depletion system uses the dynein/dynactin motor protein and specialized dynein binding partners on kinetochores, composed of the RZZ complex (*R*od, *Z*W10 and *Z*wilch) and Spindly (Barisic et al. 2010; Chan et al. 2009; Clute and Pines 1999; Gassmann et al. 2008, 2010; Griffis et al. 2007; Howell et al. 2001; Karess 2005; Yamamoto et al. 2008). However, experimental evidence suggest that these proteins cannot be the only silencing system, as cells lacking Spindly still silence the checkpoint upon chromosome alignment (Gassmann et al. 2010). Studies both in *S. cerevisiae* and *S. pombe* demonstrate that checkpoint inactivation requires the protein phosphatase 1, indicating the need for a counterbalance to the checkpoint kinases, in particular to the Aurora B type of protein kinases (Pinsky et al. 2009; Vanoosthuyse and Hardwick 2009). Another study in *S. cerevisiae* proposed that, while the spindle assembly checkpoint inhibits APC/C^{Cdc20} activity, the checkpoint protein Mps1 is in turn a target of the APC/C, indicating that these activities counteract one each other in a double negative

feedback loop (Palframan et al. 2006). This led to a model in which the checkpoint efficiently inhibits APC/C^{Cdc20} due to the combination of preformed cytoplasmic MCC and unattached kinetochores emitting the "wait anaphase" signal. As kinetochores are bound by microtubules, the negative signal is reduced and APC/C activity increases, leading to the destruction of Mps1 and possibly other checkpoint proteins. Such a mechanism might be particularly important to permanently inactivate the spindle checkpoint once cells have initiated anaphase. A conceptually similar model has been proposed in human cells, with this time Cdc20 as the critical element of checkpoint maintenance and silencing. Jonathan Pines and coworkers found that the Cdc20 protein is constantly synthesized during mitosis, but that at the same it is rapidly degraded in a spindle-checkpoint dependent manner. Importantly, expression of a nondegradable Cdc20 led to an override of the spindle checkpoint machinery, strongly suggesting that Cdc20 concentration is the critical element that controls checkpoint silencing (Nilsson et al. 2008). Finally, the $p31^{comet}$ protein was proposed as a physiological negative regulator of the spindle assembly checkpoint. At the biochemical level, $p31^{comet}$ binds to the closed Mad2 conformation, as it mimics the structure of the open Mad2 conformation, and thus acts as a competitive inhibitor of open-closed Mad2-binding (Mapelli et al. 2006; Xia et al. 2004). However, $p31^{comet}$ depletion led to a weak defect in checkpoint inactivation, raising the question of the importance of this mechanism for spindle checkpoint silencing (Habu et al. 2002; Xia et al. 2004).

As several, confluent pathways have been suggested to cooperate in the spindle checkpoint activation, it is tempting to speculate that several mechanisms are also required for its silencing, providing a tight control over this essential transition. One puzzling fact though is that in contrast to the highly conserved checkpoint activation mechanisms, the silencing pathways are less well conserved: dynein is not present at kinetochores in fungi, no $p31^{comet}$ orthologue could be identified in yeast, and protein phosphatase 1 has not been involved in checkpoint silencing in vertebrates so far. Moreover, even though we have now identified several molecular players in this system, the nature of the diffusible checkpoint inhibitor proposed is unclear at this stage (Rieder et al. 1997).

4.8 Conclusion

The spindle assembly checkpoint has been defined as the crucial mitotic checkpoint mechanism 20 years ago. This has led to a very intense field of research and to the discovery of very elegant regulatory mechanisms controlling this signaling cascade. However, after all this time we still have only a very partial molecular view of the signaling system itself and the ways it is integrated more generally into the cell cycle machinery. From our subjective viewpoint we believe that for a better understanding of the checkpoint we will need to unravel in the future two critical crossroads between the checkpoint and the cell cycle: First we need to understand how the checkpoint kinases Mps1, Aurora B, Bub1, and BubR1 translate a mechanical

signal of unattached kinetochores into an active Mad2/BubR1 inhibition signal, a question that is very diffuse at this stage. Second, we will need to better define the inhibitory mechanisms that control checkpoint silencing, including the putative feedback loops emanating from the cell cycle that guarantee that such a step is irreversible once cells have initiated anaphase.

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Chapter 5
Cell-Size-Dependent Control of Organelle Sizes During Development

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Abstract During development, cells differentiate into diverse cell types with different sizes. The size of intracellular organelles often correlates with the size of the cell, which may be important for cell homeostasis. The nucleus is a well-known example of an organelle whose size correlates with cell size. However, the mechanical basis of the correlation is unknown. The lengths of the mitotic spindle and contractile ring are emerging as model system to investigate the cell-size-dependent control mechanisms of organelle size. Mechanistic models are proposed for the cell-size-dependent control of these organelles. Understanding the cell-size dependency of organelle sizes is expected to impact not only on the morphogenesis of the individual organelle, but also on cell homeostasis, cell cycle progression, and cell differentiation.

5.1 Introduction

5.1.1 Diversity in Cell Size

The cell is the basic unit of organization of life. Cells divide and differentiate to develop into multicellular organisms. While there are diverse types of cells, cells share common characteristics. From the viewpoint of space, size variation in cells is much narrower than size variation in organisms (e.g., the size of cells in an elephant or mouse is similar, although the number of cells is different). A common explanation for the relatively constant size of cells involves the upper size limit of cell surface area to volume ratio in diffusion-based systems (Vogel 2004). Even though the size of cells is relatively constant, there is still diversity in cell size within individual organisms (Altman and Katz 1976; Conlon et al. 2001; Saucedo and

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Edgar 2002). Extreme examples are the oocyte (about 130 μm in diameter, in humans) and sperm (5 μm in length of sperm head). Metabolically active tissues, such as the liver, have large cells. In contrast, immune tissues have small cells possibly to increase the number of nuclei per unit volume (Schmidt and Schibler 1995). Small cells seem to be favored in the construction of complex tissues like the brain (Roth et al. 1994). Therefore, the size of cell is an important parameter to be controlled upon cell differentiation. Molecular mechanisms to control cell size are beyond the scope of this chapter and have been well documented in several excellent review articles (Conlon and Raff 1999; Jorgensen and Tyers 2004; Saucedo and Edgar 2002; Stocker and Hafen 2000; Umen 2005).

5.1.2 Correlation Between Organelle Size and Cell Size

In cells of different sizes, the sizes of some intracellular organelles vary. (In this chapter, we are using a broad definition of the term organelle to describe subcellular structures with specific function, not only for membrane-bound ones like the nucleus, but also for spindles and the contractile ring.) The correlation of organelle size with cell size has been previously observed particularly for nuclear size (Conklin 1912; Wilson 1925). Extensive quantifications in yeast cells indicated that the ratio between nuclear volume and cell volume (nuclear-to-cytoplasmic ratio, N/C ratio) is constant over a wide range of cell sizes (Jorgensen et al. 2007; Neumann and Nurse 2007). The mitotic spindle is another organelle whose size correlate with cell size. The spindle aligns a pair of sister chromatids carrying genetic information and segregates each of them into daughter cells (Dumont and Mitchison 2009; Goshima and Kimura 2010). The size of the metaphase spindle (Greenan et al. 2010; Wühr et al. 2008), extent, and speed of spindle elongation (Hara and Kimura 2009) correlate with cell size. Furthermore, during cytokinesis, constriction speed of the contractile ring (Carvalho et al. 2009) also correlates with cell size. These observations suggest that organelle size is regulated in a cell-size-dependent manner.

The correlation between cell size and organelle size may be important to perform basic and common cellular processes in cells of different sizes. If the size of the mitotic spindle was too small compared with the size of the cell, cell division might not proceed properly because the spindle is important for defining the position of the plane of cell division (von Dassow 2009). In cancer cells, the N/C ratio takes abnormal value (Zink et al. 2004), suggesting the importance of the correlation between nuclear size and cell size in cell homeostasis. In addition, the ratio between cell size and organelle size can be a critical parameter for the cell to proceed into next developmental stage. The mid-blastula transition (MBT) is a transition of developmental stage from rapid synchronous cleavages to slower asynchronous divisions. The MBT is triggered when the cells reach a specific ratio between DNA content and cytoplasm volume (Edgar et al. 1986; Newport and Kirschner 1982a). Therefore, understanding the regulatory mechanism of organelle size in relation to

cell size is expected to affect not only the morphogenesis of individual organelles, but also cell homeostasis, cell cycle progression, and cell differentiation.

5.1.3 *Cell-Size-Dependent Regulatory Mechanisms of Organelle Size*

Various molecules have been identified whose inactivation or hyperactivation changes the size of organelles (Guo et al. 2008; Rafelski and Marshall 2008; Tam et al. 2007; Yan et al. 2005). For example, nuclear size decreases upon inactivation of nuclear pore complex subunits or a nuclear scaffold protein, lamin (Webster et al. 2009). However, the mechanism to set an organelle to a specific size is poorly understood. A straightforward way to set the desired length of an organelle is to use a molecular ruler protein, which has a length that equals the length of the organelle (Katsura 1990; Marshall 2004). This mechanism was first discovered for the control of the length of the lambda phage tail (Katsura and Hendrix 1984), and this mechanism may be used for bacterial flagellar hooks and sarcomere actin filaments in muscles (Rafelski and Marshall 2008). However, the application of the molecular ruler proteins is limited by the fact that most organelles and cells are far larger than individual molecules. Therefore, a possible strategy to control organelle size is a dynamic balance mechanism in which size is determined by the balance of assembly and disassembly of organelles (Rafelski and Marshall 2008). The control mechanisms of the size of the mitotic spindle are extensively studied. The balance of outward and inward forces, generated by the polymerization and depolymerization of microtubules and sliding of molecular motors along microtubule, defines the length of the spindles (Dumont and Mitchison 2009; Goshima et al. 2005; Mogilner et al. 2006). In addition, concentration gradients of Ran-GTP and related molecules based on diffusion from chromosomes are proposed to function as rulers to set the size of the spindle (Bastiaens et al. 2006). Microtubules can be set to a certain length by length-dependent depolymerizers, which contribute to set the length of the mitotic spindle (Varga et al. 2006, 2009).

Cell-size dependency adds another level of complexity to the issue of organelle size control. How can a cell "recognize" its own size (using molecules much smaller than cell size) and fine-tune the assembly and disassembly of organelles such that the balance point becomes proportional to cell size? Because of this complexity, must we still accept the correlation between cell size and organelle size "as something given and not at present further analyzable" as described by Theodor Boveri in 1905 for the constant N/C ratio (Wilson 1925)? As discussed by Wallace Marshall, size control of three-dimensional structures is complicated, and size (length) control of one-dimensional structures often serve as good models to consider (Marshall 2004). In this chapter, we first introduce cell-size-dependent controls of a three-dimensional structure, the nucleus, as a classical and well-investigated subject. Later, we discuss emerging topics such as the control of the length of spindles and the contractile ring, which can be simplified to a problem of one-dimensional structure (Fig. 5.1).

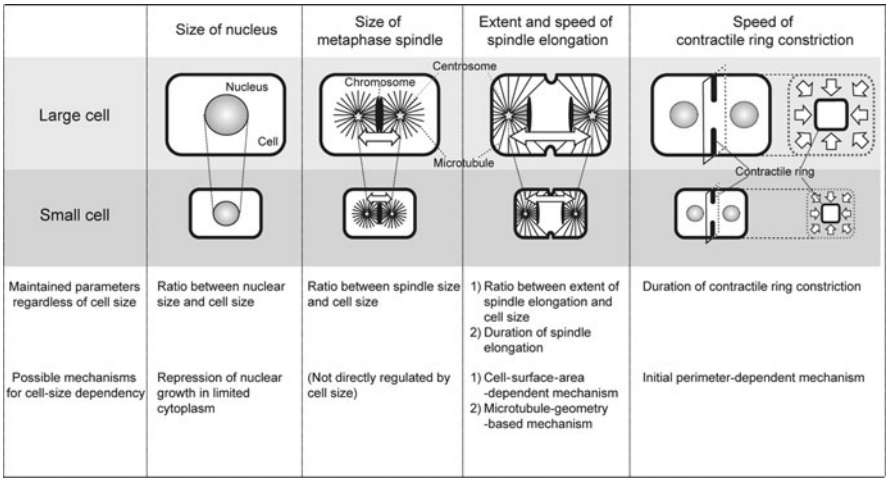

Fig. 5.1 Intracellular organelles whose size correlates with cell size. The size of the nucleus correlates with cell size, and thus the nuclear-to-cytoplasmic ratio is maintained during cell proliferation or differentiation. The length of the metaphase spindle correlates with cell size during embryogenesis. Both the speed and the extent of spindle elongation during anaphase correlate with and depend on cell size. During cytokinesis, the speed of contractile ring constriction depends on cell size

5.2 Nucleus

5.2.1 Nuclear-to-Cytoplasmic Ratio (N/C Ratio)

The size of the nucleus has a strong correlation with cell size (Fig. 5.1). In 1903, Richard Hertwig proposed the concept of the karyoplasmic ratio in which the ratio between nuclear volume and cytoplasmic volume tends to be constant (Wilson 1925). Correlation between cell size and nuclear size has been well investigated using experiments where the cell and nucleus sizes are changed by changing DNA content. In 1905, Theodor Boveri demonstrated that "the size of the nuclei at any given stage is directly proportional to the number of chromosomes that they contain" using haploid, diploid, tetraploid, and dispermic larvae of the sea urchin *Paracentrotus* (Wilson 1925). Cell size changed accordingly when the ploidy was changed as observed in yeast, tetrahymena, plant, sea urchin, amphibian, and mice (Frankhauser 1945; Henery et al. 1992; Henery and Kaufman 1992; Schmidt and Schibler 1995; Seyfert et al. 1984; Wilson 1925). In addition, species with larger genomes generally have larger cells and nuclei (Cavalier-Smith 1978; Gregory 2001; Jovtchev et al. 2006; Organ et al. 2007).

A correlation between nuclear volume and cytoplasmic volume has also been demonstrated without changing DNA content. When a murine hepatocyte was enlarged by overexpression of the c-Myc gene, the nucleus was enlarged accordingly (Kim et al. 2000). During the embryogenesis of *Caenorhabditis elegans* (later

than the ~16-cell stage), the nuclear size correlated well with blastomere size, which decreased as embryogenesis proceeds (Hara and Kimura 2009). The N/C ratio was quantified in detail in yeast and was shown to be constant for over ~2.5-fold changes in cell size for the yeast *Saccharomyces cerevisiae* (Jorgensen et al. 2007) and over ~35-fold changes in the *Schizosaccharomyces pombe* (Neumann and Nurse 2007). It should be mentioned that the N/C ratio might not be strictly regulated to take a constant value among different cell types in multicellular organisms. While the volumes of nuclei and cytoplasm are both larger in liver cells compared with thymus cells, the N/C ratio is not constant, and the ratio in liver cells is lower than in thymus cells (Schmidt and Schibler 1995).

5.2.2 How Does Ploidy Affect Nuclear Size and Cell Size?

As mentioned above, experiments changing the ploidy (DNA content) have provided major support for the concept that cells maintain constant N/C ratio. The change in ploidy (the increase or decrease) results in coordinated modification in both nuclear volume and cell volume. To investigate the mechanisms of how cells maintain constant N/C ratio, addressing the question of how ploidy affects nuclear size and cell size should be helpful. Which is the primary effect of ploidy change: nuclear volume or cell volume? Intuitively, it seems straightforward that ploidy directly affects nuclear size by changing the amount of DNA. A popular theory for control of nuclear size is the nucleoskeletal theory, in which nuclear size is determined by the amount of DNA, the compaction rate of DNA, or the amount of nuclear membrane components (Cavalier-Smith 1982). However, there is little experimental evidence demonstrating that DNA content directly defines nuclear size. For example, DNA duplication at S phase does not double nuclear size (Jorgensen et al. 2007).

Alternatively, DNA content may primarily affect cell size, instead of nuclear size. The correlation between DNA content and cell size was observed in cells without nuclei such as red blood cells (Gregory 2001). A plausible explanation for the direct regulation of cell size by DNA content is that cells with larger DNA content have a longer cell cycle duration and thus have more time to grow in size (Gregory 2001). Once the DNA content sets the cell size, then the nuclear size may be set according to the cell size. The mechanisms to set nuclear size in a cell-size-dependent manner are discussed in Sect. 5.2.3.

A close relationship between DNA content and cell volume has been demonstrated through cell differentiation. In specific cell types during differentiation, ploidy is increased through endoreplication, which results in the production of large cells, such as the larval salivary gland in *Drosophila*, epidermal cells of plant, as well as muscle fibers, megakaryocytes, liver cells, and giant trophoblast cells in mammals (Edgar and Orr-Weaver 2001; Jorgensen and Tyers 2004; Schmidt and Schibler 1995; Traas et al. 1998). MBT is an event during embryonic development when rapid synchronous cleavages switch to slower asynchronous divisions.

MBT takes place at cycle 13 of cell division in *Xenopus* and cycle 10 in starfish. MBT occurs earlier if DNA content is increased by inducing tetraploidy, polyspermic fertilization, or adding extra DNA (Mita and Obata 1984; Newport and Kirschner 1982a, b). Importantly, decreasing cytoplasmic volume also induced an earlier MBT (Kobayakawa and Kubota 1981; Mita 1983; Newport and Kirschner 1982a). In contrast, decreasing DNA content by decreasing ploidy or chromosome rearrangements resulted in a later MBT (Lu et al. 2009). These results collectively support the concept that the MBT is triggered when the cells reach a specific DNA content versus cytoplasmic volume. The mechanism to detect the specific ratio is considered to involve titration of cell components in egg by DNA (Newport and Kirschner 1982a), and histones are good candidates for such components (Prioleau et al. 1994). The mechanism to monitor the ratio between DNA content versus cytoplasmic volume may be used for the control of cell size according to DNA content (ploidy).

5.2.3 *Possible Mechanisms to Maintain a Constant N/C Ratio: Cell Cycle Control and Cytoplasmic Regulation*

The correlation between the volume of the nucleus and the cell strongly suggests a mechanism to monitor and maintain the N/C ratio. One possible hypothesis involves adjustment of the N/C ratio by the cell cycle (Jorgensen and Tyers 2004). In this hypothesis, the cell cycle enters S phase when the N/C ratio becomes lower than a desired value due to cell growth. During S phase, the N/C ratio may increase due to DNA replication. The N/C ratio may decrease in G2 phase due to cell growth and the cell may enter M phase when the ratio becomes lower than the desired value. This hypothesis explains how the N/C ratio is maintained near a constant value. However, detailed quantification of N/C ratios during G1 phase in *S. cerevisiae* revealed that the N/C ratio is rather constant and unlikely to define the timing of the cell cycle, at least for S phase entry (Jorgensen et al. 2007).

The mechanism by which cell size regulates cell cycle progression into M phase led us to speculate a mechanism by which N/C ratio regulates the cell cycle. In fission yeast, an inhibitor of mitotic entry, Pom1, forms a concentration gradient that is high at the cell ends and low at the cell center. When the cell length reaches a certain value, the level of Pom1 becomes lower than the threshold value at the cell center, which induces cell division (Martin and Berthelot-Grosjean 2009; Moseley et al. 2009). The proposed mechanism measures cell size using the gradient of protein diffusion as a ruler and determines the timing of M phase entry in a cell-size-dependent manner. Although this mechanism does not involve N/C ratio at this moment, there may be a chance that N/C ratio is involved. It is possible, yet completely speculative, that the amount of critical protein, like Pom1, depends on the volume of the nucleus, and thus N/C ratio affects the timing of M phase entry. Consistent with this idea, it has been assumed that cell division is induced earlier or later when the nucleus is small or large, respectively, by an unknown mechanism (Wilson 1925).

Another important observation in *S. pombe* provided a clue to reveal the mechanism to maintain constant N/C ratio (Neumann and Nurse 2007). The growth of nuclear volume after cell division seems to depend on the cytoplasmic volume. A detailed time course analysis in *S. pombe* revealed that even if two nuclei share a common cytoplasm, the nucleus surrounded by a larger cytoplasmic subvolume grows faster than the other. An observation in *C. elegans* blastomeres is consistent with the idea that the cytoplasmic volume controls nuclear size. During embryogenesis in *C. elegans*, the nuclear volume decreases as the cell gets smaller (Hara and Kimura 2009). Since the cell volume is predetermined by the size of the eggshell and the number of cleavages, nuclear growth may be restricted when the cell volume is limited. Therefore, it is likely that a mechanism exists in which a small cytoplasm restricts the overgrowth of the nucleus, which would maintain a constant N/C ratio, although the mechanistic nature of the regulation is still unclear.

5.3 Metaphase Spindle

5.3.1 The Length of the Metaphase Spindle Correlates with Cell Size but has an Upper Limit

The size of the metaphase spindle is an emerging example of an intracellular organelle that correlates with cell size (Fig. 5.1). The spindle is a molecular machine responsible for chromosome segregation. A bipolar spindle is formed in metaphase and elongates during anaphase. The relationship between cell size and mitotic spindle size at metaphase was systematically measured in *Xenopus laevis* and *C. elegans* embryos. In *X. laevis*, whose embryo diameter is as large as 1 mm, the size of the metaphase spindle was proportional to the cell size during later stages of embryogenesis where cells become smaller. During early embryogenesis, where cells are large, the size of the metaphase spindle appears to be constant, suggesting an upper limit in the size of the metaphase spindle (Wühr et al. 2008). In *C. elegans*, whose embryo is about 50 μm long, similar trends were observed. The correlation between the metaphase spindle size and the cell size was evident in smaller cells (Hara and Kimura 2009). In larger cells, the correlation is not as strong but is still significant and no clear upper limit in the size of the metaphase spindle was found (Greenan et al. 2010; Hara and Kimura 2009).

5.3.2 Does Cell Size Control Metaphase Spindle Size?

While the metaphase spindle size correlates with the cell size, cell size is not considered to directly regulate the size of the metaphase spindle. First, cell extracts, which do not possess information on cell size, are sufficient to reproduce the size difference in vitro between mitotic and meiotic spindles or between the metaphase

spindles of *X. laevis* and those of *Xenopus tropicalis* (Brown et al. 2007; Mitchison et al. 2005; Wühr et al. 2008). Second, the size of the meiotic spindle, which may be controlled by a similar mechanism as that of mitotic spindle, does not correlate with the size of the cells (oocytes) (Wühr et al. 2008). Instead of the cell size, the balance of forces produced by microtubules and motors inside the cell and the spatial gradient of Ran-GTP diffusing from the chromosome determines the spindle size (Dumont and Mitchison 2009; Kalab and Heald 2008; Mogilner et al. 2006; Subramanian and Kapoor 2009; Kalab et al. 2011). During *C. elegans* embryogenesis, the amount of TPXL-1 protein, ortholog of microtubule-associated protein TPX2, per centrosome correlates with the size of the metaphase spindle (Greenan et al. 2010). The diffusion of the TPXL-1 protein along microtubules is proposed to set the length of the spindle. The amount of DNA also affects spindle size. The size of the meiotic spindle in mouse and mitotic spindle in *X. laevis* correlated well with the number of chromatids (Kubiak et al. 1992, Wühr et al. 2008). The spindles with different sizes were reconstituted in cell extracts by using different amount of DNA (Brown et al. 2007; Dinarina et al. 2009). The DNA amount may affect the balance of forces to elongate and shorten the spindle or may affect the distribution of the Ran-GTP gradient. These observations indicate that not cell size but other factors are major determinant of the size of the metaphase spindle.

However, given the strong correlation between cell size and metaphase spindle size, we still believe that cell size controls metaphase spindle size in some cases. For example, the cell size may play a major regulatory role in small cells because the proportionality is evident in later embryonic stages where cell size is small. The above experiments suggesting no strict correlation have not been conducted at later embryonic stages.

5.4 Spindle Elongation

5.4.1 Spindle Length After Anaphase Elongation Correlates with Cell Size

After a complete bipolar spindle is formed during metaphase, the pole-to-pole distance of the spindle is increased, and each set of chromosomes moves toward each spindle pole during anaphase. This process of spindle elongation correlates with the cell size, as measured in the *C. elegans* embryo (Hara and Kimura 2009) (Fig. 5.1). Even during early embryonic stages, when the nuclear size and metaphase spindle size are roughly constant, the length of elongated spindle maintains a proportional relationship with cell size. In addition, the speed of spindle elongation was quantified and shown to correlate with the cell size. Dependency both of the length and of the speed of spindle elongation on cell size, but not on developmental stage or the cell type, was demonstrated by manipulating genes to make embryonic cells large or to alter cell types. Since the spindles elongated longer and faster when cells were enlarged, it is likely that cell size regulates spindle elongation. It should be

noted that spindle elongation does not always require cell boundaries and therefore is not always dependent on cell size. For example, during meiosis or *Drosophila* syncytium embryogenesis, spindles elongate independently of the cell boundary.

5.4.2 *Mechanistic Model of Cell-Size-Dependent Spindle Elongation*

We have previously proposed a model that explains how cell size affects the process of spindle elongation in the *C. elegans* embryo (Hara and Kimura 2009) (Fig. 5.2). The model considers two mechanisms that act in concert to elongate the spindle in a cell-size-dependent manner. The existence of two mechanisms is assumed based on the results that when Gα proteins are knocked down by RNA-mediated interference (RNAi), the speed of the remaining Gα-independent elongation is independent of cell size. Therefore, we considered Gα-dependent and -independent mechanisms as qualitatively distinct mechanisms. We proposed a force-generator-limited mechanism for the Gα-dependent mechanism. For the Gα-independent mechanism,

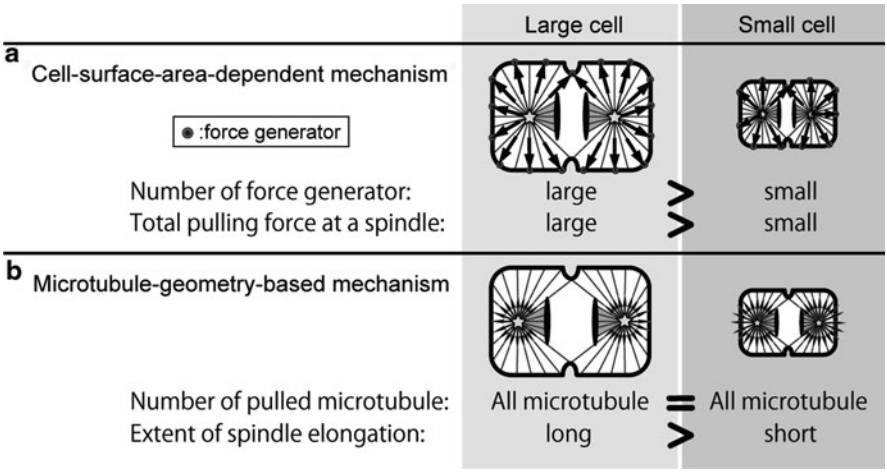

Fig. 5.2 Proposed mechanisms for cell-size-dependent elongation of the mitotic spindle. Hara and Kimura (2009) proposed that two mechanisms act in concert to elongate the mitotic spindle in a cell-size-dependent manner. (**a**) Cell-surface-area-dependent mechanism. Force generators (*gray circles*) are evenly distributed over the cortex and the estimated number of force generators is smaller than the microtubule number. Therefore, the number of pulled microtubules by force generators is proportional to the surface area, indicating that the total pulling force depends on cell surface area. (**b**) Microtubule-geometry-based mechanism. All microtubules at the spindle reach the cortex and are pulled by a constant force at a fixed contact point. This assumption allows the spindle to elongate by unbalancing the net direction of the pulling forces and to stop by balancing them. The geometry of microtubules at the time when the forces are balanced is similar regardless of cell size. Therefore, the elongation stops at a length that is dependent on cell size

we proposed a constant-pulling mechanism. We demonstrated that the combination of these two mechanisms sufficiently reproduced the quantitative feature of cell-size dependency in the extent and speed of spindle elongation in vivo.

In the Gα-dependent mechanism, the information on cell size is mediated through the surface area of the cell. A driving force for spindle elongation in the *C. elegans* embryo is thought to be generated at the cell cortex, where Gα-dependent force generators pull microtubules connected to the poles of the spindle. The number of Gα-dependent force generators on the cell cortex are estimated to be small (Grill et al. 2003), and thus the total magnitude of cortical force depends on the number of force generators on the cortex, rather than on the number of microtubules (Grill and Hyman 2005). Assuming that the density of the force generators is constant during embryogenesis, the number of force generators on the cortex is lower in smaller cells due to the decrease of the surface area. Thus, with the force-generator-limited mechanism, the elongating force is bigger in larger cells, which should result in faster and longer spindle elongation.

The Gα-independent mechanism is based on a similarity in geometry. If the number of microtubules reaching the cortex is almost constant in large and small cells, and all the microtubules that reach the cortex are pulled at a constant force, then the forces to elongate the spindle do not directly depend on the cell size, but on the angle distribution of the microtubules. When the angle distribution is the same, the force is the same regardless of the cell size, which means elongation stops when the net force on the centrosome is zero (i.e., the force is balanced) and the geometry where balanced force is achieved is similar. The initial speed of elongation would be similar if the geometry at the initiation of elongation was similar. This constant pulling mechanism results in a cell-size-dependent extent of elongation with similar initial speed. In summary, the model involving these two mechanisms is the first numerical model that well explains experimental observations.

5.4.3 The Meaning of Cell-Size-Dependent Spindle Elongation

The biological significance of spindle elongation being proportional to cell size is not clear. Intuitively, it seems beneficial for cells to separate sister chromatids to a long distance during spindle elongation to assure faithful segregation. However, chromosome segregation looks normal in cells where spindle elongation is inhibited and stops at a shorter distance in the *C. elegans* early embryo (Colombo et al. 2003; Gotta and Ahringer 2001; Gotta et al. 2003). An attractive possibility is that cell-size-dependent elongation is important for cytokinesis. Gα protein is required for aster-positioned cytokinesis in the *C. elegans* embryo (Bringmann et al. 2007), which is thought to be satisfied by full elongation of the spindle. During cytokinesis, signals sent from the aster to the cleavage furrow are important. These signals may facilitate cytokinesis only when spindle elongates to a sufficient distance to assure completion of chromosome segregation before cytokinesis (Bringmann et al. 2007; Dechant and Glotzer 2003).

5.5 Contractile Ring: Constriction Speed Correlates with Cell Size

The speed of contractile ring constriction during cytokinesis was shown to depend on cell size using early embryonic division of *C. elegans* (Carvalho et al. 2009) (Fig. 5.1). Constriction is fast in large cells and slow in small cells. This dependency on cell size enables cells to complete contraction within a constant duration regardless of cell size, which is similar to what has been observed for spindle elongation. The speed of constriction depends on cell size but not on the stage of development, as demonstrated by examining embryos in the same cell stage whose cell size is varied by means of gene manipulation (Carvalho et al. 2009).

The prevalent model of "pure string contraction" in which the myosin II motor slides the actin filament on the cell cortex cannot explain the cell-size dependency of contractile ring constriction. Another model to explain this relationship has been proposed (Carvalho et al. 2009) (Fig. 5.3). In this model, the cell memorizes the initial length of cortical ring using the assembly of cytoskeletal proteins, which controls the rate of constriction. Carvalho et al. hypothesized that the constriction units, which are small cytoskeletal structures with a fixed initial size, align as the

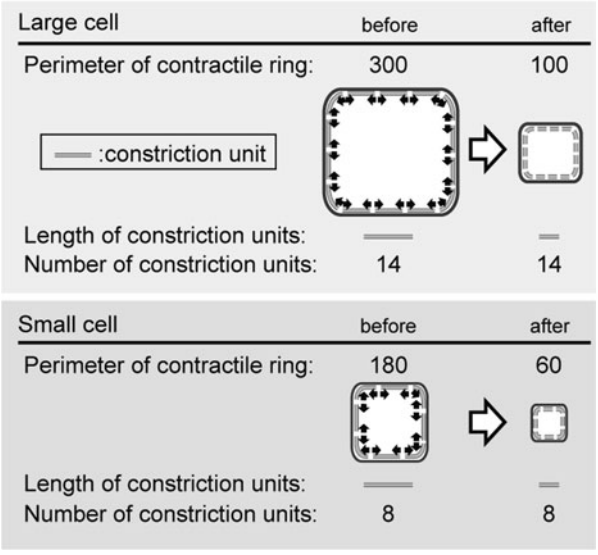

Fig. 5.3 A proposed mechanism for cell-size-dependent constriction of the contractile ring. A model proposed by Carvalho et al. (2009) for cell-size-dependent constriction of the contractile ring. The cell size (the initial perimeter of contractile ring) before the constriction is "memorized" by fixing the length of the hypothetical units of constriction regardless of cell size. The number of units is proportional to the initial perimeter of contractile ring. When these units constrict simultaneously, the net speed of constriction depends on the number of units and thus the initial perimeter of the cell

contractile ring. Since the number of the units depends on the cell size (i.e., perimeter of contractile ring), and each unit constricts with a constant speed, the net speed of constriction will be proportional to cell size. Actin filament is a candidate component of the structural memory in the *C. elegans* embryo supported by FRAP (fluorescent recovery after photobleaching) experiments. The turnover of the actin filament on the contractile ring was not evident, suggesting that the actin filament has a role in structural memory. This report sheds new light on the mechanism of cytokinesis that considers the scalability with cell size.

5.6 Conclusion

The relationship between the cell size and the size of organelles is one of the basic cellular principles that was first observed for the nucleus about 100 years ago. However, the mechanisms to control the organelle sizes in cell-size-dependent manners are still mysterious despite the tremendous progress in molecular and cell biology in the last century (Jorgensen et al. 2007; Neumann and Nurse 2007). Recently, the relationship between the length of the mitotic spindle, the contractile ring, and the cell size has been studied (Carvalho et al. 2009; Greenan et al. 2010; Hara and Kimura 2009; Wühr et al. 2008). Comprehensive models that explain how cell size affects spindle length and the contractile ring have been proposed (Carvalho et al. 2009; Hara and Kimura 2009). In this aspect, the control of the spindle length and the contractile ring became ahead of the cell-size-dependent control of nuclear size. Since the length of elongated spindle and the perimeter of contractile ring are monodimensional parameters, it is simpler to measure the size and construct mechanistic models, compared with three-dimensional nucleus (Marshall 2004). In particular, detailed time course measurement for spindle elongation and contractile ring constriction made an important contribution to investigate the regulatory mechanisms.

There still are many challenges to understand the nature of cell-size-dependent control of organelle size. First, the size of a limited number of organelles has been quantified in relation to the cell size. It is intriguing to examine whether the size of other organelles, in particular for those exist as more than one per cell, also correlates with the cell size. Second, regardless of the cell-size dependency, our knowledge of mechanisms that control the size of organelles is very limited. Further progress in quantification and genetic analyses of the sizes of various organelles is expected. The issue of the cell-size dependency does not necessarily come after we understand the mechanism of the size control. Analysis of cell-size dependency may provide a hint to understand mechanisms that control organelle size. Third, both the cell and intracellular structures are not static; their sizes change during the cell cycle, developmental stages, or environmental changes. In particular, size control is strongly linked with the regulation of the cell growth. Therefore, the time should be taken into consideration, thus adding again more complexity. Finally, we would like to answer the question as to how regulation at the cellular level can be correlated

with higher order levels of organs and individuals. The structural organization of cells has an impact on functions of organs and individuals. The genome size of birds is generally small compared with other species. One possible reason is that small genome size leads to a small cell size, which in turn contributes to small body size that aids in flying (Organ et al. 2007). The spatial organization of chromatin inside the nucleus in rod photoreceptor cells is optimized for vision in mammals (Solovei et al. 2009). The relationship between cell size and intracellular organization can be a restriction for an organism to develop into a complex multicellular structure. How organisms cope with or utilize the rules of cell-size dependency will offer a new viewpoint to understand the commonality and diversity of multicellular organisms.

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Chapter 6
The First Cell Cycle of the *Caenorhabditis elegans* Embryo: Spatial and Temporal Control of an Asymmetric Cell Division

Maria L. Begasse and Anthony A. Hyman

Abstract Throughout the development of an organism, it is essential that the cell cycle machinery is fine-tuned to generate cells of different fate. A series of asymmetric cell divisions leads to lineage specification. The *Caenorhabditis elegans* embryo is an excellent system to study various aspects of the early embryonic cell cycle. The invariant nature of the rapid cell divisions is the key feature for studying the effects of small perturbations to a complex process such as the cell cycle. The thorough characterization of the asymmetric first cell division of the *C. elegans* embryo has given great insight on how the oscillations of the cell cycle coordinate with the cytoplasmic rearrangements that ultimately lead to two developmentally distinct daughter cells.

6.1 Introduction

The mechanisms underlying the duplication and division of cells have stimulated research ever since Virchow postulated that cells can only come from preexisting cells (omni cellulae e cellula) in 1858. Earlier, in 1839, Schleiden and Schwann had recognized the importance of cells as the basic building blocks of life. From 1970s onward, groundbreaking work was done to understand the regulation of the cell cycle in genetically tractable eukaryotes, such as yeast, where many of the key players in the cell cycle were first described (Hartwell et al. 1970, 1974; Nurse et al. 1976; Nurse and Thuriaux 1980). The characterization of genes essential for a cell to divide was complemented by the discovery of temporally regulated protein synthesis, which was made possible by working in biochemical accessible organisms such as frog and sea urchin embryos (Masui and Markert 1971; Evans et al. 1983). With these two methods at hand, the model of the eukaryotic cell cycle based on the activity of cyclin dependent kinases (CDKs) was established. Over the years,

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many details of the regulatory pathways were described in a variety of organisms, and it has been found that the core regulatory principles are extremely well conserved among eukaryotic species. The discovery of the cell cycle machinery is reviewed in a great series of Nobel Prize lectures (Hartwell 2002; Hunt 2002; Nurse 2002). The question remaining is how the cell cycle regulators can be adopted throughout development to give rise to different tissues. Before we can comprehend the role of the cell cycle in cell fate regulation, we first need to understand how the cell cycle machinery mediates the morphological changes a cell undergoes during an asymmetric cell division.

6.1.1 Temporal Control: The Idea About a Cell Cycle Clock

The eukaryotic cell cycle is often described as a clock, with the different phases of the cell cycle occurring at certain times. Each clock is made up of an oscillator that defines the time periodicity, a controller that corrects the frequency of the oscillator, a counter that translates the oscillations to a more convenient unit, and an indicator that tells the time. In the cell cycle clock, the expression of cyclins sets the oscillations, a checkpoint or entrainment mechanism acts as a controller, the CDKs correspond to the counter, they translate the oscillations of the cyclins to biochemical changes in the cell, and the morphology of the cell tells us in which phase the cell cycle is, it tells the time. In most cell types, different species of cyclins oscillate at an internal frequency, and a mechanism is required to entrain the oscillations to activate the CDKs in the proper order. In some cell types, such as yeast, strong checkpoints were observed that are able to halt the cell cycle clock and restart it only when the oscillations are synchronized again. In other systems, there is only evidence for a subtle controller that can coordinate events but is not able to halt all aspects of cell division. Because of the differences observed in various cell types, different models were proposed on how a minimalist cell cycle clock could work. We discuss three models that imply different controller mechanisms.

The first model on cell cycle regulation was based on results from genetic studies in yeast (Nasmyth 1996; Stern and Nurse 1996). This model suggests that an increasing activity of a certain CDK will reach a threshold level at which it crosses a checkpoint (Fig. 6.1a). Recently, it has been shown for human cells that mitotic events are indeed ordered by their dependence on different thresholds of CDK activity (Gavet and Pines 2010). This view of the cell cycle is termed the ratchet model as each checkpoint acts as a switch from one phase to the next by irreversible protein degradation (reviewed by Reed 2003). These switches are called checkpoints because the cell will halt every aspect of cell division until the switch has been triggered (Kastan and Bartek 2004). An alternative hypothesis is that the cell cycle is composed of a set of independent clocks that are coordinated by checkpoints (Fig. 6.1b). This idea was initially proposed for the cell cycle of the prokaryote *Escherichia coli* (Jones and Donachie 1973). Nordström et al. (1991) supported this view of the *E. coli* cell cycle and suggested that the cell cycle of

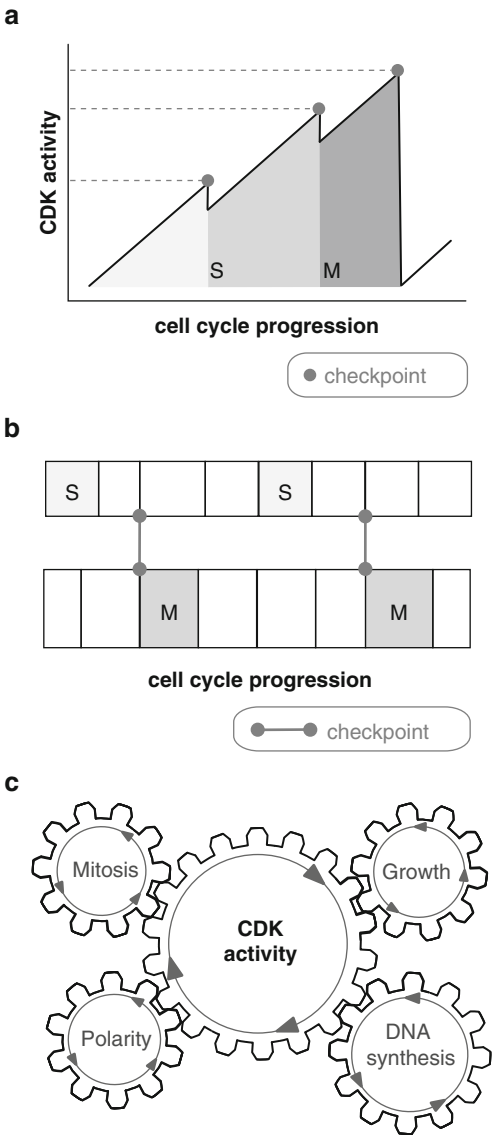

Fig. 6.1 Schematic illustrations of cell cycle clock models. (**a**) The ratchet model assumes that the phases of the cell cycle are initiated by overcoming thresholds of CDK activity. The threshold is passed by satisfying a checkpoint. This is a point of no return, ensuring that every event only happens once per cell cycle (Stern and Nurse 1996). (**b**) The model of independent clocks proposes that the different phases of the cell cycle run in parallel and are kept in synchrony by the activity of cell cycle checkpoints (Boye and Nordström 2003). (**c**) The phase-lock model is based on independent clocks that are entrained by the oscillations of a master regulator (Lu and Cross 2010)

higher organisms is also regulated by multiple independent clocks (Boye and Nordström 2003). In their model, the checkpoints act to synchronize events that run in parallel and do not necessarily depend on the same signals. The third model is the only one that does not depend on strict checkpoints as a controller unit. Lu and Cross (2010) proposed a phase-lock model for the cell cycle of a eukaryote, the budding yeast *Saccharomyces cerevisiae*. Phase locking presumes the presence of multiple independent oscillators, which run in parallel at their own frequencies.

In contrast to the former model, these clocks depend on each other, they act like slave clocks that are entrained by a master clock (Fig. 6.1c). In this model, each peripheral oscillator could be responsible for a different phase of the cell cycle. In eukaryotic cells, the CDK/cyclin system is the most likely candidate to provide a reliable oscillator capable of synchronizing the peripheral clocks and ensuring a stable order of events (Pomerening et al. 2003).

It is possible to distinguish these three models experimentally. Perturbation of the first and second model could lead to a complete arrest of the cell. Distinct events could only be decoupled by disruption of the checkpoint mechanisms. If non-checkpoint proteins are perturbed, the second and third model could result in a deceleration of the cell cycle but only the third model could lead to the decoupling of events as observed in many embryonic systems, such as the first cell cycle of the *Caenorhabditis elegans* embryo (see Sect. 6.1.3).

6.1.2 An Elegant Model: C. elegans

The free-living, bacterivorous nematode *C. elegans* is an excellent model organism for the genetic analysis of cell cycle regulation during embryonic development. The first two rounds of cell division are highly reproducible in their temporal and spatial sequence (Fig. 6.2). All consecutive embryonic divisions have been characterized (Deppe et al. 1978). Any discrepancy from the stereotypical cell divisions can be

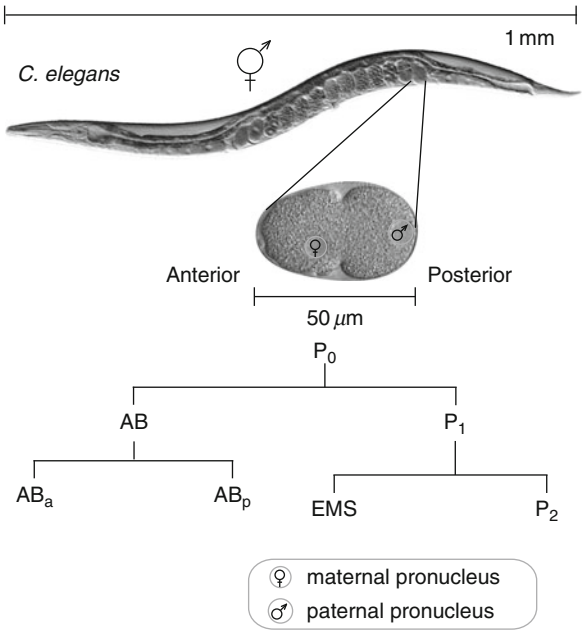

Fig. 6.2 Nomarski micrographs of a *C. elegans* hermaphrodite and a one-cell stage embryo (P_0). The lineage up to the four-cell stage is depicted. The cell divisions of P_0 and P_1 are asymmetric. The division of P_1 is initiated later than the symmetric division of AB

detected as a phenotype if the system is experimentally compromised. Another advantage as a genetic model organism comes from its mode of reproduction. Genetic homogeneity can be achieved by self-fertilization of the hermaphrodites, while genetic crosses can be set up with males that are infrequently occurring in the population (Brenner 1974; Sulston and Brenner 1974). Two breakthroughs for *C. elegans* as a model organism came in 1998 when it became the first animal to have its genome fully sequenced (*C. elegans* Sequencing Consortium 1998). At the same time, RNA interference was adopted as a tool to deplete cellular protein levels in *C. elegans* (Fire et al. 1998) and opened the possibility of genome wide screens (Fraser et al. 2000; Gönczy et al. 2000; Sönnichsen et al. 2005). The early embryo proved to be ideal for studying the effects of small perturbations such as single gene knockdowns. The size (roughly 50-μm long, with a diameter of 30 μm) and transparency of the *C. elegans* embryo make it easily accessible for light microscopy. This is a clear advantage for studying the morphological changes that a cell undergoes throughout the cell cycle. Individual proteins can be fluorescently tagged, and their distribution can be followed through the cell cycle by time-lapse microscopy. Through recent technological advance in the generation of transgenic lines, it is promising that the activity of any gene or promoter of interest can be tracked throughout the development of the worm (Sarov et al. 2006; Frøkjaer-Jensen et al. 2008; Murray et al. 2008). The first division of the *C. elegans* embryo is asymmetric, providing an opportunity to study the coordination between the CDK/cyclin rhythms and the series of polarization events leading to the segregation of cell fate determinants. Many players essential to the complex process of asymmetric cell division were uncovered by using the *C. elegans* embryo as a model organism.

6.1.3 Regulation of the Embryonic Cell Cycle in C. elegans

The first divisions of the *C. elegans* embryo are fast and do not require growth of cells. The cell cycle in the early embryo is only comprised of consecutive rounds of DNA synthesis (S phase) and cell division during mitosis (M phase), with no gap phase until the 28-cell stage (Edgar and McGhee 1988). Although the cell cycles of the embryo progress in a highly stereotypical fashion, it is clear that various processes essential to cell division can be uncoupled. The fact that endoreduplication cycles, the duplication of DNA without segregation of sisters, exist in later stages in the development of the embryo suggests that various aspects of cell division can be separated (van den Heuvel 2005). Specific experimental disturbance confirmed that the individual steps in the cell cycle in *C. elegans* are not strictly dependent on each other. If one aspect of the cell cycle like chromosome segregation is artificially blocked in the one-cell embryo, it does not prevent a later phase of the cell cycle; the cytokinesis furrow will still dissect the cell even though it has to cut through the DNA and spindle (van der Voet et al. 2009). Another example is that DNA and centrosome duplication proceed normally in the absence of cytokinesis (Chase et al. 2000). In a classic experiment, Schierenberg and Wood (1985)

showed that enucleated blastomeres of the one-cell embryo divided with typical timing. These examples indicate that at least the nuclear and the cytoplasmic cell cycle can be uncoupled to a certain extent in the early embryo.

Furthermore, these experiments indicate that the checkpoint mechanisms present in the embryo are not sufficient to halt all aspects of cell cycle progression. The lack of definite checkpoints in the one-cell *C. elegans* embryo makes it an ideal model system to study the finer details of the cell cycle. In other systems with strong checkpoint mechanisms, most perturbations of the cell cycle will lead to a checkpoint dependent arrest and give rise to a number of phenotypes limited by the number of checkpoints. Thus, studying the cell cycle in *C. elegans* has given great insight into some peripheral regulatory mechanism, but the system that synchronizes all aspects of the cell cycle is still poorly understood. In this review, we discuss various features of the first cell division of the *C. elegans* embryo individually and also emphasize steps that coordinate the different processes essential to the cell cycle.

6.2 The Nuclear and Centrosome Cycle

6.2.1 Meiosis and DNA Synthesis Phase

Before fertilization, the *C. elegans* oocyte is arrested at diakinesis of meiosis I in the gonad of the hermaphrodite. The oocytes are kept in a quiescent state by the activity of CKI-2, an inhibitor of the S-phase initiating CDK-2/cyclinE complex (Buck et al. 2009). The inhibition of CDK-2 by CKI-2 also seems to be the mechanism that eliminates the maternal centrosomes (Kim and Roy 2006). It is important that the set of centrosomes, which enters the oocyte at fertilization together with the paternal nucleus, will form the only microtubule-organizing center in the one-cell embryo. Upon fertilization, the activation of the oocyte triggers several events. First, meiosis I and II proceed, resulting in the extrusion of two polar bodies. Next an incompletely understood centrosome signal initiates a change in cortex activity at the site of sperm entry, leading to polarity establishment (see Sect. 6.3.1). Proper timing of this centrosome signal requires an active CDK-2/cyclinE complex (Cowan and Hyman 2006). From the activation of the oocyte onward, the CDK-2 complex is thought to be constantly active during early embryonic development (Fig. 6.3a) (Brodigan et al. 2003). The onset of cytoplasmic streaming by the asymmetric activation of the actomyosin cortex slightly precedes the migration of the maternal pronucleus toward the paternal pronucleus (Fig. 6.3b). Polarity establishment coincides with the DNA synthesis phase of the cell cycle (Edgar and McGhee 1988). Before the pronuclei migrate toward each other, the haploid chromosomes within both nuclei are replicated. The DNA damage checkpoint is silenced in the one-cell embryo (Holway et al. 2006). Stalling DNA replication delays cell cycle progression in the first cell division, which leads to defects in the

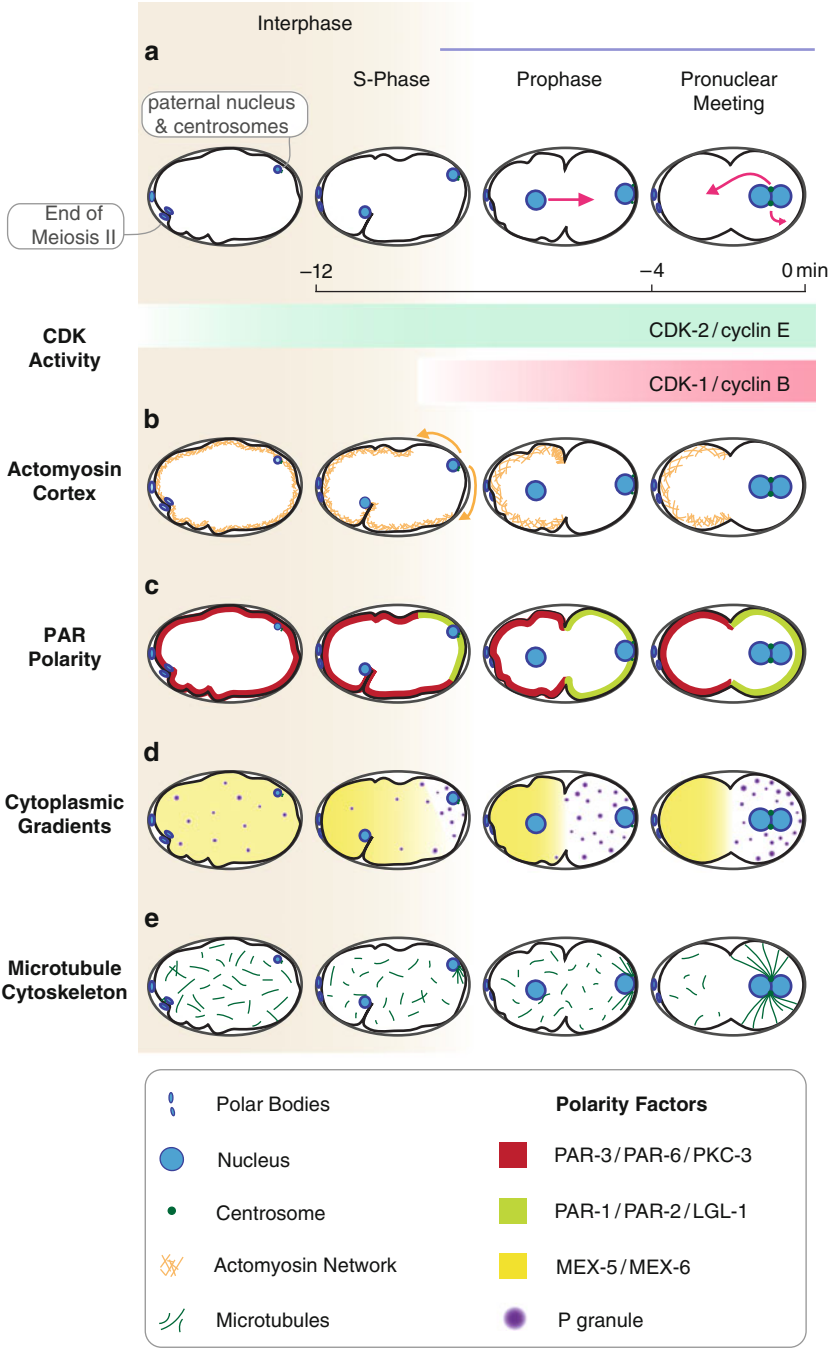

Fig. 6.3 (*continued*)

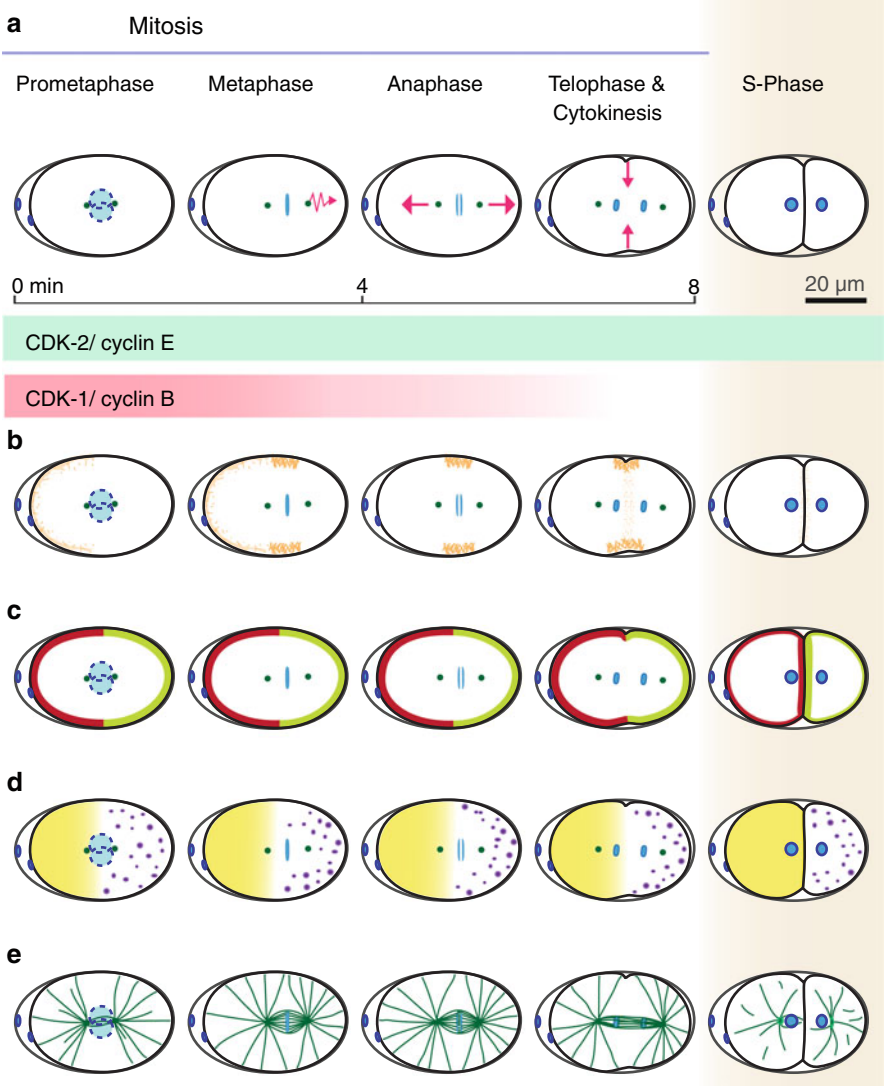

Fig. 6.3 Outline of the mitotic cell cycle in the one-cell *C. elegans* embryo. (**a**) The nuclear cycle and the activities of the cyclin dependent kinases are depicted. Timing is relative to prometaphase at 20°C. (**b**) The dynamics of the cortical actomyosin network (*orange*). (**c**) The cortex of the embryo is patterned by the anterior (*red*) and posterior (*green*) PAR proteins. (**d**) The distribution of the cytoplasmic cell fate determinants is downstream of PAR polarity. The somatic determinants MEX-5/-6 (*yellow*) segregate to the anterior and the germline specific P granules (*purple*) to the posterior. (**e**) The microtubule cytoskeleton (*green*) reorganizes into the mitotic spindle to segregate the sister chromatids. The anterior of the embryo is to the *left*, the posterior to the *right*. *Arrows* indicate motion

asymmetric cell division and hence it is not a true checkpoint (Encalada et al. 2000). The lack of a delay mechanism in case of DNA damage ensures that the cell cycle timing is not disrupted and S-phase completes before pronuclei meet in the posterior of the cell. DNA damage checkpoint activation becomes apparent in the two-cell embryo (see Sect. 6.6).

6.2.2 Prophase and Prometaphase

The increase of CDK-1 activity during mitotic prophase is accompanied by chromosome condensation in the nuclei (Hachet et al. 2007; Portier et al. 2007). The activation of CDK-1 in the early embryo is dependent on the phosphatase CDC-25.1 (Ashcroft et al. 1999; Rivers et al. 2008). At the same time, daughter centrioles are nucleated off the existing centrioles within the centrosomes (reviewed by Müller-Reichert et al. 2010). The mitotic kinase Aurora B (AIR-2) recruits the condensin complex to the chromosomes and is required for the phosphorylation of histone H3 during prophase, which temporally regulates DNA condensation (Kaitna et al. 2002; de Carvalho et al. 2008). Upon phosphorylation, the *C. elegans* homologue of CENP-A, centromere histone H3 (HCP-3), relocalizes from the center of the chromosomes to their surface, a process called centromere resolution. HCP-3 decorates almost the entire length of the chromosomes, typical for the establishment of holocentric kinetochores, which assemble around the centromeres (Moore et al. 2005). At the time of pronuclear meeting, the polo-like kinase PLK-1 can be detected at the centrosomes and has been implicated in promoting centrosome maturation (Chase et al. 2000). After pronuclear meeting, the nucleocentrosomal complex migrates to the middle of the cell and rotates. Timely regulation of nuclear envelope break down (NEBD) requires the cell cycle regulators PLK-1, CDC-25, and CDK-1 (Chase et al. 2000). Furthermore, it has been shown that the association of centrosomes with the nuclei is critical for timely NEBD (Hachet et al. 2007; Portier et al. 2007). Centrosomes are thought to act as signaling hubs that coordinate certain aspects of cell cycle progression. The maturation of centrosomes by Aurora A kinase (AIR-1) is essential for the simultaneous dissociation of the nuclear envelopes of both pronuclei (Hannak et al. 2001; Hachet et al. 2007; Portier et al. 2007). The coupling of centrosome maturation to nuclear envelope breakdown ensures that both sets of chromosomes are located between the spindle poles to be efficiently captured by spindle microtubules during prometaphase (Fig. 6.3e).

6.2.3 Metaphase and Anaphase

After NEBD, the spindle microtubules attach to the kinetochores on the chromosomes and arrange the condensed DNA onto the metaphase plate. Premature

chromosome segregation is prevented by cohesin, which holds the sister chromatids together. Cell cycle dependent activation of the catalytic function of separase cleaves the cohesin molecules all along the chromosomes and allows segregation of sister chromatids at the onset of anaphase. Two of the ultimate targets of the Anaphase Promoting Complex/Cyclosome (APC/C), an ubiquitin E3-ligase, are the inactivation of CDK-1 as well as the activation of separase (McCarthy Campbell et al. 2009). A study by van der Voet et al. (2009) has shown that association of CDK-1 with cyclinB1 is required for full condensation of chromosomes and for proper alignment at the metaphase plate, while cyclinB3 (CYB-3) is essential for the segregation of sister chromatids. The direct target of the CDK-1/CYB-3 complex has not yet been identified. Interestingly, inhibition of chromosome segregation by CYB-3 depletion does not activate a checkpoint to prevent cytokinesis. The activation of separase not only leads to sister chromatid segregation but also plays a role in centriole separation and regulation of spindle dynamics (Sullivan et al. 2001; Tsou et al. 2009). In *C. elegans*, the catalytic activity of separase is essential for sister chromosome separation, while it has a noncatalytic function in the completion of cytokinesis. Bembenek et al. (2010) described a separase mutant, which successfully segregates chromosomes but leads to cytokinesis failure. This indicates, for the first time, that the various functions of separase during exit of mitosis are likely to depend on different cell cycle signals.

Following successful sister chromatid segregation and initiation of cytokinesis, the nuclear envelopes reform in the two daughter cells during telophase. Centriole and centrosome dynamics in the one-cell *C. elegans* embryo have recently been reviewed (Müller-Reichert et al. 2010). For a review on nuclear envelope dynamics, see Gorjánácz et al. (2007). After telophase, the two-cell embryo is ready for a new division cycle, which is equipped with one nucleus and two centrioles per cell.

6.3 Asymmetric Cell Division: PAR Polarity

The first cell division of the *C. elegans* embryo is asymmetric, producing one larger anterior cell and one slightly smaller posterior cell (reviewed by Cowan and Hyman 2004, 2007; Munro and Bowerman 2009). Making two cells with different volumes is not the only challenge of the asymmetric cell division in the one-cell embryo. In order for the embryo to develop into an adult organism, cell fate determinants need to be segregated into one or the other half of the cell before the first division. The anterior cell will give rise to purely somatic tissue. The posterior cell will go on to give rise to the germline of the adult organism, among other tissues, and needs to inherit the germ cell factors, which are initially distributed throughout the oocyte. The prerequisite for an asymmetric cell division is the polarization of the parent cell. Asymmetry is initiated by the segregation of anterior and posterior PAR (partitioning defective) domains (Fig. 6.3c). Polarization of the one-cell embryo requires the PDZ domain proteins PAR-3 and PAR-6, as well as the atypical protein

kinase C (PKC-3) to form the anterior domain. The serine threonine kinase PAR-1, the ring-finger protein PAR-2, and the tumor suppressor LGL-1 establish the contrasting posterior domain (Hoege et al. 2010). The PAR-3/PAR-6/PKC-3 complex will thereafter be called the anterior PAR-6 domain, and the localization of PAR-1, PAR-2, and LGL-1 will be referred to as the posterior PAR-2 domain. The cytoplasmic proteins PAR-5, a member of the regulatory 14-3-3 protein family and PAR-4, a serine-threonine kinase, are also required for stable polarity domains in the embryo although their functions are less well understood.

Once the PAR polarity is established, it is essential to keep the cell polarized to ensure the accurate distribution of other factors, which ultimately gives rise to daughter cells with different fate. On the one hand, the PAR polarity regulates cortical factors to position the spindle slightly posterior before cytokinesis, leading to two differently sized daughters. On the other hand, it signals to cytoplasmic components that segregate the fate determinants, ensuring that the daughters will form different types of tissue.

6.3.1 Polarity Establishment Phase

In the *C. elegans* embryo, the onset of polarity is triggered by the sperm, which enters the oocyte in the process of fertilization. The site of sperm entry designates the posterior of the embryo (Goldstein and Hird 1996). The one-cell embryo continues to be unpolarized during meiosis. At meiosis II, the PAR-2 complex is weakly associated with the cortex. At the end of meiosis II, PAR-2 falls off the cortex and PAR-6 decorates the whole cortex (Fig. 6.3c). The onset of anterior–posterior (AP) polarity is not apparent until the start of mitotic prophase when the cortex contractility becomes asymmetric and PAR-2 forms a posterior domain, thereby replacing PAR-6 in this region (Cuenca et al. 2003). The mechanism by which sperm entry initiates polarity establishment half an hour after sperm–egg fusion is incompletely understood. The asymmetric activation of the actomyosin network has been suggested as an important factor for the timely regulation of polarity onset. A spatial cue that is dependent on the centrosome is thought to lead to the exclusion of the activating RHO-1 guanine exchange factor (GEF) ECT-2 (Cowan and Hyman 2006; Motegi and Sugimoto 2006). The GAP (GTPase activating protein) CYK-4 has also been proposed to play a role in the asymmetric activation of the cortex (Jenkins et al. 2006). In this model, the regulation of RHO-1 activity through spatial localization or exclusion of RHO inhibitors or activators has been proposed to induce the retraction of the active actin cytoskeleton toward the anterior. However, due to a lack of tools to image the activity level of RHO-1, this model remains controversial. The cortical actomyosin flow from the posterior pole correlates with the displacement of the PAR-6 domain toward the anterior, allowing the complementary PAR-2 domain to establish on the noncontractile cortex at the posterior (Munro et al. 2004).

6.3.2 Polarity Maintenance Phase

The contractility of the anterior cortex ceases as the nuclei migrate toward the center of the cell. The boundary between the cortical polarity domains needs to be maintained at the center of the cell until mitotic metaphase. While RHO-1 is the major regulator of the actomyosin network during polarity establishment, maintaining two stable domains depends on the interaction between the anterior PAR proteins and the rho family GTPase CDC-42 (Schonegg and Hyman 2006; Kumfer et al. 2010). The active form of CDC-42 interacts with PAR-6 and is enriched on the anterior cortex, possibly by the opposing activity of the GAP CHIN-1 localized at the posterior cortex (Gotta et al. 2001; Aceto et al. 2006; Kumfer et al. 2010). At the cortex, PAR-6 is thought to interact with the kinase PKC-3, which can phosphorylate PAR-2 to keep it off the anterior cortical domain (Hao et al. 2006). It has been proposed that PKC-3 is able to phosphorylate PAR-1, while PAR-1 phosphorylates the anterior protein PAR-3, constructing a feedback loop. This model is based on cell polarity studies in other organisms, mainly *Drosophila melanogaster* (Benton and St Johnston 2003; Hutterer et al. 2004). The *Drosophila* polarity model can be mapped onto *C. elegans*, considering that the *C. elegans* specific polarity protein PAR-2 performs the same function as LGL in flies (Hoege et al. 2010). The other polarity proteins discussed here are conserved. The ability of the anterior complex to phosphorylate the components of the posterior PAR complex and vice versa is at the heart of mutual antagonism, the process that is thought to allow maintenance of the polarity boundary. The cytoplasmic 14-3-3 protein PAR-5 functions to ensure a sharp boundary between the anterior and posterior cortical complexes but mechanism by which it does so is not yet clear. 14-3-3s are known to bind to phosphorylated proteins, and it has been speculated that they are able to remove phosphorylated PAR proteins from the cortical region, thereby increasing the turnover of proteins at the cortex.

If polarity establishment was blocked in early prophase by inhibiting cortical contractility, there is an alternative mechanism to form the two opposing domains during the maintenance phase (Zonies et al. 2010). The signal initiating the accumulation of PAR-2 on the posterior cortex at this time point in the cell cycle has yet to be revealed. The expansion of the PAR-2 domain in the absence of strong cortical flows is dependent on the activity of CDC-42 (Zonies et al. 2010). This indicates that the antagonistic interaction between the posterior and anterior PAR complexes allows the PAR-2 domain to push back the PAR-6 domain. The position of a PAR boundary established in the absence of flows would, therefore, depend on the balanced activity between the antagonistic complexes. On the basis of our current understanding of PAR polarity, it is likely that the redistribution of the PAR domains during early prophase is due to passive transport by cortical flows while the delayed onset of polarity in the absence of strong cortical contractility as well as the maintenance of the established domains is due to an active mechanism involving mutual antagonism between anterior and posterior PARs.

6.3.3 Repositioning the PAR Boundary to Match the Cytokinesis Furrow

The final position of the PAR polarity boundary correlates with the site of the asymmetric position of the cytokinesis furrow (Schenk et al. 2010). Just before the sister chromatids segregate to opposite poles of the one-cell embryo at anaphase, the spindle is displaced slightly toward the posterior (see Sect. 6.5.3) (Labbé et al. 2004; McCarthy Campbell et al. 2009). At metaphase, the shift in spindle position induces a reorganization of the actomyosin cortex (Werner et al. 2007). The formation of the cytokinesis furrow depends on a spatial activation of RHO-1, which is required to assemble the actomyosin based contractile ring. During the establishment phase, positioning the boundary between the anterior and the posterior PAR complexes can be artificially adjusted by changing the activity of RHO-1 (Schonegg et al. 2007; Werner et al. 2007).

The exact position of the polarity boundary at cytokinesis is important, since the PAR proteins ultimately mediate the distribution of cytoplasmic cell fate determinants.

6.4 Cytoplasmic Segregation of Cell Fate Determinants

The PAR protein complexes pattern the cortex and thereby establish the polarity of the embryo; however, this is not sufficient to form daughter cells with different fates. The segregation of cytoplasmic germline factors from determinants of the somatic lineage in the one-cell embryo is essential for proper development. The exclusive inheritance of P granules, germline specific ribonucleoprotein (RNP) aggregates, and free germline proteins to the posterior daughter, conditions the cells to follow their fate (Strome and Wood 1982). The differential segregation of fate determining factors leads to the induction of specific cell cycle control in the two-cell embryo (Budirahardja and Gonczy 2008; Rivers et al. 2008).

6.4.1 A Gradient in MEX Proteins Determines the Somatic Lineage

Protein and RNA segregation in the cytoplasm of the one-cell embryo is dependent on PAR polarity and on an anterior–posterior gradient of MEX (muscle excess) proteins (Schubert et al. 2000; Cuenca et al. 2003). As the PAR-6 complex retracts after polarity onset, it cosegregates the active form of MEX-5 and MEX-6 (MEX-5/-6) toward the anterior (Fig. 6.3d). These CCCH finger proteins are required for the asymmetric distribution of all cell fate determinants, and they inhibit the expression of germline factors in the somatic daughter cells. The MEX proteins

were identified in a mutant screen for aberrant muscle cells (Schubert et al. 2000). The observed defect was that the muscle specific transcription factor SKN-1 was not restricted to the posterior daughter cell, which implied the regulatory role of the MEX proteins on generating cytoplasmic asymmetry. The activity of MEX-5/-6 is regulated in a cell cycle dependent manner. Maternally provided MEX-5 is evenly distributed in the oocyte, so are the germline specific zinc-finger protein PIE-1 and the P granules (Schubert et al. 2000; Cuenca et al. 2003). At meiosis II, CDK-1 activates the DYRK kinase MBK-2, which subsequently marks oocyte specific proteins for degradation (Stitzel et al. 2006; Cheng et al. 2009). For a discussion of the oocyte-to-embryo transition and the regulation of MBK-2, see Parry and Singson (2011). The cell cycle dependent activation of MBK-2 leads to a priming phosphorylation of MEX-5/-6 (Nishi et al. 2008). During polarity establishment, PLK-1 and PLK-2 bind to the primed MEX-5/-6 and activate them. As a result of active MEX-5/-6 being sequestered to the anterior half, PIE-1 and other germline factors get restricted to the posterior half of the one-cell embryo (Nishi et al. 2008). Interestingly, PLK-1 segregates to the anterior half together with MEX-5/-6, which ultimately leads to an unequal inheritance of PLK-1/-2 to the daughters. The next three divisions of the P lineage continue to be asymmetric, producing one somatic and one germline daughter (Fig. 6.2). These divisions of the germline blastomere show that MEX-5/-6 and PLK-1 are always enriched in the somatic daughter cell. MEX-5/-6 abundance diminishes in somatic daughters after cell division (Schubert et al. 2000), while PLK-1 protein levels stay high in somatic cells (Nishi et al. 2008), indicating that MEX-5/-6 are only transiently required to deplete the somatic cytoplasm of germline factors, while high levels of PLK-1 might be required for the regulation of the somatic cell cycle. In the posterior, MEX-5/-6 was shown to assemble with P granules (Schubert et al. 2000; Tenlen et al. 2008). The association of determinants of somatic tissues with P granules could be relevant by ensuring that minimal amounts of somatic determinants are inherited by the P lineage.

6.4.2 Spatial Regulation of Protein Mobility Generates Segregation of Cytoplasmic Components

We are slowly starting to understand how the partitioning of the anterior and posterior PAR domains influences the antagonistic distribution of cytoplasmic factors. As for the cell cycle in general, the differential distribution of cytoplasmic components also greatly depends on the activity and spatial distribution of kinases and phosphatases. The PAR-1/PAR-4 dependent phosphorylation status of MEX-5/-6, for example, has been reported to be important for the asymmetric segregation to the anterior (Tenlen et al. 2008). It has also been shown that cytoplasmic flows, generated during polarity establishment, are neither sufficient nor necessary to segregate cell fate determinants. The current models for the distribution of cytoplasmic factors are based on spatial regulation of the mobility of these factors in the

embryo (Tenlen et al. 2008; Brangwynne et al. 2009; Daniels et al. 2010). An alteration in mobility can be brought by phosphorylation. Tenlen et al. (2008) proposed that phosphorylation of MEX-5 at a specific site would enhance its mobility in the posterior increasing the likelihood that it crosses the boundary into the anterior cytoplasm. In the anterior, the mobility of MEX-5 is reduced, limiting its chance to move back to the posterior. This model proposes that a difference in diffusive mobility would drive the AP gradient of MEX-5. It is still unclear if the asymmetric distribution of MEX-5 requires asymmetric binding sites to improve its enrichment in the anterior (Tenlen et al. 2008; Daniels et al. 2010). In the case of the RNP rich P granules, a slightly different diffusion based model has been proposed. Brangwynne et al. (2009) illustrate that P granules behave like liquid droplets in the cytoplasm, and hence they can be in a dissolved, or in a condensed form. In this model, the MEX-5 dependent dissolution of P granules in the anterior increases the mobility of proteins and RNAs, while the condensation into droplets in the posterior serves to decrease the mobility of soluble components. The segregation of cell fate determinants, achieved by differential diffusion mechanisms, is dependent on polarity establishment of the cortical PAR proteins.

6.5 Microtubule Dynamics Throughout the Cell Cycle

The mitotic spindle has to faithfully orchestrate the segregation of chromosomes to the daughter cells. In order to do so, the structure of the microtubule network needs to be dramatically altered throughout the cell cycle (Fig. 6.3e). For proper development of an organism, it is essential that the shape of the spindle correlates with the size of cells (Hara and Kimura 2011). The length of the mitotic spindle at metaphase of the early *C. elegans* embryo is regulated by the size of the centrosomes (Greenan et al. 2010).

6.5.1 Interphase and Prophase

In the one-cell embryo, the microtubules exhibit the fastest growth rates at interphase and early prophase but they stay short and lack directionality (Srayko et al. 2005). Upon centrosome maturation in late prophase, the microtubule network in the cell drastically alters its structure. Long, microtubules emanate from centrosomes, forming asters that span the whole cell soon after pronuclear meeting. Microtubules are thought to anchor to the cell cortex where force is generated to pull the centrosomes anterior toward the center of the cell and then rotate along the AP axis (Park and Rose 2008). This process also centers the pronuclei, which are tightly attached to the centrosomes (reviewed by Gorjánácz et al. 2007).

6.5.2 Prometaphase

At this stage of the cell cycle, the centrosomes are fully mature and induce the disintegration of the nuclear envelope. In the early stages of nuclear envelope breakdown, fenestrae form in the membrane, allowing the nucleoplasm to mix with the cytoplasm and microtubules to grow toward the chromosomes (Gorjánácz et al. 2007; Müller-Reichert et al. 2010). The mitotic spindle is established by a biased growth of microtubules toward the chromosomes. This process depends on the small GTPase RAN-1 but not on de novo microtubule formation at the kinetochores (Srayko et al. 2005). For more details on the functions of RAN gradients in meiosis and mitosis, see Kaláb et al. (2011). The assembly of a microtubule rich spindle between the centrosomes ensures efficient capture of kinetochores and alignment of the chromosomes on the metaphase plate.

6.5.3 Posterior Displacement of the Spindle During Metaphase and Anaphase

Before the onset of anaphase, the whole spindle starts rocking and is displaced toward the posterior, preparing for the asymmetric division of the one-cell embryo. The regulation and mechanisms of spindle displacement in *C. elegans* has been a focus of intense research over the last decade. The asymmetric positioning of the spindle places the cytokinesis furrow off center resulting in two daughter cells of different size. Displacement of the spindle is tightly linked to anaphase onset and both processes depend on the inactivation of CDK-1 (McCarthy Campbell et al. 2009). *C. elegans* has become the prime model to study the forces that are exerted on a spindle during mitosis, as Grill et al. (2001) introduced the technique of local microtubule severing by directed laser light. With this method, distinct sections of the spindle microtubules were cut or centrosomes were disintegrated, which allowed the indirect determination of forces by measuring the rates at which the remaining parts of the spindle move. Application of this method allowed the dissection of the pathway leading to an asymmetric cell division. The cell cycle components that activate the force-generating complex (FGC) on the cortex, which promotes spindle displacement, are largely unknown. The phosphatase PPH-6 has been implicated in the localization of the FGC and in the timing of force generation during anaphase (Afshar et al. 2010). As mentioned above, cortex-generated forces mediate the centration of the nucleocentrosomal complex during prophase. The force generating Gα (GOA-1 and GPA-16) signaling complex acts downstream of the PAR polarity and is redistributed from an anterior enrichment at prophase to a posterior cortical localization at metaphase (Park and Rose 2008). Microtubule-based forces and an asymmetric position of the spindle can only be achieved upon proper localization of Gα and its associated proteins GPR-1/-2 (G protein regulator) and the coiled-coil protein LIN-5 (Colombo et al. 2003; Gotta et al. 2003;

Srinivasan et al. 2003). LIN-5 can bind to both the Gα/GPR complex as well as the microtubule motor dynein, thereby anchoring the astral microtubules to the cortex and achieving force generation (Couwenbergs et al. 2007; Nguyen-Ngoc et al. 2007). At anaphase, the cortex is patterned with low GPR-1/-2 and LIN-5 at the anterior domain, a band of LET-99 slightly posterior to the center of the cell and a posterior domain enriched in GPR-1/-2 and LIN-5 (Colombo et al. 2003; Bringmann et al. 2007; Wu and Rose 2007; Krueger et al. 2010). In order to move the spindle, the FGC needs to link the microtubules to the cortex or membrane of the embryo. It has been shown recently that the actomyosin network at the cortex stabilizes the anchoring of the FGC at the membrane (Redemann et al. 2010). By weakening of the actomyosin network, single force generating events could be observed, as microtubules were able to pull membrane invaginations toward the centrosomes. Spindle severing experiments, analysis of centrosome movements as well as observation of individual force generation events came to the conclusion, that the asymmetric distribution of force generators results in a net asymmetry of cortical pulling forces that enables the posterior positioning of the spindle at metaphase and anaphase (Grill et al. 2003; Pecreaux et al. 2006; Redemann et al. 2010).

6.5.4 The Role of the Central Spindle During Anaphase

The destruction of cyclin B by the APC/C deactivates CDK-1 at anaphase onset. As discussed, this leads to the segregation of sister chromatids and centrioles (see Sect. 6.2.3), and ultimately evokes cytokinesis. It also induces a remarkable change in spindle morphology. As the chromosomes separate, the central spindle forms between the opposite poles and limits the rate of spindle elongation (Grill et al. 2001). For a functional central spindle, the nonkinetochore microtubules need to form stable bundles. Bundling of microtubules requires the activity of the kinesin-like protein ZEN-4 (CeMKLP-1) and the microtubule-associated protein SPD-1 (Verbrugghe and White 2004). ZEN-4 is inactivated prior to anaphase onset by an inhibitory CDK-1 mediated phosphorylation. Upon anaphase onset, the phosphatase CDC-14 removes the inhibitory modification that prevented the association of ZEN-4 with microtubules (Mishima et al. 2004). Localization of ZEN-4 to the central spindle depends on Aurora B and the inner centromere protein ICP-1 (Kaitna et al. 2000; Severson et al. 2000). ZEN-4 functions in the centralspindlin complex (ZEN-4 and CYK-4), which not only stabilizes the central spindle but is also required for the midzone to signal to the cytokinesis furrow and for the completion of cytokinesis (Bringmann and Hyman 2005). The capacity of the central spindle to position the contractile actomyosin ring is most likely through the ability of CYK-4 to recruit the RHO-1 activator ECT-2, which in turn recruits myosin to pattern the cortex around the spindle midzone (Werner et al. 2007).

6.5.5 *Two Consecutive Signals Determine the Cleavage Plane of the Embryo*

It has been established that two successive signals position the cytokinesis furrow in the one-cell embryo (Dechant and Glotzer 2003; Bringmann and Hyman 2005; Werner et al. 2007). Both signals are mediated by the microtubule cytoskeleton. The first signal comes from an interaction between the astral microtubules and the cortex. The separation of the asters at anaphase onset temporally couples chromosome segregation with the initiation of the cytokinesis furrow (Lewellyn et al. 2010). The ingression of the furrow is mediated by the constriction of an actomyosin ring. Furrow ingression based on the astral signal alone is faster than wildtype, indicating that the central spindle sends a retardation signal to the actomyosin network. The activity of the myosin light chain NMY-2 determines the speed of furrow ingression (Bringmann and Hyman 2005). This indicates that the second furrow-positioning signal, which depends on the central spindle, is able to correct the position of the cleavage plane by influencing actin dynamics.

6.6 The Differential Segregation of Cell Cycle Regulators Determines Cell Cycle Timing and Cell Fate of the Daughter Cells

The development of an organism with many different tissues requires the differentiation of the stem cell-like, one-cell embryo into various cell lineages. The asymmetric first division determines the future developmental program of the whole worm as it leads to the segregation of germline factors to one daughter exclusively. The specific timing of the cell cycle is tightly linked to cell fate (Bao et al. 2008; Lange et al. 2009). Schierenberg and Wood demonstrated in 1985 that the lineage specific timing in the *C. elegans* embryo is dependent on the nature of the cytoplasm and not a differential control by the nuclei. As discussed above, the asymmetric first division gives rise to two cells with distinct cytoplasmic protein compositions (see Sect. 6.4).

The larger anterior, somatic cell AB divides around 2 min prior to the smaller, posterior germline progenitor P_1. It is known that PLK-1, CDC-25, and CDK-1 are required for timely breakdown of the nuclear envelope in the first mitosis of the embryo (Chase et al. 2000). These cell cycle regulators have been implicated in mediating the differential timing between the AB and the P_1 cell, as the embryo goes from the two- to the four-cell stage (Fig. 6.2). From this set of cell cycle regulators, only PLK-1 is distributed in an asymmetric fashion. It has been proposed that the kinase PLK-1 promotes the nuclear accumulation of the phosphatase CDC-25. Activation of CDC-25 triggers CDK-1 function, leading to DNA condensation and the initiation of mitosis. At this point, it is not clear if PLK-1 activity is required upstream of CDK-1 or if these two kinases function in parallel. Sixty-percent

of the difference between the cell cycle timing of AB and P_1 can be accounted for by the unequal distribution of PLK-1 (Budirahardja and Gonczy 2008; Rivers et al. 2008). The kinase PLK-1 is enriched in the somatic AB cell, giving rise to a faster import of CDC-25 into the nucleus of the anterior cell. It has also been shown that the posterior cell is more sensitive to the levels of PLK-1 and CDC-25, while it seems that there is an excess of these cell cycle regulatory proteins in the anterior daughter. It is plausible that the timing of cell division in the early embryo is regulated by a balance between PLK-1, CDC-25, and CDK-1, with different emphasis on one of these proteins in different lineages. Bao et al. (2008) have shown that CDC-25 levels are rate limiting for the cell division timing of P_3 and the E lineage but not for any other lineage in the developing worm. The remaining 40% in the difference in cell cycle timing between AB and P_1 comes from a differential activation of the DNA replication checkpoint (Brauchle et al. 2003). The regulation of cell cycle timing in a DNA checkpoint dependent manner is specific to the germline precursors. It is reasonable that DNA replication is monitored more carefully in cells that give rise to the germline to ensure healthy offspring.

6.7 Conclusion

The development of the nematode *C. elegans* is highly reproducible (Sulston and Horvitz 1977; Deppe et al. 1978). The exact timing and stereotypic order of events in the one-cell *C. elegans* embryo allow for the precise description of the major morphological changes during a cell division cycle. The detailed characterization of different events with high time resolution encourages new studies on possible links between seemingly independent parts of the cell cycle and provides a base for further hypothesis driven research. The continuous advance in the genetic tools available for *C. elegans* provides opportunities to answer long-standing questions such as which mechanism regulates the cell cycle clock and how is an asymmetric cell division coordinated with the cell cycle.

The mapping of cell divisions and cell fates throughout the worm's development is the perfect prerequisite to study the regulation of cell cycle control throughout its development. It has been recognized that the temporal regulation of the cell cycle is critical for cell fate determination as each lineage has its own rhythm of divisions. The challenge is the identification of the key regulators for the individual steps of the cell cycle and of development without loosing sight of the complexity of cell division and cell–cell interactions. It will be interesting to learn how the cell cycle, as we understand it today, is modified to produce cells with different developmental fate.

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Chapter 7
EGG Molecules Couple the Oocyte-to-Embryo Transition with Cell Cycle Progression

Jean M. Parry and Andrew Singson

Abstract The oocyte-to-embryo transition is a precisely coordinated process in which an oocyte becomes fertilized and transitions to an embryonic program of events. The molecules involved in this process have not been well studied. Recently, a group of interacting molecules in *C. elegans* have been described as coordinating the oocyte-to-embryo transition with the advancement of the cell cycle. Genes *egg-3*, *egg-4*, and *egg-5* represent a small class of regulatory molecules known as protein–tyrosine phosphase-like proteins, which can bind phosphorylated substrates and act as scaffolding molecules or inhibitors. These genes are responsible for coupling the movements and activities of regulatory kinase *mbk-2* with advancement of the cell cycle during the oocyte-to-embryo transition.

7.1 Introduction

7.1.1 C. elegans, a Model Organism

During the 1960s, Dr. Sydney Brenner, Nobel Laureate, began searching for a new model organism. He put forward the free-living, nonparasitic nematode, *Caenorhabditis elegans* as an ideal model (Brenner 1974). The worms are tiny, only 1 mm fully grown, reproductively fecund, producing approximately 300 progeny, and simple to culture in bulk as they feed on *E. coli* bacteria and so can be colonized on Petri dishes (Fig. 7.1). They possess a transparent cuticle that allows live imaging of their internal processes, through both light and fluorescent microscopy. Transgenic animals are relatively easy to produce, by injection of DNA into the germline, by use of the MOS transposon recombination system, or through microparticle bombardment that can be successfully used to generate transgenic arrays (Granger et al. 2004; Kadandale et al. 2009; Praitis 2006). By this method, GFP- and RFP-tagged

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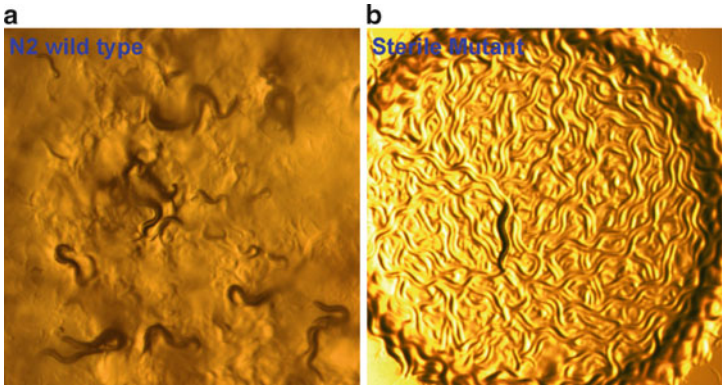

Fig. 7.1 (**a**) N2 wild-type population with worms in various stages of development. (**b**) An adult sterile hermaphrodite

proteins can be introduced and expressed under the direction of either their own or a tissue-specific promoter. Quite uniquely, the lineage of every somatic cell in the organism, both male and hermaphrodite, can be traced from fertilization to adult (Sulston and Horvitz 1977). In addition, *C. elegans* represents the first multicellular organism to have a completely sequenced genome, and now the genomes of several other species of *Caenorhabditis* and other nematodes have subsequently been published, making identification and study of orthologues easy (Coghlan et al. 2006; Sulston and Horvitz 1977).

Much pioneering work has been completed in the humble *C. elegans*, including seminal work in apoptosis and cell death, RNAi (RNA mediated interference), and fluorescent protein tagging (GFP) (Chalfie et al. 1994; Fire 1998; Hedgecock et al. 1983). *C. elegans* also presents an excellent model for the examination of mitosis and meiosis (Greenstein 2005; Kimble and Crittenden 2005; L'Hernault 2006).

7.1.2 C. elegans Reproductive Tract and Gametes

As a species, *C. elegans* exists primarily as hermaphrodites, with males arising approximately 1 in 1,000 births from nondisjunction of the X chromosome. Hermaphrodites are self-fertile, while males produce only sperm and are capable of fertilizing hermaphrodites to produce outcross progeny (Fig. 7.2a, b) (Ward and Carrel 1979). The hermaphrodite germline fills the body cavity in a bi-lobed tube. The germline begins with two DTC's (distal tip cells) one on the anterior and posterior dorsal axis (Austin and Kimble 1987). The DTC's provide a niche for the germ cells, which proliferate from dedicated germ line progenitor stem cells (Kimble and Crittenden 2005). Distal germ cells are maintained at mitosis by LAG-2, a protein ligand secreted by the DTC (Hubbard and Greenstein 2000; Kimble and Crittenden 2005). Regulation of cell cycle in the *C. elegans* germ

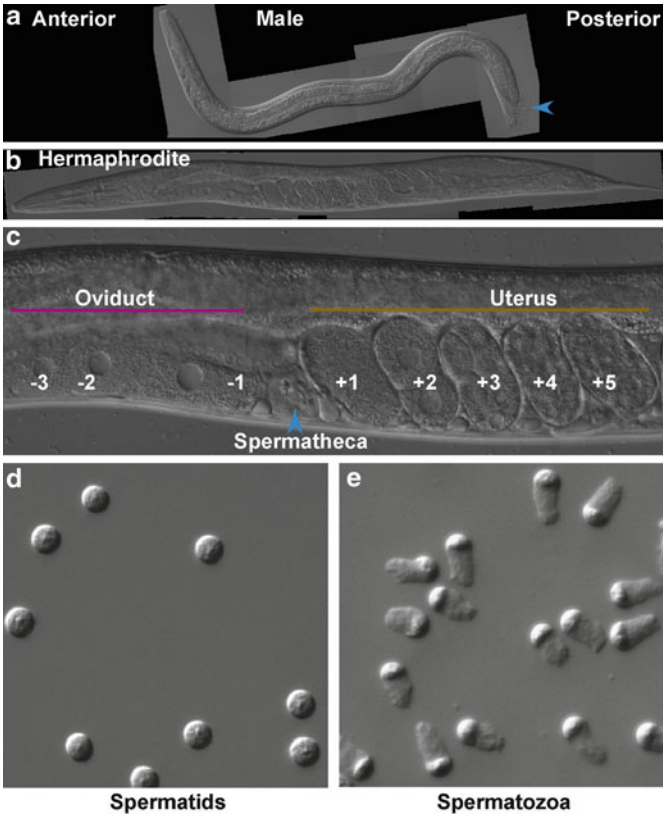

Fig. 7.2 (**a**) Adult male, wild-type N2 worm. (**b**) Adult hermaphrodite, wild-type N2 worm. (**c**) Close-up of adult hermaphrodite proximal germline. At left are proximal most oocytes, at center the spermatheca containing mature spermatozoa, at left fertilized and developing embryos. (**d**) Inactive spermatids dissected from a wild-type N2 male. (**e**) Activated spermatozoa dissected from a wild-type N2 male, and activated with pronase

cells is necessarily precise, and can be clearly visualized by DNA staining in fixed specimens or by live imaging of fluorescent-tagged histones. Distal germ cells progress in syncitium with one another in an organ called the rachis. As germ cell nuclei begin to differentiate, the germ cells begin to enter prophase of meiosis I in the transition zone which demarcates the change from mitosis to meiosis (Hubbard and Greenstein 2000; Kimble and Crittenden 2005). Well-defined regions of the rachis correspond sequentially to pachytene, diplotene, and diakinesis of prophase I (Hubbard and Greenstein 2000). *C. elegans* hermaphrodites produce first sperm during their last larval phase of development and then switch permanently to the production of oocytes. The amoeboid sperm are stored in each of two spermathecas, one corresponding to the anterior and posterior gonad arms (Fig. 7.2) (L'Hernault 2006). As oocytes approach the bend of the gonad arm, they individuate from the rachis and begin to accumulate yolk. The male of the species produces only sperm,

beginning in its last larval stage. Male germ cells develop in a single gonad arm extending from a single distal tip cell to the seminal vesicle (Klass et al. 1976). The male spermatids are stored unactivated, as round nonmotile spermatids, and introduced to the hermaphrodite through mating structures in the tail region. After their introduction through the hermaphrodite vulva, male-derived sperm quickly acquire motility, move to the spermatheca, and outcompete hermaphroditic self-sperm for fertilization of the oocytes (Ward and Carrel 1979).

In the *C. elegans* hermaphrodite, the movement and development of sperm and oocytes are precisely coordinated. Major sperm protein (MSP) is a core component of the sperm cytoskeleton; the protein is also secreted as a signal to both oocytes and the somatic germline (Harris et al. 2006; Kuwabara 2003). As oocytes progress sequentially closer to the spermatheca, they are often referred to numerically by position with the oocyte closest to the spermatheca referred to as -1 (Figs. 7.2 and 7.3). Oocytes receive the MSP signal from sperm such that the -1 oocyte is stimulated to undergo nuclear-envelope breakdown (NEBD) and cytoskeletal rearrangement in preparation for ovulation (Harris et al. 2006). The oocyte and sperm also signal to the overlying somatic sheath cells, a type of smooth muscle covering the oviduct, and the spermathecal valve dilates and forces the spermatheca to engulf the -1 oocyte (Figs. 7.2 and 7.3). Thus the -1 oocyte is ovulated, an event which occurs approximately every 23 min when sperm are abundant (McCarter et al. 1999; McNally and McNally 2005). Hermaphrodites produce a fixed number of sperm during the last larval stage; in the absence of sperm, the process of ovulation drops off dramatically occurring approximately once every 10 h (McCarter et al. 1999). However, introduction of sperm through fertilization by a male or artificial insemination causes resumption of robust ovulation rates and fertilization (LaMunyon and Ward 1994). Returning to our discussion of the cell cycle, the -1 oocyte undergoes NEBD and simultaneously reenters the meiotic cell cycle at metaphase I of meiosis I approximately 5 min before ovulation will occur (Fig. 7.3) (McCarter et al. 1999; McNally and McNally 2005).

The first ovulation events push the spermatids from the oviduct into the spermatheca (Fig. 7.2) (L'Hernault 2006). As the subsequent oocytes enter the spermatheca, they are surrounded by the amoeboid *C. elegans* spermatozoa. Molecules including *spe*-9 on the surface of the spermatozoa and *egg-1* and *egg*-2 on the egg are necessary for sperm/egg interaction and sperm entry (Kadandale et al. 2005; Putiri et al. 2004; Singson et al. 1998). When sperm are abundant, fertilization occurs quickly, and the newly fertilized embryo exits the spermatheca to complete embryogenesis.

In wild-type hermaphrodites, six pairs of sister chromatids, corresponding to the six *C. elegans* chromosomes, undergo meiosis I and extrude a polar body containing the excess genetic material (Fig. 7.3) (McNally and McNally 2005). Then six pairs of chromatids undergo the meiosis II division and extrude a second polar body. Finally, the female chromosomes decondense at the anterior pole of the embryo to form the female pronucleus, while on the posterior pole the male chromosomes decondense to form the male pronucleus. Both pronuclei will

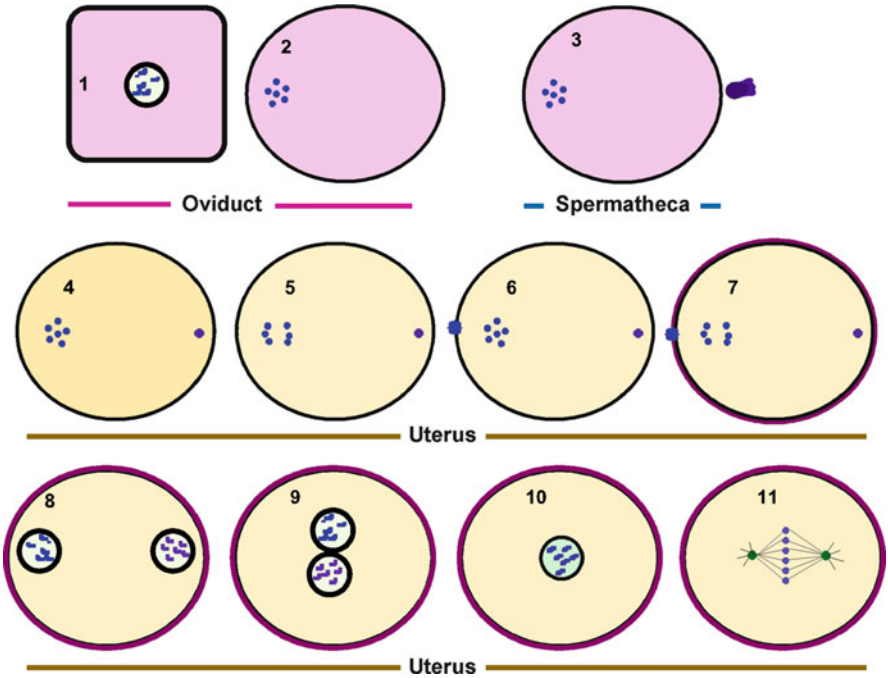

Fig. 7.3 The events of the oocyte-to-embryo transition. (1) The proximal most (-1) oocyte. (2) As the proximal most oocyte approaches the spermatheca it undergoes NEBD and cytoskeletal rearrangment. The chromosomes condense. (3) Contractions of the somatic gonad sheath and spermatheca dilation lead to ovulation of the oocyte. After spermathecal entry, the egg is rapidly fertilized by a single sperm. (4) Metaphase I of meiosis I. The chromosomes are arranged in a characteristic pentagonal array. The sperm DNA marks the presumptive posterior of the embryo. (5) Anaphase I of meiosis I. The chromosomes separate and a single polar body is extruded. (6) Metaphase II of meiosis II. (7) Anaphase II of meiosis II. By this stage a robust chitin rich eggshell has been extruded. (8) The male and female DNA decondense and form pronuclei. (9) Cytoskeletal rearrangements bring the pronuclei together, they fuse. (10) The one cell embryo contains a single nucleus. (11) The beginning of mitotic divisions

eventually fuse to form a single diploid nucleus, and the mitotic divisions of embryonic development will begin.

The processes that occur just prior to fertilization and before the start of embryogenesis are referred to collectively as the oocyte-to-embryo transition (Fig. 7.3) (Govindan and Greenstein 2007; Yamamoto et al. 2006). During these critical developmental stages, the oocyte completes a maternally provided program of development and switches to an embryonic one. This process occurs in many organisms, and yet the molecules involved in this process have not been well studied. It has long been recognized that the oocyte-to-embryo transition occurs in the absence of transcription (Evsikov and Marin de Evsikova 2009). Thus, cellular changes rely on translation of stored maternally provided mRNAs, modification of maternally provided proteins (adenylation, phosphorylation, etc), and the targeted

destruction of maternal proteins (Evsikov and Marin de Evsikova 2009). Yet, what mechanisms drive these changes? Complex machinery must coordinate a common set of events including resumption of meiosis and advancement through the cell cycle, the block to polyspermy, cytoskeletal rearrangements, the cortical granule reaction, apposition of the male and female pronuclei followed by their fusion in many nonmammalian species, and establishment of embryonic polarity (Fig. 7.3) (Ducibella and Fissore 2008). In mammals, the most studied regulatory mechanism involves the oscillation of intracellular calcium levels in the early embryo.

C. elegans presents an excellent model organism for parsing the molecular underpinnings of the ooctye-to-embryo transition. Every stage from undifferentiated stem cell, to fertilization competent egg, to developed embryo can be viewed progressively in vivo under light or fluorescence microscopy (Fig. 7.2c). Many fluorescent and antigenic markers are available for cellular compartments and components, including nuclear, plasma membrane, Golgi, and endosomal (Hadwiger et al. 2010; Sato et al. 2006). Genetic deletion mutants are freely available for study, in addition to the ease of RNAi in the germline. A primary strength of RNAi in the *C. elegans* germline is the ability to perform RNAi on larval stage hermaphrodites, knocking down their maternal complement of protein, and examining their germline function at adult stages. This allows for the investigation of many genes that would otherwise be embryonic lethal.

In addition to carrying out the same basic developmental processes as mammals and other organisms during the oocyte-to-embryo transition, *C. elegans* undergo the resumption of meiosis, cytoskeletal rearrangement, ovulation, fertilization, the block to polyspermy, completion of meiosis including the extrusion of two polar bodies, extrusion of a chitinous, trilamellar eggshell, formation of a female and male pronucleus, fusion of the two pronuclei, and the initiation of the mitotic divisions (Fig. 7.3) (McNally and McNally 2005). The genetic requirements for many of these processes have been partially explored. Much is known about how the *C. elegans* oocyte is signaled to undergo maturation and ovulation, fertilization, how meiotic and mitotic spindles are formed, how the chitinous eggshell is formed, and how the embryonic polarity is initially formed, but these subjects are well reviewed elsewhere (Gonczy and Rose 2005; Greenstein 2005; Johnston et al. 2006; Oegema and Hyman 2006; Sundaram 2006). The subject of our research has been the coupling of the progression of the oocyte-to-embyro transition to the entry of sperm and to the advancement of the cell cycle. Toward this end, we have elucidated several members of a gene module required for egg activation and the oocyte-to-embryo transition.

7.2 The Oocyte-to-Embryo Transition in *C. elegans*: EGG-3

The first lynchpin of the ooctye-to-embryo transition gene module is *egg-3* (F44F4.2), a gene required for maternal effect viability (Maruyama et al. 2007; Stitzel et al. 2007). This gene was identified in a screen of fertility genes performed by the Sugimoto lab (Maeda et al. 2001; Maruyama et al. 2007). A hallmark of loss

of function mutant genes required for function of sperm, late oocytes, or early embryos is the production of many unshelled, nondeveloping oocytes (Singson et al. 2008). Mutations in genes required for sperm activation (spermiogenesis) or sperm/oocyte fusion prevents fertilization from occurring. In this situation, when observing DAPI-stained embryos newly released from the spermatheca, the oocyte nuclei are visible, but the corresponding sperm nuclei are absent. However, sperm entry occurred in *egg-3* embryos, and sperm nuclei were found localized to the presumptive posterior of the embryo as in a wild type embryo. Studies of *egg-3* were carried out in tandem with a knockout supplied by National Bioresource Project for the Experimental Animal "Nematode *C. elegans*" (NBP) Japan *egg-3* (*tm1191*) and with knockdown by soaking-based RNAi. Results in all cases were identical. Knockdown of *egg-3* resulted in oocyte-specific lethality (Maruyama et al. 2007). Spermatogenesis and oogenesis were phenotypically normal, and no defects were visible until the one-cell stage of the embryo.

In *egg-3* mutant hermaphrodites, the first visible defects occur just after fertilization. A severe defect is observed in *egg-3* when the embryo fails to extrude a polar body at the conclusion of meiosis I (Maruyama et al. 2007). This leads to an abnormal embryo in which 12 pairs of sister chromatids undergo the division of meiosis II, before failing to extrude the second polar body. The female chromosomes eventually decondense to form a female pronucleus, which will fuse with the male pronucleus. However, as with other fertility defects, the DNA undergoes constant rounds of mitosis without cytokinesis leading to the formation of endomitotic embryos (Maruyama et al. 2007).

As previously mentioned, a highly visible marker for hermaphrodite fertility is the presence of the chitin containing eggshell. In *C. elegans*, the eggshell is trilamellar with a thin vitelline layer facing outside, a chitin containing center layer, and an interior proteolipid layer, which will eventually house the first polar body (Johnston et al. 2006). In wild type, eggshell deposition begins just after fertilization, with the chitin layer being robustly detectable during meiosis I (Fig. 7.3). Eggshell deposition fails both in sperm and oocyte mutants, including mutants causing deficiencies during the ooctye-to-embryo transition such as *egg-3,* which lacks a detectable chitin layer at the conclusion of meiosis I (Johnston et al. 2006; Maruyama et al. 2007; Singson et al. 1998).

The third notable defect in *egg-3* mutants occurs during cytoskeletal rearrangement after fertilization. In wild-type embryos, the actin cytoskeleton rearranges after fertilization such that a thick focal cap of actin forms under the plasma membrane directly over the site of sperm entry (Maruyama et al. 2007). Subsequently in wild-type embryos, the actin cap disperses throughout the posterior half of the embryo. In *egg-3* mutant embryos, an actin cap forms as normal at the site of sperm entry. However, the actin filaments disperse abnormally throughout both posterior and anterior halves of the embryo (Maruyama et al. 2007). The consequences of this aberrant dispersal to the embryo are unknown.

The protein structure of EGG-3 was analyzed for further clues to its activity, and was found to contain the sequence of a protein-tyrosine phosphatase (Maruyama et al. 2007; Stitzel et al. 2007). However, protein-tyrosine phosphatases require the

presence of three key amino acid residues for their catalytic activity, an aspartate, a glutamate, and a cysteine, and upon closer examination the EGG-3 protein lacked two of these residues: the aspartate and the glutamate. This sequence change indicated that the EGG-3 protein was most likely catalytically inactive, but should retain its ability to bind phosphorylated substrates. This made EGG-3 a member of a growing group of proteins referred to as protein-tyrosine phosphate-like (PTPL), or as antiphosphatases (pseudophosphatases). Previously examined molecules of this class include the Pasticcino2 gene in Arabidopsis, which is required for the regulation of cell cycle through direct interaction with cyclin dependent kinase (CDK), mouse and human genes such as *Ptpla* which is required for development, and another *C. elegans* gene, *sdf-9* which is involved in dauer formation (Bellec et al. 2002; Da Costa et al. 2006; Jensen et al. 2007; Uwanogho et al. 1999). The structure of PTPL domains suggests two intriguing possibilities for regulating the activity of other genes. In the first scenario, the PTPL containing protein could bind to a phosphorylated target protein and then hold that protein as part of a complex with other proteins acting as a molecular scaffold (Govindan and Greenstein 2007). In the second scenario, the PTPL containing protein could bind to a phosphorylated target protein and then limit the target protein's access to active phosphatases acting as a competitive inhibitor. Determination of EGG-3's role as either scaffold or inhibitor would require further molecular analysis.

In order to examine the localization of the *egg-3* protein, GFP and RFP (mcherry) fusion tagged proteins were generated by microparticle bombardment. Fusions for tissue-specific expression were driven by the *pie-1* promoter, which expresses in the germline of *C. elegans* hermaphrodites. It was determined that EGG-3 localizes to the cytoplasm directly below the plasma membrane, often referred to as the cortex, of developing oocytes (Fig. 7.4) (Maruyama et al. 2007; Stitzel et al. 2007). After fertilization, and during meiosis I, the protein undergoes a dynamic shift in localization, moving to cytoplasmic puncta and then being degraded (Fig. 7.4) (Maruyama et al. 2007; Stitzel et al. 2007). The dynamic spatial localization of EGG-3 indicated that the protein's purpose might shift during meiosis I.

In order to determine the epistatic relationship between *egg-3* and the developmental pathway, we examined its interactions with other genes either involved in the oocyte-to-embryo transition or with similar patterns of localization. The associations between EGG-3 and many other genes were analyzed, with the predominant associations occurring with CHS-1 and MBK-2.

Gene *chs-1* (T25G3.2) was selected for analysis due to its integral role in the extrusion of the chitinous eggshell after fertilization, a process that fails in *egg-3* mutants (Johnston et al. 2006; Zhang et al. 2005). The gene encodes a large multipass transmembrane protein, chitin synthase, which is responsible for catalyzing the production of chitin from UDP-N-acetylglucosamine (UDP-GlcNAc). This chitin is required as a critical component of the center layer of the trilamellate eggshell. In addition to the requirement for CHS-1 during eggshell production, knockdown of CHS-1 through gene-specific RNAi leads to defects during meiosis and polar body formation, identical to those seen in *egg-3* mutant hermaphrodites (Johnston et al. 2006).

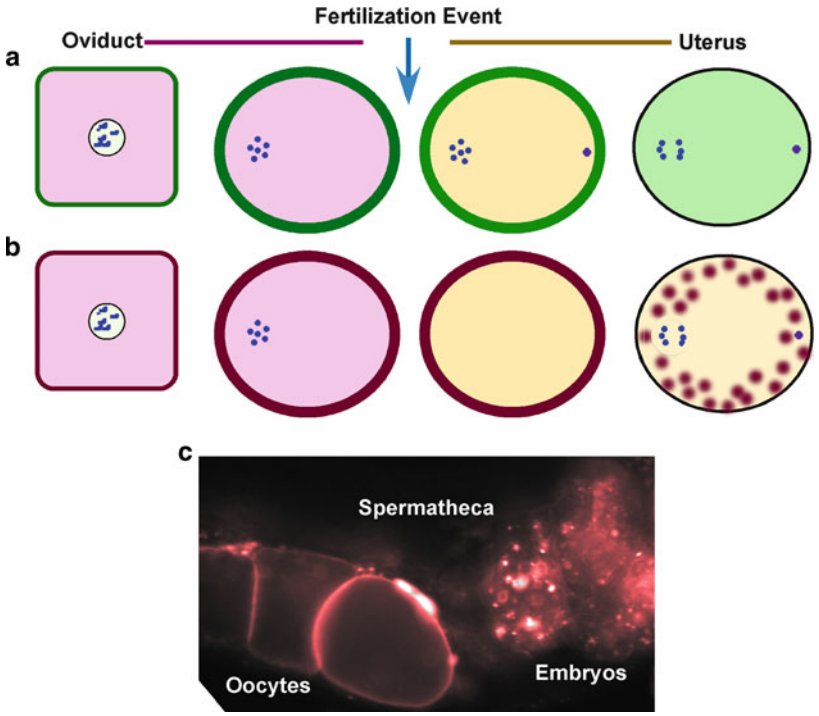

Fig. 7.4 (**a**) The EGG-4 and EGG-5 proteins rings the oocyte cortex. Again, the heaviest confluorescence occurs as the oocyte undergoes nuclear envelope breakdown. The protein remains at the cortex until the transition between metaphase I and anaphase I of meiosis I. At that point the protein washes across the cell in a distinct wave, becoming diffusely cytoplasmic. (**b**) The EGG-3, CHS-1, and MBK-2 proteins rings the oocyte cortex. The heaviest confluorescence occurs as the oocyte undergoes nuclear envelope breakdown. The protein remains at the cortex until the transition between metaphase I and anaphase I of meiosis I. At that point the protein forms discrete cortical puncta. (**c**) The hermaphrodite reproductive tract centered on the proximal most oocytes, spermatheca, and newest embryos. The animal contains a RFP:EGG-3 fusion protein

Also chosen for close examination was gene *mbk-2*, a kinase important for degrading maternal proteins during the oocyte-to-embryo transition and known to have an identical pattern of protein localization to EGG-3 (Raich et al. 2003). In an example of coordinate regulation, *mbk-2* is responsible for phosphorylating and marking for degradation *mei-1*, *oma-1* and *oma-2*, and *mex-5* and *mex-6* proteins important for early postfertilzation processes, which must be destroyed before the transition to later embryonic development (Nishi and Lin 2005; Pellettieri et al. 2003; Quintin et al. 2003; Stitzel et al. 2007). The *mei-1* gene encodes an orthologue of the catalytic subunit of katanin, which acts as a microtubule severing protein during meiosis but not mitosis. Thus, the MEI-1 protein has a function tightly linked to the oocyte-to-embryo transition, as its function is required during the completion of meiosis, but is deleterious to the functioning of the mitotic cycles, which mark the beginning of embryonic development (Pellettieri et al. 2003;

Quintin et al. 2003; Yang et al. 2003). Similarly, the *oma-1* and *oma-2* genes, which are redundant, encode zinc finger proteins, which are necessary for setting up correct cell fate specification during early embryogenesis (Detwiler et al. 2001; Lin 2003; Pellettieri et al. 2003). Persistence of the OMA-1 or OMA-2 proteins past the first mitotic division lead to adoption of the incorrect cell fates and embryonic lethality, again making the precise and timely degradation of OMA-1 and OMA-2 critical to early embryonic development (Lin 2003). Finally, the MEX-5 and MEX-6 proteins are partially redundant proteins required for polarization at the one cell stage of the embryo. The MEX-5 and MEX-6 proteins are localized to the anterior cytoplasm of the embryo where they control the localization of other maternal proteins such as PIE-1, POS-1, and MEX-1 (Nishi et al. 2008; Schubert et al. 2000). Therefore, the highly precise temporal and spatial action of *mbk-2* is necessary to progression through the oocyte-to-embryo transition. However, knockdown of *mbk-2* leads to less severe embryonic defects than depletion of *egg-3* or *chs-1*. In the absence of *mbk-2*, the embryo completes a phenotypically normal meiosis and extrudes the chitin-containing eggshell. In *mbk-2* hermaphrodites, embryos die from the combined defects caused by aberrant functioning of MEI-1, OMA-1, OMA-2, MEX-5, and MEX-6 (Stitzel et al. 2007).

The spatial and temporal localizations of the three proteins, EGG-3, CHS-1, and MBK-2 were examined using integrated GFP- or RFP-tagged fusion proteins, and found to be identical during late oogenesis and early embryogenesis, indicating that a direct interaction between the proteins was possible (Maruyama et al. 2007; Stitzel et al. 2007). Epistasis analysis was performed in order to elucidate whether the localizations of the three proteins were dependent on one another. Using gene-specific RNAi, it was determined that the localizations of EGG-3 and CHS-1 fusion proteins were interdependent, such that knocking down EGG-3 by RNAi lead to aberrant localization of CHS-1 and vice versa (Maruyama et al. 2007). The localization of MBK-2 was dependent on the presence of both EGG-3 and CHS-1 (Maruyama et al. 2007). While neither EGG-3 nor CHS-1 localizations were affected by the absence of MBK-2 (Maruyama et al. 2007). This result was anticipated because knockdown of MBK-2 leads to less severe defects than knockdown of EGG-3 or CHS-1, as meiosis is completed as in wild type, and the chitin containing eggshell is formed. Concurrently, it was shown that EGG-3 and MBK-2 have a direct physical interaction by coimmunoprecipitation of either protein (Stitzel et al. 2007). However, the codependence of these proteins does not speak to their precise dynamic localization, which in the case of MBK-2 is critical for its temporal catalytic activity. Therefore, it was critical to determine the trigger for dynamic relocalization of these proteins. A double-tagged strain containing RFP:EGG-3 and GFP:HIS (histone H2B) was constructed, and live imaging with this strain showed that the redistribution of EGG-3, and by extension CHS-1, and MBK-2 occurred somewhere between metaphase I and anaphase I of meiosis I (Maruyama et al. 2007). In order to verify the timing of this event, anaphase promoting complex (APC) component *mat-1* was depleted by RNAi in GFP:EGG-3, GFP:CHS-1, and GFP:MBK-2 backgrounds (Maruyama et al. 2007; Stitzel et al. 2007). Not surprisingly, abrogation of an APC subunit arrested the fertilized embryos at metaphase of

meiosis I and caused retention of all three proteins at the cortex. Therefore, the dynamic redistribution of EGG-3, CHS-1, and MBK-2 relies on the advancement of the cell cycle (Maruyama et al. 2007; Stitzel et al. 2007). Conversely, depletion of EGG-3, CHS-1, or MBK-2 did not have an effect on the depletion of GFP:CYB-1, which is an integral part of the machinery regulating entry into meiosis I. This indicated that EGG-3, CHS-1, and MBK-2 were not required for cell cycle progression during meiosis.

Both EGG-3 and CHS-1 are required for the localization of MBK-2 during development; therefore, it remained to determine the precise molecular interactions between the proteins. It was determined that both the EGG-3 and MBK-2 proteins could be coimmunoprecipitated by the other protein, indicating a direct physical interaction (Stitzel et al. 2007). In addition, it was shown that while depletion of APC component MAT-1 lead to retention of MBK-2 at the cortex, simultaneous depletion of MAT-1 and EGG-3 shifted the MBK-2 protein from the cortex to the cytoplasm, allowing it to carry out its biochemical activity (Cheng et al. 2009; Stitzel et al. 2007). The implications of this experiment are twofold. First, this indicates that the EGG-3 protein plays a direct role in holding the complex at the cortex until signaled by cell cycle progression to redistribute. Second, this indicates that EGG-3 does not directly regulate the biochemical activity of MBK-2, rather EGG-3 only regulates the spatial and temporal localization of MBK-2. Finally, simultaneous depletion of CDK-1 and EGG-3 did not alter the timing of degradation of MBK-2 target MEI-1 (Cheng et al. 2009). This indicates that CDK-1 does not regulate MBK-2 through the scaffolding molecule EGG-3.

7.3 A New Player in the Complex: EGG-4/5

It was clear that EGG-3 alone could not account for the complex regulation of MBK-2. While abrogation of EGG-3 caused premature degradation of MBK-2 targets, it did not alter MBK-2's catalytic activity (Stitzel et al. 2007). Although mislocalization of CHS-1 might be key in preventing extrusion of the chitinous eggshell, none of the proteins elucidated thus far could be pegged absolutely as the cause of failure during polar body extrustion (Johnston et al. 2006; Maruyama et al. 2007; Stitzel et al. 2007). In addition, it was as yet unknown what factors might link EGG-3, CHS-1, and MBK-2 to the advancement of the cell cycle. Therefore, further protein-tyrosine phosphatase-like proteins were examined in the hopes of finding a second such gene, which might play a role during the oocyte-to-embryo transition. This led to the identification of proteins EGG-4 and EGG-5 (Cheng et al. 2009; Parry et al. 2009).

Genes *egg-4* (T21E3.1) and *egg-5* (R12E2.10) were identified as PTPL motif containing proteins based on their homology with *egg-3* and other antiphosphatases (Parry et al. 2009). Outside the PTPL domains, EGG-4 and EGG-5 amino acid sequences do not contain any other obvious homologies. The genes bear a high degree of sequence similarity being 99.2% identical and containing only a single

significant alteration in amino acid. The activity of the genes was examined both through deletion alleles available from NBP Japan, and through the use of soaking based RNAi. Individually, the *egg-4* or *egg-5* genes retain partial fertility, producing broods that are 76–78% of wild type sizes (Parry et al. 2009). A double mutant containing both the *egg-4* and *egg-5* deletion alleles was constructed, and found to be completely sterile (Parry et al. 2009). The partial redundancy in function of the genes coupled with their close proximity on the chromosome (0.3 cM apart), their high degree of sequence similarity, and the presence of only a single homologue in other species of *Caenorhabditis* supports the hypothesis that genes *egg-4* and *egg-5* represent a gene duplication and that the genes are predominantly redundant.

Knockdown of *egg-4/5* leads to oocyte-specific lethality (Parry et al. 2009). The *egg-4/5* hermaphrodites produce phenotypically normal sperm and oocytes, which meet and fertilize in the spermathecal compartment as in wild type. However, no cell divisions occur after fertilization and the fertilized but nondeveloping embryo becomes filled with endomitotic nuclei identical to those observed in *egg-3* mutants (Maruyama et al. 2007; Parry et al. 2009). Most *egg-4/5* defects phenocopy the oocyte-to-embryo transition defects seen in *egg-3* and *chs-1* mutants. Knockdown of EGG-4/5 prevents extrusion of the polar bodies, leading to an abnormal meiosis in which 12 sister chromatids undergo the second meiotic division. This, in turn, gives rise to a fertilized nondeveloping embryo, which contains multiple endomitotic nuclei in syncitium (Maruyama et al. 2007; Parry et al. 2009). In addition, a chitin staining assay shows that knockdown of EGG-4/5 also prevents formation of the tri-lamellar eggshell. Knockdown of EGG-4/5 also causes defects in the actin cytoskeleton, which are more severe than the defects present in EGG-3 mutants, in which a robust actin cap does not form at the site of sperm entry, but remains diffuse across the posterior end of the plasma membrane and eventually diffuses across both anterior and posterior poles (Parry et al. 2009).

A defect thus far unique to knockdown of EGG-4/5 is the entry of multiple sperm into a single oocyte (polyspermy). Polyspermy has never been documented before in *C. elegans* hermaphrodites, indicating that the block to polyspermy in this species is generally quite robust. However, at a rate of 15% in EGG-4/5 RNAi knockdown mutants and 25% in EGG-4/5 double mutants, two or more sperm nuclei were visible in a single egg cytoplasm. Little is known about the block to polyspermy in *C. elegans,* making the *egg-4/5* genes a rare peak into the functioning of this process.

In order to determine the localization of EGG-4/5, GFP-tagged fusions of the EGG-4 and EGG-5 proteins were constructed by microparticle bombardment as with the EGG-3 fusion proteins. In addition, peptide antibodies were raised against the EGG-4 and EGG-5 proteins to confirm localization of the protein fusions (Cheng et al. 2009; Parry et al. 2009). It was anticipated that the EGG-4/5 proteins would colocalize with EGG-3, which held true for the proteins during late oogenesis when the proteins localize to the cortex of the developing oocyte (Fig. 7.4). However, rather than forming discrete cortical puncta at the transition between metaphase I and anaphase I of meiosis I, the EGG-4/5 proteins either become diffusely cytoplasmic or are degraded (Fig. 7.4) (Parry et al. 2009). The difference

in EGG-4/5 localization after fertilization could reflect divergent functions after fertilization from EGG-3. Further, if EGG-4/5 forms a complex with EGG-3, CHS-1 and MBK-2 in developing oocytes, the proteins must part ways during the first meiotic division.

Once again it was determined that preventing advancement of the cell cycle past metaphase I by depleting APC component MAT-1 lead to aberrant retention of GFP:EGG-4 and GFP:EGG-5 to the oocyte cortex (Cheng et al. 2009; Parry et al. 2009). Furthermore, depletion of cyclin-dependent kinase CDK-1 also caused aberrant retention of EGG-4/5, EGG-3, CHS-1, and MBK-2 to the cortex (Cheng et al. 2009). The *cdk-1* gene is required for meiotic reentry of the oocyte at metaphase I and is antagonized by kinase *wee-1* (Burrows et al. 2006). Contrary to the effects of depleting MAT-1 or CDK-1, depletion of WEE-1 caused premature relocalization of EGG-4/5, EGG-3, CHS-1, and MBK-2 away from the cortex (Cheng et al. 2009; Stitzel et al. 2007). Finally, depletion of EGG-4/5 had no effect on the degradation of GFP:CYB-1, and thus no effect on advancement of the cell cycle through meiosis (Parry et al. 2009). Therefore, the dynamic redistribution of EGG-4/5 is cell cycle dependent. Conversely, advancement of the cell cycle occurs in the absence of EGG-4/5.

The EGG-3, CHS-1, and MBK-2 proteins all experience a complex interdependence on one another; therefore, the EGG-4/5 proteins might similarly be required for the localization of one or more of the other proteins. In order to examine this possibility, the localizations of EGG-3, CHS-1, and MBK-2 GFP- or RFP-tagged fusion proteins were examined in wild-type and EGG-4/5 RNAi knockdown backgrounds (Parry et al. 2009). It was determined that the presence of EGG-4/5 is required for the dynamic relocalization of EGG-3 and CHS-1 during the first meiotic division. Similar to the requirement for the functioning of the APC during redistribution, depletion of EGG-4/5 caused aberrant retention of EGG-3 and CHS-1 to the cortex after fertilization. This differs from the interdependence between EGG-3 and CHS-1 in which each gene is required for the localization of the other at the cortex during oocyte development (Maruyama et al. 2007; Parry et al. 2009). In addition, the localization of MBK-2 during late oogenesis and early embryogenesis requires EGG-4/5 as well as EGG-3, and CHS-1, and conversely depletion of MBK-2 has no effect on localization or dynamics of EGG-4/5 (Maruyama et al. 2007; Parry et al. 2009). Again, knockdown of MBK-2 causes less severe phenotypes than knockdown of EGG-4/5, complementing the results of the epistasis analysis.

This accumulated wealth of epistatic relationships would not be complete without proof of direct physical interactions between molecules. The yeast-two-hybrid system was used to investigate interactions between EGG-4/5, EGG-3, and MBK-2 (Parry et al. 2009). All three molecules were examined in both context of bait and prey. It was determined that EGG-4 and EGG-3, EGG-4 and MBK-2, and EGG-3 and MBK-2 interacted with one another (Parry et al. 2009). The interactions between EGG-4 and MBK-2 and EGG-3 and MBK-2 were corroborated by evidence from the Seydoux lab, which showed that these molecules interact in a coimmunoprecipitation assay (Cheng et al. 2009). It had already been shown that abrogation of MAT-1 led to retention of EGG-4/5, EGG-3, CHS-1, and MBK-2 at

the plasma membrane (Cheng et al. 2009; Maruyama et al. 2007; Parry et al. 2009; Stitzel et al. 2007), while simultaneous abrogation of both MAT-1 and EGG-3 released MBK-2 into the cytoplasm (Cheng et al. 2009; Stitzel et al. 2007). Therefore, if EGG-3 were acting as a putative scaffolding molecule for EGG-4/5, CHS-1, and MBK-2, what molecular function was EGG-4/5 performing in this complex? Again the answer could be found in the link between the dynamic distributions of EGG-4/5 and the advancement of the cell cycle.

Like APC component MAT-1, knocking down activity of cyclin-dependent kinase CDK-1 leads to retention of EGG-4/5, EGG-3, CHS-1, and MBK-2 at the cortex. However, as discussed above, unlike MAT-1, simultaneous depletion of EGG-3 and CDK-1 does not remove MBK-2 to the cytoplasm (Cheng et al. 2009). What then is the regulatory relationship between CDK-1 and the complex? Evidence from kinase assays and coimmunoprecipitation assays shows that cyclin-dependent kinase CDK-1 is capable of phosphorylating MBK-2 at S68 (Cheng et al. 2009). Furthermore, wild-type GFP:MBK-2 but not an unphosphorylatable form GFP:MBK-2(S68E) was able to rescue the lethality of *mbk-2(pk1427)* (Cheng et al. 2009). Therefore, CDK-1-dependent phosphorylation of MBK-2 is necessary for its cellular activity. However, CDK-1 becomes active during late oogenesis before the oocyte has made its resumption of meiosis I, and as previously mentioned it is critical that MBK-2 does not carry out its catalytic functions in developing oocytes or the very early embryo. A mechanism must be in place to prevent premature activity of MBK-2.

The role of inhibitor for MBK-2 seems to be fulfilled by EGG-4/5. As mentioned above, EGG-4 and MBK-2 were found to interact both by yeast-two hybrid, coimmunoprecipitation assay, and GST/FLAG pull down assay (Cheng et al. 2009). The specific phosphorylation of the MBK-2 active loop also increased the efficiency of the binding reaction between EGG-4/5 and MBK-2. Additionally, evidence from in vitro kinase assays also indicates that MBK-2 kinase activity, phosphorylating target MEI-1, is inhibited by the presence of EGG-4/5 (Cheng et al. 2009). Therefore, rather than acting as a scaffolding molecule like fellow PTPL protein EGG-3, EGG-4/5 seems to behave as a mixed inhibitor for MBK-2 (Tonks 2009).

7.4 Perspectives

The process of completing the transition between fully developed, mature oocyte, and fertilized developing embryo requires precise spatial and temporal control of molecular events. In short order, the *C. elegans* oocyte is contacted by a protein signal from sperm in the nearby spermatheca to complete the process of maturation, including nuclear envelope breakdown, cytoskeletal rearrangment, and resumption of meiosis I from prophase to metaphase I. As maturation is completed, the oocyte immediately enters the spermatheca where it should be fertilized by a single sperm before passing into the uterus. What follows is a highly complex series of events that allow the newly fertilized embryo to complete its transition from maternal

program to embryonic program. The complex module of proteins described in these pages is required for coupling the precise timing of necessary transitory events to the advancement of the cell cycle. The genes *egg-3, chs-1,* and *egg-4/5* are minimally required for production of the chitin eggshell during meiosis, an error-free meiosis and polar body extrusion, and in the case of *egg-3* and *egg-4/5* a normal formation and/or distribution of the cytoskeletal actin cap. All of these genes, *egg-3, chs-1,* and *egg-4/5*, are also required for the precise spatial and temporal localization of regulatory kinase *mbk-2*. Because *mbk-2* is required to mark several time sensitive maternal proteins for degradation, its localization and activity must be precisely restricted during the oocyte-to-embryo transition. To this end, several interrelated regulatory mechanisms have been elucidated. First, it has been determined that the redistribution of *egg-3*, *chs-1*, *mbk-2*, and *egg-4/5* depends on the advancement of the cell cycle past metaphase I and that the redistribution is signaled in an EGG-3-dependent manner. It has also been shown that the activity of MBK-2 depends on phosphorylation by cyclin-dependent kinase CDK-1, a protein that becomes active during late oogenesis. However, it is critical that MBK-2 does not have access to its targets until the completion of meiosis. Therefore, a second regulatory protein, in the form of EGG-4/5, is required for holding MBK-2 and preventing its premature activity. This complex protein module, EGG-3, EGG-4/5, CHS-1, MBK-2, and CDK-1, represents a complex and novel method of regulating the transition between maternal oocyte programming and embryonic programming. Given the high degree of complexity inherent in this model, it is predicted that other molecular regulators and effectors must be active during the oocyte-to-embryo transition. Genetic and biochemical analyses are currently underway to seek out new players in this highly dynamic process.

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Chapter 8
Cell Cycle in Ascidian Eggs and Embryos

Alex McDougall, Janet Chenevert, Karen W. Lee, Celine Hebras, and Remi Dumollard

Abstract In ascidians the cell cycle machinery has been studied mainly in oocytes while ascidian embryos have been used to dissect the mechanism that controls asymmetric cell division (ACD). Here we overview the most specific and often exceptional points and events in cell cycle control in ascidian oocytes and early embryos. Mature stage IV eggs are arrested at metaphase I due to cytostatic factor (CSF). In vertebrates, unfertilized eggs are arrested at metaphase II by CSF. Meta II-CSF is mediated by the Mos/MEK/MAPK/Erp1 pathway, which inhibits the ubiquitin ligase APC/C^{cdc20} preventing cyclin B destruction thus stabilizing MPF activity. CSF is inactivated by the fertilization Ca^{2+} transient that stimulates the destruction of Erp1 thus releasing APC/C^{cdc20} from inhibition. Although many of the components of CSF are conserved between the ascidian and the vertebrates, the lack of Erp1 in the ascidians (and indeed other invertebrates) is notable since the Mos/MAPK pathway nonetheless mediates Meta I-CSF. Moreover, since the fertilization Ca^{2+} transient targets Erp1, it is not clear how the sperm-triggered Ca^{2+} transient in ascidians (and again other invertebrates) stimulates cyclin B destruction in the absence of Erp1. Nonetheless, like mammalian eggs, sperm trigger a series of Ca^{2+} oscillations that increases the rate of cyclin B destruction and the subsequent loss of MAPK activity leading to meiotic exit in ascidians. Positive feedback from MPF maintains the Ca^{2+} oscillations in fertilized ascidian eggs ensuring the eventual loss of MPF stimulating the egg-to-embryo transition. Embryonic cell cycles in the ascidian are highly stereotyped where both the rate of cell division and the orientation of cell division planes are precisely controlled. Three successive rounds of ACD generate two small posterior germ cell precursors at the 64 cell stage. The centrosome-attracting body (CAB) is a macroscopic cortical structure visible by light microscopy that causes these three rounds of ACD. Entry into mitosis activates the CAB causing the whole mitotic spindle to rotate and migrate toward the cortical CAB leading to a highly ACD whereby one small cell is formed that

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inherits the CAB and approximately 40 maternal *postplasmic/PEM* RNAs including the germ cell marker *vasa*.

8.1 Introduction to Ascidians and the Urochordates/Tunicates

Ascidians belong to the subphylum urochordata (or Tunicates) and are now thought to be the closest extant relatives of the vertebrates (Fig. 8.1 and Delsuc et al. 2006; Singh et al. 2009). Adult ascidians are sessile marine invertebrate filter feeders, which in their adult form do not resemble chordates (Fig. 8.1a).

However, ascidians form a tadpole larva (Fig. 8.1e), which has chordate structures such as a notochord and dorsal nerve tube. Ascidians have been favorites of embryologists for more than a century (Chabry 1887). A number of excellent reviews are available for those interested in ascidian embryonic development (see review by Satoh 1994, 2003; Corbo et al. 2001; Kumano and Nishida 2007; Sardet et al. 2007; Lemaire et al. 2008; Sobral et al. 2009). Since the development

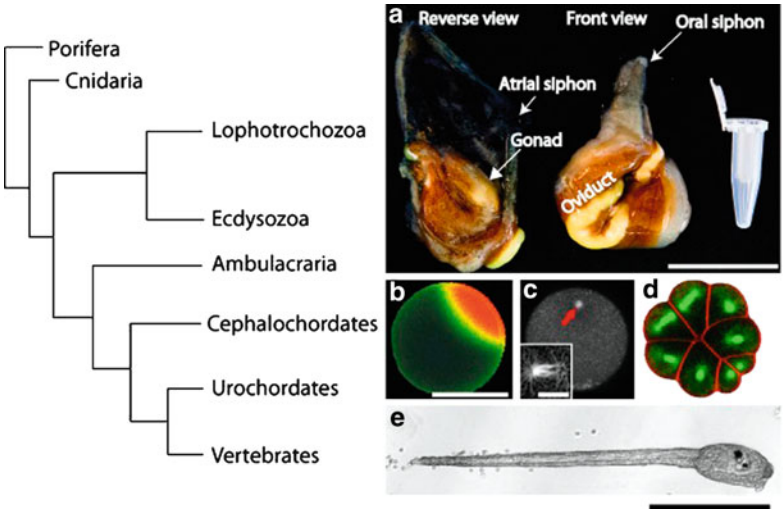

Fig. 8.1 Urochordates and the ascidian *Phallusia mammillata*. The urochordates are the closest living relative of the vertebrates [adapted from Singh et al. (2009)]. (**a**) The two sides of *Phallusia* following removal from its tunic (the tunic is a protective layer made of cellulose and chitin). The gonad (comprising ovary and testes) is found on the opposite side of the animal from the prominent oviduct. Mature eggs give the yellow color to the oviduct. The sperm-duct is obscured by the oviduct. Full animals contain around 100,000 eggs and about 1 mL of sperm. *Scale bar* = 5 cm. (**b**) Beginning of the sperm-triggered Ca_i^{2+} wave at fertilization (elevated Ca_i^{2+} is *red*). (**c**) Meiotic spindle (*red arrow*) labeled with the microtubule binding domain of MAP7 coupled to EGFP. (**d**) Confocal image of a 16 cell stage embryo whose microtubules are labeled *green* with MAP7::EGFP and the plasma membrane with the *red* lipophilic dye FM4-64. *Scale bar* = 100 μm for (**b**) and 5 μm for (**c**) *inset*. (**e**) Brightfield image of a tadpole larva approximately 15 h after fertilization. *Scale bar* = 100 μm

of the fate map over a century ago (Conklin 1905), many resources have been produced by the ascidian community. The initial effort for the development of online resources came from Satoh's group (Kyoto University, Japan) which culminated in the sequencing and extensive annotation of the full *Ciona intestinalis* genome by JGI (http://genome.jgi-psf.org/ciona4/ciona4.home.html, see Dehal et al. 2002). This revealed that the nonduplicated genome of *Ciona* (160 Mb) has approximately 16,000 genes. Other extremely useful databases are housed at Aniseed (http://aniseed-ibdm.univ-mrs.fr/, see Tassy et al. 2006 and the spectacular 3D virtual embryo at aniseed), Ghost (http://ghost.zool.kyoto-u.ac.jp/, see Satou et al. 2005) and DBTGR (http://dbtgr.hgc.jp/, see Sierro et al. 2006). Extensive film archives showing ascidian development (http://biodev.obs-vlfr.fr/recherche/biomarcell/, see Sardet et al. 2007) and 3D reconstruction of different embryonic stages are also available (http://chordate.bpni.bio.keio.ac.jp/faba/1.1/top.html, see Hotta et al. 2007). In addition to these online resources, germline transgenesis (see review by Sasakura 2007), electroporation (Corbo et al. 1997), knockdown using morpholinos (see Christiaen et al. 2009 for a review), blastomere manipulation (see Nishida and Sawada 2001 and other articles by Nishida), a large collection of "Gateway" reporter constructs (Roure et al. 2007), live cell imaging from mRNA encoding GFP reporter constructs in eggs (Levasseur and McDougall 2000), and early embryos (Prodon et al. 2010) are methods that are routinely applied in the ascidian model. Three species of ascidian are commonly used: *Ciona intestinalis* which is cosmopolitan and is the most popular, the Japanese ascidian *Halocynthia roretzi* which has large eggs and embryos that are ideal for micromanipulation and the European ascidian *Phallusia mammillata* which has completely transparent eggs and embryos that are ideal for imaging. *Ciona* and *Phallusia* both belong to the same order (Enterogona) while *Halocynthia* belongs to the Pleurogona. In this review we will focus on what is known about how the cell cycle is controlled from oogenesis and fertilization through early embryonic development.

8.2 Cell Cycle During Oogenesis and Fertilization

8.2.1 Oogenesis

In the ascidians, four stages of oogenesis have been described based on oocyte size, yolk content and pigmentation, cortical endoplasmic reticulum (cER) distribution, mitochondrial distribution, and DNA condensation status as well as follicular cell morphology (Fig. 8.1 and Prodon et al. 2006; Swalla et al. 1991; Jeffrey and Capco 1978). Stage I oocytes are previtellogenic and most likely represent preleptotene to pachytene stage oocytes (Fig. 8.2). Stage II is the vitellogenic stage during which oocyte volume increases dramatically.

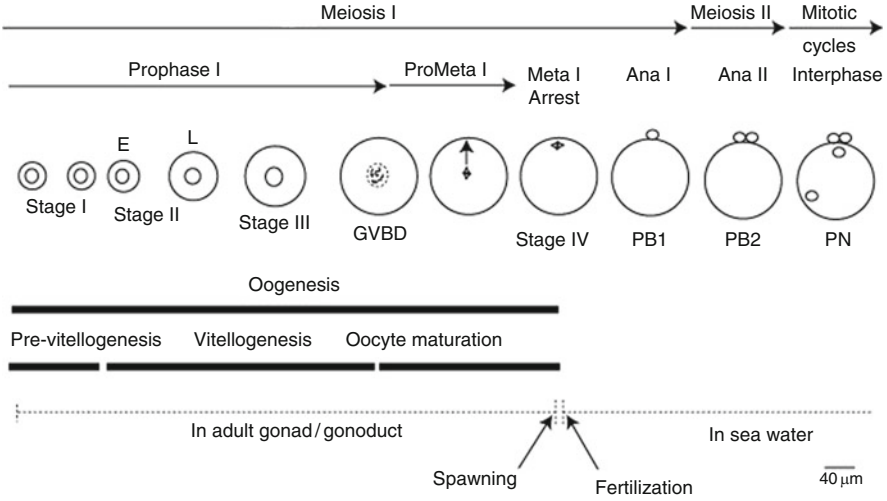

Fig. 8.2 Oogenesis and egg activation are depicted. The stages of meiosis are shown on the *top* (prophase I through to anaphase II). During prophase I the stage I oocytes are likely those undergoing the process of crossing over (preleptotene to pachytene). Once the oocytes reach stage II (diplotene) they arrest and accumulate yolk during vitellogenesis (*E*, *early* and *L*, *late*). Stage III oocytes continue to grow until they undergo GVBD. Following GVBD oocyte maturation occurs during which time the chromosomes and first meiotic spindle migrate to the oocyte cortex. Mature stage IV oocytes arrest in metaphase I and are spawned ready for fertilization. Germinal vesicle breakdown (GVBD), polar body (PB), and pronucleus (PN) fertilization. Fertilization triggers the formation of two small polar bodies (PB1 and PB2) followed by formation of the male and female pronuclei (PN stage)

8.2.2 Oocyte Maturation

Stage III oocytes are fully grown germinal vesicle (GV) oocytes at the diakinesis stage of prophase, which have a centrally located GV and a subcortical mitochondrial layer (termed the myoplasm), and are ready to mature (Conklin 1905, see Prodon et al. 2006). Stage III oocytes do not display an animal-vegetal axis and the mitochondria-rich myoplasm lines the whole of the egg subcortex (Swalla et al. 1991; Prodon et al. 2006). Stage III oocytes removed from the ovary mature spontaneously in sea water (Sawada and Schatten 1989; Sakairi and Shirai 1991; Lambert 2008; Prodon et al. 2009). Because germinal vesicle breakdown (GVBD) is very rapid in stolidobranch (a suborder of the Pleurogona) ascidians such as *Molgula* and *Boltenia* and takes up to 2.5 h in phlebobranch (a suborder of the Enterogona) ascidians such as *Ciona* and *Phallusia* it has been suggested that preMPF (maturation promoting factor) is likely present in the faster maturing group and that cyclin B synthesis is likely required in the slower maturing group (Lambert 2008). Like nemerteans (Stricker and Smythe 2001), but unlike mammals (see Mehlmann 2005 for a review), an increase in cAMP induces GVBD in the ascidian *Boltenia villosa* (Lambert 2008). Recently it has been shown that in

Ciona intestinalis oocytes a fall in cAMP levels induces GVBD as in mammalian oocytes (Silvestre et al. 2010). After GVBD, an actin-dependent mechanism is required for spindle migration toward the prospective animal pole (Prodon et al. 2006) as in mouse oocytes (Leader et al. 2002; Azoury et al. 2008; Schuh and Ellenberg 2008).

8.2.3 Sperm-Triggered Calcium Oscillations and Exit from Metaphase I Arrest in Ascidians

8.2.3.1 Metaphase I Arrest

c-Mos is a key Ser/Thr protein kinase in oocytes and is well known for its role in inducing CSF (cytostatic factor) arrest in unfertilized eggs (Sagata et al. 1989; Masui 2000 and Fig. 8.3). c-Mos is present throughout the Eumetazoa (Cnidaria: Amiel et al. 2009; Mollusc: Shibuya et al. 1992; Echiura: Gould and Stephano 1999; Echinoderms: Tachibana et al. 1997; Urochordates: Russo et al. 2009; Vertebrates: Hashimoto et al. 1994; Verlhac et al. 1996). Erp1 relays the signal from the Mos/MAPK cascade to inhibit the anaphase promoting complex/cyclosome (APC/C^{cdc20}) thus inducing metaphase II arrest by preventing destruction of cyclin B in vertebrates (Rauh et al. 2005; Schmidt et al. 2005; Shoji et al. 2006 and Fig. 8.3). How mature ascidian eggs arrest at metaphase I is intriguing since the *Ciona intestinalis* genome does not contain Erp1 (see Fig. 8.3).

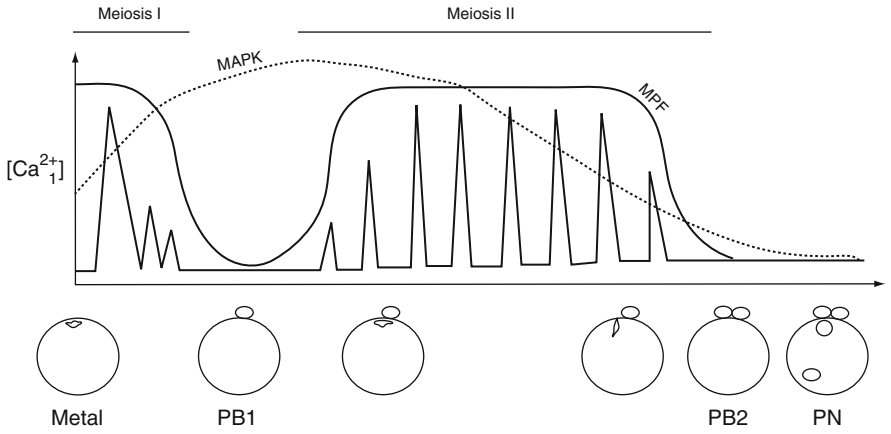

Fig. 8.3 Meta I-CSF and egg activation in ascidian eggs. Meta I-CSF is likely composed of the Mos/ERK pathway causing an inhibition of APC/C^{cdc20}, thus preventing cyclin B destruction. However, Erp1 is not present in ascidians (*dashed box*). We therefore propose that egg activation does not involve CaMKII (which operates via Erp1) and instead suggest that calcineurin (CN) is likely involved in ascidian egg activation

Sperm-triggered Ca^{2+} increases are a universal feature of egg activation (see reviews by Stricker 1999 and Whitaker 2006). In *Xenopus* eggs the sperm-triggered Ca^{2+} transient activates a kinase (calmodulin-dependent kinase II or CaMKII, Lorca et al. 1993) and a phosphatase (calcineurin or CN, Mochida and Hunt 2007; Nishiyama et al. 2007). CN and CaMKII are both required to fully activate the APC/C^{cdc20} and trigger exit from metaphase II arrest (Fig. 8.3). CaMKII phosphorylates Erp1 opening up a cryptic phosphorylation site for XPlk1 causing the proteolytic destruction of Erp1 via the β-Trcp pathway (Schmidt et al. 2005). However, it is not known whether CN or CaMKII are involved during egg activation in ascidians or other invertebrates. Because ascidians do not contain Erp1 we speculate that CaMKII is not involved in egg activation in the ascidians. On the contrary, since CN directly stimulates the APC/C we would surmise that CN is involved during egg activation in the ascidian (Fig. 8.3) and perhaps other invertebrate eggs that are stimulated to exit metaphase I by a Ca^{2+} signal (see Fig. 8.1b).

Egg activation requires more than APC/C^{cdc20} activity since the Mos/MAPK signaling cascade also has to be turned off. During egg activation in the mouse the loss of MAPK activity is required to exit meiosis and form pronuclei (Moos et al. 1995). c-Mos contains N-terminal Pro and Ser residues forming part of an N-terminal degron that controls the switch between c-Mos stability during metaphase and destruction during interphase (Sheng et al. 2002). MPF induces c-Mos stability by direct phosphorylation of Ser-3 in the N-terminal degron in *Xenopus* oocytes (Castro et al. 2001). Loss of MPF activity therefore favors c-Mos instability and leads to the proteolytic destruction of c-Mos. Since it is ERK1/2 that mediates the effect of the Mos/MAPK pathway, ERK1/2 activity has also to be switched off once c-Mos is degraded and this requires dephosphorylation of the conserved Thr or Tyr in the TEY motif of ERK1/2. In *Xenopus* oocytes protein phosphatase 2A (PP2A) causes Thr dephosphorylation of the TEY motif of ERK1/2, which is sufficient to inactivate ERK1/2 in intact oocytes (Sohaskey and Ferrell 1999). Our unpublished observations suggest that the inactivation of MPF is not a sufficient stimulus for inducing the loss of MAPK activity in ascidians caused by ERK1. However, a role of PP2A appears to be conserved between *Xenopus* and the ascidian since we find that ERK dephosphorylation depends on PP2A following egg activation (Levasseur et al. in preparation).

8.2.3.2 Sperm-Triggered Ca^{2+} Oscillations Trigger Egg Activation

Ca^{2+} oscillations are often triggered by sperm as is the case in many invertebrate species and in mammalian eggs (see reviews by Sardet et al. 1998; Dumollard et al. 2002). These Ca^{2+} oscillations are associated with the meiotic cell cycle (see review by Sardet et al. 1998; Stricker 1999). This correlation between the meiotic cell cycle and the presence of Ca^{2+} oscillations is not fortuitous since sperm-triggered Ca^{2+} oscillations are maintained when exit from the meiotic cell cycle is blocked in mouse (Jones et al. 1995) and ascidian eggs (Levasseur and McDougall 2000). In ascidians, sperm entry triggers two series of periodic Ca^{2+} waves that occur during

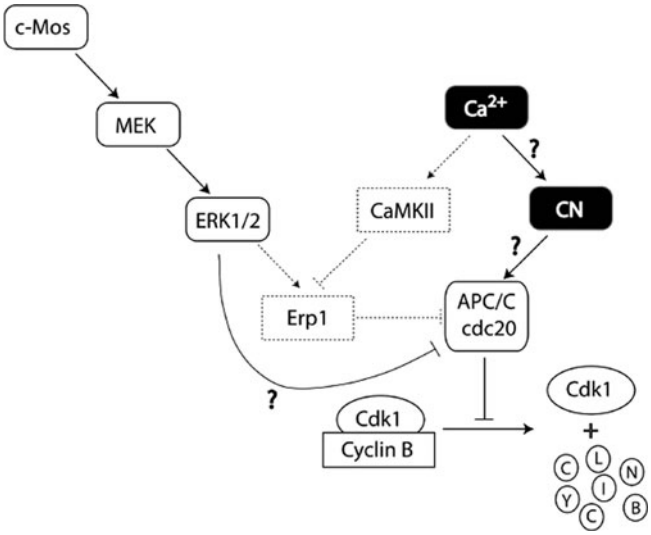

Fig. 8.4 Sperm-triggered Ca^{2+} oscillations, MPF activity, and MAPK activity are shown following fertilization. Meiosis I and II and the morphology of the egg/zygote are shown. The calcium oscillations correlate with meiosis I and II and the phases of MPF activity. Extrusion of the first and second polar bodies is indicated (PB1 and PB2) followed by formation of the pronuclei (PN)

meiosis I and II (Fig. 8.3 and Speksnijder et al. 1989a, b; McDougall and Levasseur 1998 and reviewed in McDougall 2001). These Ca^{2+} oscillations are required for meiotic exit (McDougall and Sardet 1995) and lead to the loss of MPF activity (Russo et al. 1996; McDougall and Levasseur 1998; Levasseur 1998) and an increase in the rate of cyclin B destruction (Levasseur and McDougall 2000). The two series of meiotic Ca^{2+} waves in ascidian eggs are triggered by two different localized Ca^{2+} wave pacemakers (McDougall and Sardet 1995; Dumollard and Sardet 2001). The pacemaker driving meiosis I Ca^{2+} waves is located in a cortical ER-rich domain surrounding the sperm aster and the pacemaker driving meiosis II lies in a cortical ER domain in a vegetal constriction called the contraction pole (Speksnijder et al. 1993; McDougall and Sardet 1995; Dumollard and Sardet 2001). How MPF regulates the two Ca^{2+} wave pacemakers associated with each phase of meiosis is not yet known (Levasseur et al. 2007). Both phases of Ca^{2+} waves occur when the MPF activity is elevated during meiosis I and II and pause for about 5 min when the MPF activity is low (McDougall and Levasseur 1998 and Fig. 8.4). The periodic Ca^{2+} waves finally stop just before extrusion of the second polar body when both the MPF and MAPK activities are low. Remarkably, by maintaining the MPF activity of the egg elevated the sperm-triggered Ca^{2+} waves keep pulsing across the fertilized egg indefinitely (Levasseur and McDougall 2000 and review by McDougall 2001). Because the egg can respond to the Ca^{2+}-releasing second messenger inositol 1,4,5-trisphosphate ($InsP_3$) during the pause between the two pacemakers (Fig. 8.4), we suggested that MPF regulates $InsP_3$ production rather

than the sensitivity of $InsP_3$ receptors (McDougall and Levasseur 1998). $InsP_3$ receptors do eventually become desensitized upon entry into first interphase once both MAPK and MPF activities have decreased (Levasseur and McDougall 2003). The positive–negative feedback loop between MPF and the sperm-triggered Ca^{2+} waves therefore ensures that the egg-to-embryo transition occurs.

8.3 Embryonic Cell Cycles in the Ascidian

8.3.1 The Duration and Number of Cell Cycles Are Precisely Controlled

Not much is known about how the cell cycle is controlled during embryogenesis in the ascidians. However, the embryo displays a highly stereotyped pattern of cell division where both the rate of cell division and the orientation of cell division planes are precisely controlled. Both the rapid rate of cell division and the precise orientation of the cleavage plane are necessary for the embryo to develop rapidly with minimal proliferation. Indeed, ascidians form a tadpole larva composed of less than 2,700 cells that hatches about 12 h after fertilization (Satoh 2003). Gastrulation starts at the 110 cell stage by the invagination of 10 endodermal cells (see review by Swalla 1993 and book by Satoh 1994, page 57). At this stage, four lineages are determined and almost all cells are already fate-restricted (see Kumano and Nishida 2007 for a review).

8.3.1.1 The Rate of Cell Division

After meiotic completion, the mitotic cell cycles of the cleaving ascidian embryo show several features in common with early mitotic cell cycles found in *Xenopus* or zebrafish embryos. Like these and other embryos including mammals, the first cell cycle is longer than the subsequent ones (see Fig. 8.5 and Kubiak et al. 2008 for a review).

From the 2 cell stage to the 16 cell stage (cell cycles 2–4), the embryo displays synchronous and rapid divisions but with a slight decrease in cell cycle speed (Fig. 8.5). The progressive slowing of the cell cycle is due to an increase in interphase length (from 8 min at the 2 cell to 35 min at the 16 cell stage) whereas the duration of mitosis stays constant lasting approximately 15 min (Dumollard et al. unpublished observations). At the fifth cell cycle (16 cell stage), asynchronous cell cycles are observed when the eight animal cells divide after the eight vegetal cells giving rise to a brief 24 cell stage. This is due to the fact that mitosis begins later in the eight cells (Chenevert et al. unpublished observations). Such asynchrony follows the onset of zygotic transcription, which for the earliest genes begins at the eight cell stage (Shimauchi et al. 1997; Miya and Nishida 2003). Zygotic transcription of

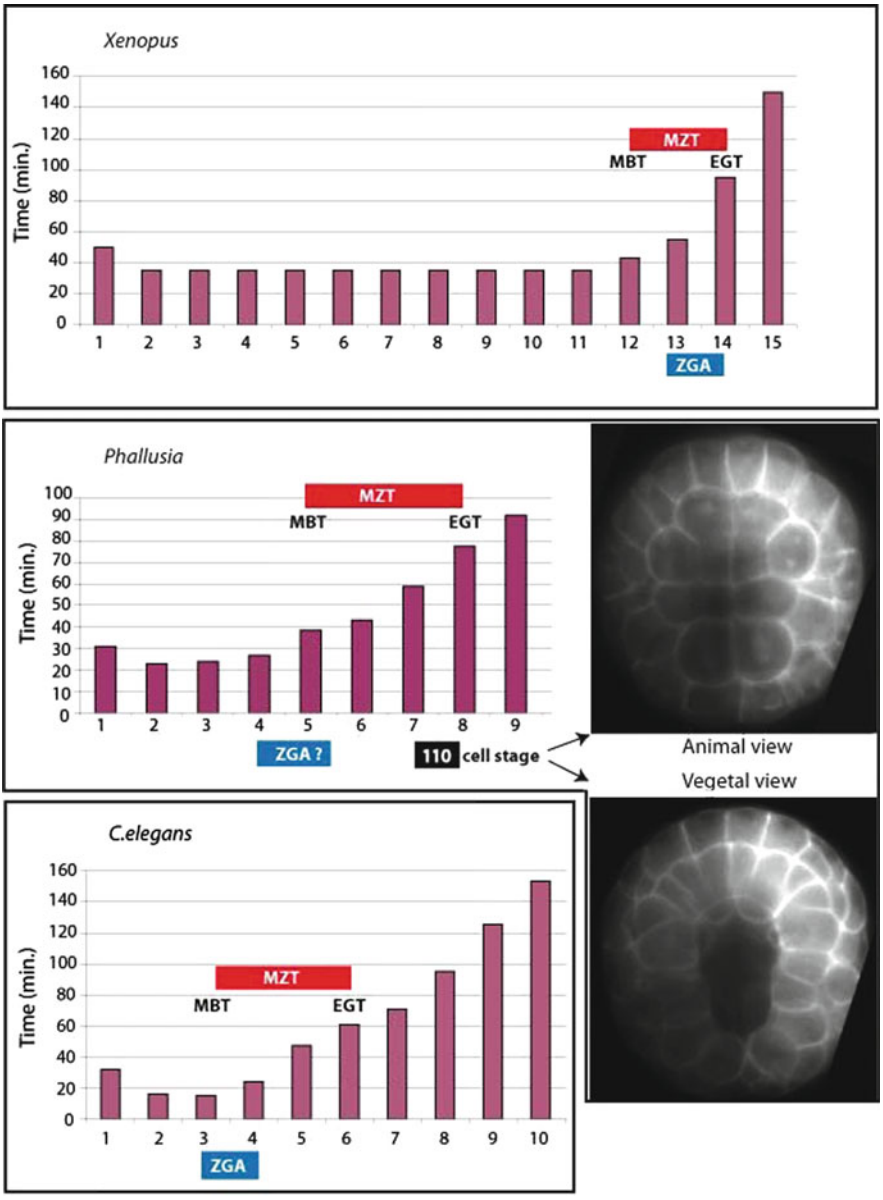

Fig. 8.5 Cell cycle duration in *Xenopus*, *Phallusia*, and *C. elegans* embryos. Midblastula transition (MBT), early gastrula transition (EGT), midzygotic transition (MZT), and zygotic genome activation (ZGA) are indicated. The *x*-axis shows the cell cycle number such that 1 is the 1 cell stage, 2 the 2 cell, and 3 the 4 cell stage, etc. The plasma membrane marker PH::EGFP shows the individual cells at the 110 cell stage in *Phallusia* at the onset of gastrulation. The vegetal view shows the formation of the blastopore

transcription factors increases progressively from the 16 cell stage onwards (Imai et al. 2004). There is not therefore an abrupt mid blastula transition (MBT) in the ascidian embryo as has been described in *Xenopus* (Newport and Kirschner 1982a, b), zebrafish (Kane and Kimmel 1993) and *Drosophila* cellularization (Foe and Alberts 1983). *C. elegans* embryos might also display an abrupt MBT (Bao et al. 2008 and Fig. 8.5). Nonetheless, in the ascidian as in *Xenopus*, *C. elegans* and *Drosophila*, gastrulation begins two or three cell cycles after zygotic genes are first expressed and cell cycle time begins to lengthen (Fig. 8.5).

Asynchrony is further amplified in the vegetal half of the embryo where the cells that will form the primary muscle, notochord, and endoderm precursors are located. Ascidian embryos from different orders (*Phallusia* and *Halocynthia*) display the same distinctive 110 cell stage embryo indicating that the mechanisms regulating both the rate and the orientation of cell divisions are well-conserved in solitary ascidians (Fig. 8.5). This asynchrony in the rate of cell division is maintained after gastrulation and further amplified when some cells stop dividing at the neurula stage such as the muscle and notochord lineages whilst others such as the endoderm or epidermis continue to divide during early tailbud stage. The net result of this is that a tadpole larva composed of ~2,600 cells is produced containing precisely 36 muscle cells, 40 notochord cells, 330 central nervous system cells, about 500 endoderm cells and a large number of mesenchyme and epidermal cells (see review by Satoh 2003). The ascidian embryo will be a powerful model system to decipher how cell cycle progression is modulated in each of these lineages.

8.3.1.2 The Orientation of the Cell Division Plane and the Centrosome-Attracting Body or CAB

All cleavage planes of the early ascidian embryo are precisely oriented in a defined manner. This program is essential for the segregation of developmental determinants and the morphology of the embryo. Aside from the centrosome-attracting body (CAB)-regulated divisions (see below), very little is known about the mechanisms that control positioning of the ascidian mitotic spindles during development.

The CAB is a macroscopic structure visible by light microscopy that mediates these three rounds of asymmetric cell division (ACD) (Hibino et al. 1998). In the cell in which it resides the CAB causes an unequal cleavage and is inherited by the smaller of the two daughter cells generating two small posterior cells at the 64 cell stage that are germ cell precursors (Nishikata et al. 1999). Around 40 maternal *postplasmic/PEM* (posterior end mark) RNAs including *macho-1* and *pem-1* (Yamada 2006; Prodon et al. 2007; Paix et al. 2009) as well as the germ cell marker *vasa* (Fujimura and Takamura 2000; Paix et al. 2009) are enriched in the CAB. Postplasmic/PEM RNAs were first identified as a group of maternal RNAs that localize to the posterior end mark hence the term PEMs. These *postplasmic/PEM* RNA domains are formed during oogenesis. The *postplasmic/PEM* RNAs are localized to the CAB during the 8 cell stage and tethered there until the 110 cell

stage. During the next cell cycle some *postplasmic*/*PEM* RNAs are segregated into a somatic cell (termed B8.11) while others including the germ cell marker *vasa* are segregated into the germ cell precursor cell (B8.12). How the CAB controls the plane of cytokinesis so that one of the two daughter cells inherits these *postplasmic*/*PEM* RNAs is not entirely known. However, it is known that the CAB accumulates a submembranous layer of cortical polarity proteins PAR3, PAR6, and aPKC (Patalano et al. 2006) that are known to be involved in spindle orientation in *C. elegans* one cell embryos, *Drosophila* neuroblasts, and mammalian epithelial cells (see review by Siller and Doe 2009). Classic micromanipulation experiments demonstrated that removal of the CAB abolished ACD and that transplantation of the CAB caused unequal cleavage at an ectopic site (Nishida 1994, 1996; Nishikata et al. 1999). Finally, PEM1 protein is enriched in the CAB and is required for unequal cleavage (Negishi et al. 2007).

A dual mechanism organizes the mitotic spindle during ACD in the ascidian *Phallusia mammillata* (Fig. 8.6 and Prodon et al. 2010). Previously it had been reported that a microtubule bundle dragged the nucleus toward the CAB causing the mitotic spindle to form at an asymmetric site near the CAB (Nishikata et al. 1999). However, in *Phallusia* embryos we recently found that one pole of the mitotic spindle is attracted toward the CAB after NEB from prometaphase through metaphase (Fig. 8.6 and Prodon et al. 2010). The movement of the spindle pole toward the CAB is strongly correlated with the onset of prometaphase, but it is unknown how the cell cycle affects spindle pole migration toward the CAB. The capacity of

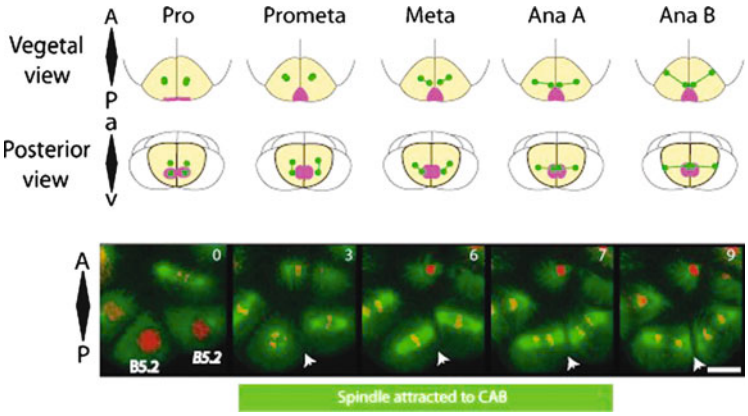

Fig. 8.6 Asymmetric cell division. *Top:* model showing vegetal and posterior views of a 16 cell stage embryo with the B5.2 cells highlighted *yellow*. The movement of the spindle poles (*green dots*) toward the CAB (*violet*) from prometaphase through metaphase is shown. Anterior (A), posterior (P), animal (a) and vegetal (v) are indicated. *Below*: confocal images displaying spindle alignment during mitotic progression in a pair of B5.2 cells at the 16 cell stage (*vegetal view*). Chromosomes are *red* (histone H2B::mRfp1), microtubules *green* (MAP7::EGFP) and the time is indicated in minutes. *Arrowheads* indicate CAB position. *Scale bar* = 20 μm

the CAB to attract the spindle pole could be an indirect consequence of MPF's affect on microtubule reorganization that occurs during prometaphase. Alternatively, the CAB may itself be rendered competent: it is known that the CAB undergoes a cell cycle-dependent shape change and becomes compact during prometaphase when it attracts the spindle pole (Patalano et al. 2006). Interestingly, once the CAB has attracted one spindle pole the opposite pole then migrates toward the site of previous cell division and we suggest the midbody remnant may be involved (Prodon et al. 2010), as has been proposed in two cell *C. elegans* embryos (Waddle et al. 1994). Recently it has been demonstrated that both macho-1 and β-catenin are also involved during ACD in the *Halocynthia roretzi* (Kumano et al. 2010). Knockdown of both β-*catenin* and *macho-1* disrupts the microtubule bundle that is proposed to drag the nucleus toward the CAB before nuclear envelope breakdown (NEB) (Kumano et al. 2010). It will also be interesting to determine how β-catenin and Macho-1 affect the movement of the mitotic spindle toward the CAB after NEB in *Phallusia* embryos.

While cell division in CAB-containing cells is the most dramatic and extensively-studied ACD during early embryonic development in ascidians, many other cells of the early embryo also undergo ACD. Quantitative measurements of 3D models reveal other unequal cleavages (Tassy et al. 2006). Also, during the acquisition of tissue identities many binary cell fate choices are made yielding two daughter cells that have different fates such as the division that produces one notochord cell and one neural precursor cell at the 44 cell stage (Picco et al. 2007).

8.4 Conclusions and Perspectives

Valuable insight into how sperm-triggered calcium oscillations in the mammalian egg are regulated in a cell cycle-dependent manner came largely from work performed in the ascidian. It will be interesting to probe further the nature of Meta I-CSF and in particular determine how the Mos/MAPK pathway inhibits the APC/C in the absence of Erp1. Are there specific metaphase I APC coactivators such as *cortex* in *Drosophila* or Slp1 in *S. pombe*? On a related note, it will be equally interesting to determine how the fertilization Ca^{2+} signal is transduced in the absence of Erp1. Is calcineurin involved and not CaMKII? In the embryo it will be intriguing to decipher how the asynchrony in cell cycle duration arises during early embryonic development. Is cell cycle asynchrony and lengthening in ascidian embryos due to the increase in nucleocytoplasmic ratio as in *Xenopus* or is it due to unequal inheritance of maternal factors as in *C. elegans*? Finally, since it is the spindle rather than the nucleocentrosomal complex that is attracted toward the CAB, it will be possible to determine whether asymmetric spindle positioning is caused by activation of the APC/C as in one cell *C.elegans* embryos or by cell cycle regulated kinases such as Aurora A in *Drosophila* neuroblasts.

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Chapter 9
Regulatory Pathways Coordinating Cell Cycle Progression in Early *Xenopus* Development

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Abstract The African clawed frog, *Xenopus laevis*, is used extensively as a model organism for studying both cell development and cell cycle regulation. For over 20 years now, this model organism has contributed to answering fundamental questions concerning the mechanisms that underlie cell cycle transitions – the cellular components that synthesize, modify, repair, and degrade nucleic acids and proteins, the signaling pathways that allow cells to communicate, and the regulatory pathways that lead to selective expression of subsets of genes. In addition, the remarkable simplicity of the *Xenopus* early cell cycle allows for tractable manipulation and dissection of the basic components driving each transition. In this organism, early cell divisions are characterized by rapid cycles alternating phases of DNA synthesis and division. The post-blastula stages incorporate gap phases, lengthening progression, and allowing more time for DNA repair. Various cyclin/Cdk complexes are differentially expressed during the early cycles with orderly progression being driven by both the combined action of cyclin synthesis and degradation and the appropriate selection of specific substrates by their Cdk components. Like other multicellular organisms, chief developmental events in early *Xenopus* embryogenesis coincide with profound remodeling of the cell cycle, suggesting that cell proliferation and differentiation events are linked and coordinated through crosstalk mechanisms acting on signaling pathways involving the expression of cell cycle control genes.

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9.1 Introduction

In vertebrates, progression through all stages of development, from oocyte to tadpole and beyond, is accompanied by alterations in the mode in which the cell cycle is regulated, the number and role of its phases, and its overall length. In this chapter, we discuss the events that precede oocyte fertilization and center our discussion on the events that drive cell progression during the early stages of *Xenopus* development.

9.1.1 Key Players Driving the Oocyte Meiotic Cell Cycle

In sexually mature *Xenopus* females, oogenesis progresses asynchronically as oocytes move from their smallest size (stage I) to their largest, fully grown size (stage VI) at a rate that is largely influenced by environmental factors and hormonal stimulation. Stage VI oocytes contain cytoplasmic stores of maternal mRNAs, ribosomes, tRNAs, and other macromolecules and organelles that are required for protein synthesis and that are sufficient to last into early embryogenesis. Immature *Xenopus* oocytes are naturally arrested at the first meiotic prophase in a G_2-like state by the action of the *m*aturation-*p*romoting *f*actor (MPF) until steroid hormones secreted by follicle cells, such as progesterone, trigger oocyte meiotic maturation (Fig. 9.1). Then, progression occurs through stages that include *g*erminal *v*esicle *b*reak*d*own (known as GVBD), formation of the metaphase I spindle, first meiotic division and extrusion of the first polar body, which ends with mature oocytes being

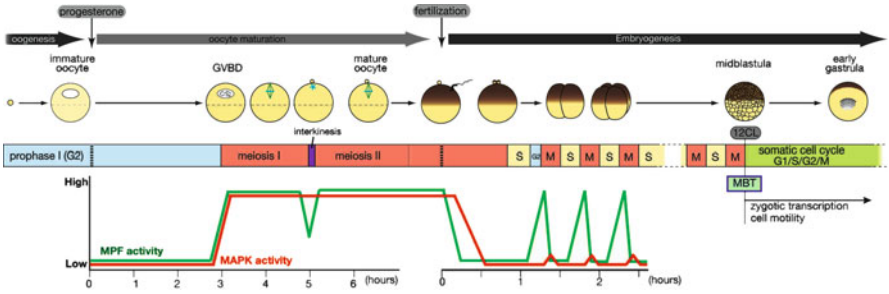

Fig. 9.1 *Xenopus* cell cycle remodeling: from oocyte to early embryonic cycles. Fully grown *Xenopus* oocytes are arrested at prophase I in a G_2-like state until a mitogenic signal triggers maturation. Progesterone (PG) promotes meiotic resumption, followed by GVBD, interkinesis, and meiosis II. Mature oocytes are arrested at metaphase II until fertilization initiates the early mitotic cleavage cycles. Completion of the 12th cleavage (12CL) marks the midblastula transition (MBT) and the stage at which embryonic cell cycles (S/M) gradually change toward a more somatic-like cycle (G_1/S/G_2/M) (Frederick and Andrews 1994). The graph shows MPF activity (*green line*) and MAPK activity (*orange line*) during oocyte maturation and in early cleavage stages. *S* (DNA synthesis)-phase, *M* mitosis, $G_{1/2}$ Gap1/2 phases

arrested at the second meiotic metaphase by the action of an endogenous cytoplasmic factor called *c*yto*s*tatic *f*actor (CSF) (Masui and Clarke 1979; Ferrell 1999; Nebreda and Ferby 2000; Karaiskou et al. 2001; Sagata 1996). Release from CSF-induced arrest occurs shortly after fertilization with eggs entering S-phase followed by mitotic cycles.

Yoshio Masui first described MPF activity, initially defined as maturation-promoting factor, in a pioneering paper published in the early 1970s (Masui and Markert 1971). In his work, Masui demonstrated that (a) production of MPF is independent of nuclear function and is entirely a cytoplasmic process, (b) there is an autocatalytic production of MPF that results from the existence of a precursor (pre-MPF) that is catalytically amplified by active MPF, and (c) initiation of oocyte maturation by progesterone depends on the synthesis of proteins that activate pre-MPF and triggers the MPF autocatalytic loop (Wasserman and Masui 1975). Today, we know that MPF is responsible for progression through not only meiotic but also mitotic cell cycles and that both its presence and autocatalytic activities have been described in organisms as diverse as starfish and mammals. Remarkably, the requirement of protein synthesis for MPF activation, which is observed in *Xenopus* oocytes, is not a universal feature. Mice and a variety of fish and some amphibian species do not require protein synthesis for MPF activation.

The MPF, later re-baptized *M*-phase *p*romoting *f*actor due to its role as a universal G_2/M regulator in eukaryotic cells, is a complex of cyclin B and its kinase counterpart Cdc2 (Nurse 1990) that, when together, drives oocytes to enter not only meiosis I but also meiosis II, although there is a transient decrease in the activity of the complex between the two meiotic divisions (Gerhart et al. 1984) (Fig. 9.1). The initial step in Cdc2 activation is binding to its cyclin B partner, forming a complex that initially possesses low kinase activity and weak affinity for substrates until a residue (Thr161) within Cdc2 located in the T-loop of the protein is posttranslationally modified by phosphorylation (Ducommun et al. 1991). The role of Thr161 in Cdc2 was apparent from the crystal structure of human Cdk2 (De Bondt et al. 1993), as well as from the finding that simple binding of the cyclin partner to Cdc2 does not fully activate the catalytic subunit, although partial activation has been detected in a limited number of cases (Desai et al. 1992; Connell-Crowley et al. 1993) and by the fact that phosphorylation-dependent association of Cdc2 with cyclin was needed for full activation (Ducommun et al. 1991). Given the high degree of primary sequence homology among all Cdks (~60%), the elucidation of the crystal structure of Cdk2 provided relevant information on how these molecules recognize their substrate, associate with their regulatory subunits, are regulated by other kinases/phosphatases, and how phosphorylation promotes structural rearrangements that influence cyclin/Cdk activity. For example, the low kinase activity in Cdk2 is likely to result from structural contraints generated by the position of the side chain residues surrounding the ATP-binding site and by the location of the T-loop, containing the Thr160 residue (the analogous residue to Thr161 of Cdc2), that blocks the protein substrate-binding cleft (De Bondt et al. 1993). Similar to what happens with other protein kinases that are activated as result of phosphorylation events, modification of Thr160/161 re-positions the T-loop stabilizing the

cyclin-binding site into an active enzyme conformation. Phosphorylation in Thr161 is catalyzed by the *C*dc2-*a*ctivating *k*inase (CAK, Solomon et al. 1993; Poon et al. 1993; Fesquet et al. 1993), an enzyme whose activity remains steady throughout the cell cycle (Brown et al. 1994). Interestingly, phosphorylation in Thr161 has been detected in inactive pre-MPF complexes of G_2-arrested oocytes suggesting that phosphorylation in this residue is needed to stabilize a functional cyclin/Cdc2 molecule but, is unlikely to play a direct role in the full activation of MPF (De Smedt et al. 2002).

An additional level of regulation over MPF activity is mediated by phosphorylation in two key residues overlapping the ATP-binding site, Thr14 and Tyr15, within the Cdc2 subunit (Gautier et al. 1989; Dunphy and Newport 1989; Ferrell et al. 1991; Jessus et al. 1991; Posada et al. 1991). In G_2-arrested oocytes, the cyclin B/Cdc2 complex is maintained in an inactive state by phosphorylation of both residues, an event that is balanced by the interplay between the Wee1 family protein kinase Myt1 and its counterpart enzyme, the Cdc25 phosphatase, which removes Thr14 and Tyr15 phosphorylations and thus allows entry into the M-phase (Nakajo et al. 2000; Liu et al. 1999; Wells et al. 1999; Strausfeld et al. 1991; Gautier et al. 1991; Kumagai and Dunphy 1991). The Wee1 kinase has long been recognized as a key regulator of Cdc2 activity and mitotic entry in a variety of organisms and, although its initial characterization was in fission yeast, it proved to be a universal regulator across species (Russell and Nurse 1987). The Wee1 enzyme behaves as a dual specificity enzyme that phosphorylates Tyr15 and to a much lesser extent Thr14 in MPF *in vitro* (Featherstone and Russell 1991; Parker et al. 1992). Further studies demonstrate that most of the key aspects that pertain to phosphorylation-mediated cyclin B/Cdc2 regulation are essentially similar in fission yeast and animal cells, with dual inhibitory phosphorylation playing a more relevant role in higher eukaryotes (Krek and Nigg 1991a, b; Norbury et al. 1991). Later identification of the Wee1 family member Myt1 kinase in *Xenopus* reconciled hypotheses that pointed toward dual control of cyclin B/Cdc2 activity by different kinases (Mueller et al. 1995). Unlike Wee1, the Myt1 enzyme was equally efficient in phosphorylating residues Thr14 and Tyr15 in Cdc2, a critical feature for G_2-arrested *Xenopus* oocytes that lack Wee1 expression before meiosis II (Nakajo et al. 2000; Murakami and Vande Woude 1998; Palmer et al. 1998). Interestingly, Myt1 activity is also regulated by phosphorylation with maximum inhibition shown during mitosis and in *Xenopus* oocyte maturation (Palmer et al. 1998).

For the G_2/M transition to occur, inactive MPF needs to be dephosphorylated by a phosphatase activity capable of removing the phosphate groups from residues Thr14 and Tyr15. The first identification of such an activity resulted from studies carried out on fission yeast that led to the characterization of a novel Cdc2 activator from the product of the *Cdc25* gene (Gould et al. 1990; Russell and Nurse 1986). Homologs of Cdc25 were later identified and further characterized in various species, establishing a universal role for Cdc25 in controlling MPF activity (Russell et al. 1989; Edgar and O'Farrell 1989; Sadhu et al. 1990). *Xenopus* extracts proved to be an excellent resource for the characterization of the Cdc25 dual phosphatase activity, its direct regulation of MPF activation, and the elucidation

of posttranslational events that tightly control phosphatase function (Gautier et al. 1991; Kumagai and Dunphy 1992; Jessus and Beach 1992; Izumi et al. 1992; Dunphy and Kumagai 1991). As a result, the two key regulators of MPF activity, Myt1 and Cdc25, are present in G_2-arrested oocytes with Myt1 exerting a negative regulation over MPF while Cdc25 remains inactive in arrested cells.

9.1.2 *Overcoming Oocyte G_2-Arrest*

To escape the G_2-arrest, a number of events need to take place to ensure that a threshold number of active MPF molecules are generated to complete the meiotic cycle. Progesterone, a natural mitogen in *Xenopus* oocytes, activates a receptor-mediated signaling cascade that leads to a reduction in the intracellular levels of cAMP with its concomitant effect in c*A*MP-dependent *p*rotein *k*inase (PKA) activity (Fig. 9.2). This initial signaling event results from progesterone binding to a newly identified, and long elusive, seven-transmembrane G protein-coupled receptor followed by inhibition of adenylyl cyclase activity and an increase in protein

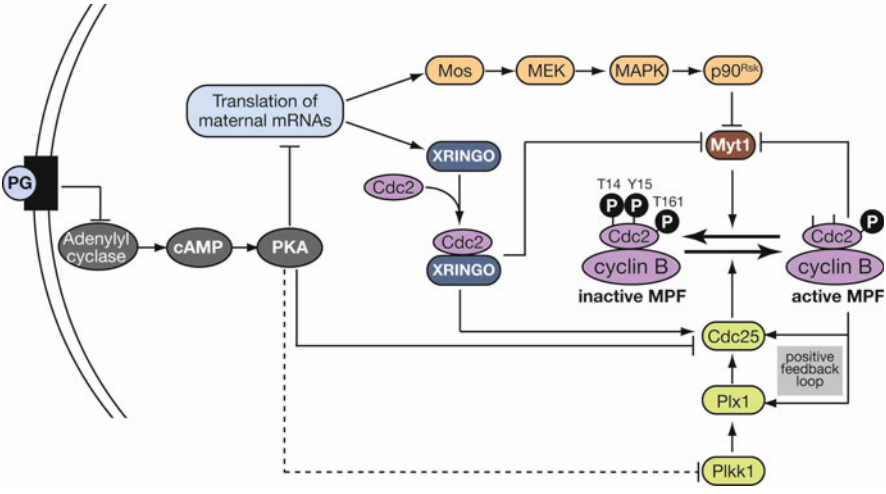

Fig. 9.2 Current model of signaling pathways acting on cyclin B/Cdc2 during meiotic G_2–M progression. In G_2-arrested oocytes, high levels of cAMP correlate with increased activity of PKA, which suppresses both the translation of maternal mRNAs and the activity of the Cdc25 dual phosphatase through an inhibitory phosphorylation on its Ser287 residue. In response to its natural mitogenic stimuli PG, the activity of the adenylyl cyclase is altered leading to a decrease in the level of cAMP and downregulation of the PKA activity. These events trigger the translation of maternal mRNAs (i.e., Mos and XRINGO) and the release of Cdc25 from inhibition in a pathway that is mediated by the polo-like kinase enzymes. Mos synthesis triggers the activation of the Mos/MEK/MAPK/$p90^{Rsk}$ pathway and inactivation of Myt1. Eventually, changes in the Myt1/Cdc25 balance drive the conversion of inactive cyclin B/Cdc2 (pre-MPF) into an active complex

synthesis (Zhu et al. 2003a, b; Finidori-Lepicard et al. 1981; Maller and Krebs 1977). But, how do these events translate into increased MPF activity?

Increasing evidence establishes a role for progesterone in triggering at least two signaling pathways that converge at the pre-MPF to MPF transition (Fig. 9.2), where one depends on protein synthesis and leads to the inhibition of the Myt1 kinase through the MAP kinase pathway (Mos/MEK/MAPK/$p90^{Rsk}$) and the other signaling event results in the phosphorylation-dependent activation of Cdc25 by the *p*olo-*l*ike kinase *1* (Plx1) and other additional kinases (Fig. 9.2). The initial production of MPF is later accelerated by what it is known as the "MPF autoamplification loop", in which MPF directly targets Cdc25 for activation while inhibiting Myt1 function.

The proto-oncogene *c-mos* is a Ser/Thr kinase that is normally absent in oocytes arrested at prophase I but is *de novo* synthesized from maternal stores of mRNA modified in its 3′-UTR shortly after progesterone addition (Sagata et al. 1988; Sheets et al. 1994, 1995; Gebauer and Richter 1997). The Mos protein functions as a MAPK kinase kinase and thus activates the dual specificity kinase MEK, which in turn acts on MAPK, resulting in the activation of the ribosomal S6 kinase, $p90^{Rsk}$ (Posada et al. 1993; Nebreda and Hunt 1993; Shibuya and Ruderman 1993; Hsiao et al. 1994) (Fig. 9.2). The importance of Mos synthesis for meiotic maturation was established by depleting eggs of the most upstream component of the signaling cascade using antisense oligonucleotides, which prevented progesterone-induced maturation (Sagata et al. 1988). Microinjection of Mos, or any of its constitutively active downstream targets in G_2-arrested oocytes, resulted in induction of GVBD even in the absence of progesterone (Sagata et al. 1989a, b; Gross et al. 2001; Huang et al. 1995). Conversely, microinjection of U0126, an MEK inhibitor that prevents MAPK activation, sustains the arrest (Gross et al. 2000). The link between the MAPK cascade and cyclin B/Cdc2 activation was strengthened by the finding that the regulatory subunit of Myt1 is an inherent substrate for the $p90^{Rsk}$ kinase and that this interaction leads to phosphorylation and downregulation of Myt1 activity (Palmer et al. 1998) (Fig. 9.2). Further experiments pointed toward Mos as a direct regulator of Myt1 and a facilitator of Myt1 inhibition (Peter et al. 2002). An additional mode of regulation came from the discovery that phosphorylation of Myt1 by XRINGO-activated CDK1 (alternative name for Cdc2) synergizes the effect of $p90^{Rsk}$ resulting in a more effective inhibition of Myt1 ensuring meiotic progression (Ruiz et al. 2010) (Fig. 9.2). Other contributions in the regulation of cyclin B/Cdc2 came from the pioneer work of the Pines and Nishida laboratories (Pines and Hunter 1994; Hagting et al. 1999; Toyoshima-Morimoto et al. 2001). Translocation is an important regulatory process in the cell that simply helps to locate a substrate and its enzyme in the right place at the right time. Pines' group elegantly showed that human cyclin B1 phosphorylation enhances its rapid translocation to the nucleus toward the end of prophase (Hagting et al. 1999), whereas Nishida's group directly implicated the *Xenopus* homolog of the Plx1 and the MAPK pathway in this event (Toyoshima-Morimoto et al. 2001). Even when all evidence pointed toward a MAPK regulation of MPF activity and G_2/M entry, a number of experimental observations suggested the need for additional players.

For example, Gross et al. established that the MAP kinase pathway is not essential for entry into meiosis I in *Xenopus*, but is required during the onset of meiosis II to suppress entry into S-phase, unexpectedly and based on the existing model, the MEK inhibitor U0126 failed to block maturation induced by progesterone. This is in agreement with previous observations that suggested Mos helps maintain high MAP kinase activity, but not MPF, in meiosis (Nebreda and Hunt 1993; Gross et al. 2000; Yew et al. 1992; Daar et al. 1993). Although these are just a few examples of the collective experimental data that questioned the unique inhibition of Myt1 as sufficient to drive maturation, there was still a fundamental flaw underlying this simple model. The fact that Cdc2 is already phosphorylated in pre-MPF complexes present in G_2-arrested oocytes makes, a priori, the inhibition of Myt1 activity an unsound response to hormone stimulation. Thus, efforts focused on identifying parallel route(s) that led the activation of Cdc25 instead.

Several mechanisms have been proposed for the activation of the dual specificity phosphatase Cdc25 in response to progesterone stimuli in *Xenopus* oocytes. Initially, a cascade of phosphorylation events triggered by progesterone and mediated by the polo-like kinase pathway activates Cdc25. *In vitro* experiments first demonstrated that Plx1 phosphorylates Cdc25 and stimulates its activity promoting mitotic progression (Kumagai and Dunphy 1996) (Fig. 9.2). Further work established a parallel between the kinetics of activation of Plx1, Cdc25, and cyclin B/Cdc2 during oocyte maturation (Qian et al. 1998a, b, 2001). *In vivo* studies showed that microinjection of Plx1 into oocytes promoted maturation even in the absence of a mitogenic signal (Qian et al. 2001), whereas Plx1 depletion inhibited progression, an event reversed by the addition of active Cdc25 (Qian et al. 1998a, b). Overall, data place Plx1 upstream of the Cdc25 phosphatase. In addition, inhibition of the MAPK pathway in cell-free oocyte extracts delayed, but did not prevent, activation of Plx1 suggesting an alternative signaling mechanism that is independent of Mos synthesis (Qian et al. 2001). The Plx1 kinase is targeted by the polo-like kinase kinase 1 (xPlkk1) enzyme, or a related kinase in mammals, defining an additional protein kinase cascade that is independent of MAPK activation and that is known to control several events in mitosis (Qian et al. 1998b; Ellinger-Ziegelbauer et al. 2000; Pahlavan et al. 2000; Roshak et al. 2000; Jang et al. 2002).

Another level of regulation of Cdc25 activity involves its direct association with the 14-3-3 protein, a member of a regulatory mechanism extensively characterized during checkpoint activation. Similarly, Duckworth et al. have shown that PKA directly phosphorylates *Xenopus* oocyte Cdc25 in a site, Ser287, proven critical for its interaction with 14-3-3, thus keeping the phosphatase sequestered until PKA activity is downregulated by a hormonal signal (Duckworth et al. 2002). While the mechanisms described above represent the best-characterized pathways involved in Cdc25 regulation, it is most likely that others, which are yet uncovered, and additional players and pathways will contribute to controlling Cdc25 function.

We now know that initial exposure to progesterone simultaneously inhibits Cdc2 phosphorylation while promoting Cdc25 activation, thus triggering the conversion of inactive pre-MPF stockpiles in G_2-arrested oocytes into MPF. To sustain progression through the cycle, it is then necessary to establish an autoamplification

loop that ensures continuous production of active MPF by targeting key regulatory reactions in the amplification cascade. One of the various feedback activation loops results from the direct phosphorylation of Cdc25 by cyclin B/Cdc2 (Jessus and Beach 1992) (Fig. 9.2). Transient interaction among these molecules was demonstrated *in vivo* and proven to require a structural region in cyclin B known as the P-box for association (Jessus and Beach 1992; Zheng and Ruderman 1993). Work from the Maller laboratory demonstrated that the activity of Cdc25 oscillates in both meiotic and mitotic cell cycles peaking in M-phase with a concomitant increase in phosphorylation and that its activity is seriously compromised by unscheduled dephosphorylation of residues targeted by cyclin B/Cdc2 (Izumi et al. 1992; Izumi and Maller 1993). Another level of regulation is exerted by polo-like kinases where a positive feedback loop between Plx1 and xPlkk1 results in each kinase being phosphorylated and activating each other (Erikson et al. 2004). Tightly tied to the action of these kinases over Cdc25 activity is the role of PP2A and PP1 phosphatases. Although the exact mechanism by which these Ser/Thr phosphatases act on Cdc25 activation and the autoamplification loop is not completely understood, evidence points toward them controlling dephosphorylation of sites needed for full activation of Cdc25, specifically those targeted by Plx1 (Izumi et al. 1992; Maton et al. 2005). Among PP1's function is the dephosphorylation of Ser287 of *Xenopus* Cdc25, a residue responsible for anchoring the enzyme to the 14-3-3 protein, thereby derepressing Cdc25 at the time when MPF is active (Margolis et al. 2003).

Like Cdc25, Myt1 activity is subject to strict control by both autophosphorylation and the action of diverse kinases including Plx1, Mos, $p90^{Rsk}$, and cyclin B/Cdc2. The current model of Myt1 regulation establishes that members of the MAPK pathway control Myt1 activity during the meiotic cell cycle (Palmer et al. 1998; Peter et al. 2002) (Fig. 9.2). However, $p90^{Rsk}$ is inactivated after fertilization as a result of Mos degradation, upon which Plx1 takes control over Myt1 activity (Inoue and Sagata 2005). Lastly, it seems feasible that cyclin B/Cdc2 targets Myt1 as well as it does for Cdc25, thus synergizing the action of the MAPK pathway and Plx1 by sustaining an inhibitory activity over Myt1.

9.1.3 Meiotic Maturation

Below is a brief overview of the proteins and pathways implicated in meiotic maturation in *Xenopus* oocytes. For a more comprehensive discussion on this topic, the authors recommend reviews by Tunquist and Maller (2003) and Philpott and Yew (2008).

In vertebrates, the follicle cells surrounding the oocyte secrete progesterone, which induces oocyte maturation, a process that follows nuclear envelope breakdown, assembly of the meiosis I spindle, asymmetrical cell division, and progression through meiosis II until the action of CSF arrests the cell in metaphase of the second meiotic division (metaphase II) (Sagata 1996; Masui and Markert 1971). All

these events are accompanied by a complete remodeling of the oocyte envelope, plasma membrane, and underneath structures, after which mature oocytes pass through the oviduct to emerge from the female frog as unfertilized eggs.

The interval between meiosis I and meiosis II (called interkinesis) is characterized by a drastic and transient reduction in MPF activity largely due to degradation of up to 50% of the total level of cyclin B during which M-phase still persists but oocytes are unable to replicate chromosomal DNA (Peter et al. 2001; Taieb et al. 2001; Iwabuchi et al. 2000). Soon after meiosis I ends, there is *de novo* synthesis and accumulation of cyclin B as entry into meiosis II occurs (Taieb et al. 2001; Ledan et al. 2001).

A number of pioneer studies established the need of protein phosphorylation for sustaining CSF arrest (Maller et al. 1977; Doree et al. 1983; Shibuya and Masui 1988; Moses and Masui 1990). However, most of the work that followed initial observations of Masui and Markert needed to wait until the development of *in vitro* techniques that allowed the use of inhibitors, recombinant proteins, and antibodies to selectively manipulate signaling molecules in egg extracts (for details on extract preparation see Murray 1991). Nevertheless, by the end of the 1980s, it was clear that kinase activity was required for the establishment and maintenance of CSF arrest (for review see Tunquist and Maller 2003 and references within). By then, Sagata's studies established Mos as a necessary component of CSF for which it satisfies the four criteria: (a) it is expressed during oocyte maturation, (b) it induces metaphase arrest, (c) it is present and active throughout meiosis until fertilization, and (d) it is inactivated shortly after fertilization (Sagata et al. 1989a, b; Watanabe et al. 1991). Thus, the presence of Mos serves two purposes: the activation and stabilization of MPF and the establishment of CSF-mediated metaphase arrest. Further studies determined that the ability of Mos to cause meiosis II arrest in oocytes was due to the kinase activities of MEK1 and MAPK as shown by injecting phosphatase-resistant thiophosphorylated wild-type protein to promote arrest, depleting the system of MEK1 using a specific antibody, monitoring MAPK activity, and preventing oocyte arrest by co-injecting anti-MEK1 antibody and recombinant Mos (Kosako et al. 1994a, b; Haccard et al. 1993). Later experiments placed the $p90^{Rsk}$ kinase downstream of MAPK activation. Injection of a constitutive active form of $p90^{Rsk}$ was sufficient to arrest the blastomere of a two-cell embryo in metaphase (Gross et al. 2001). Conversely, addition of Mos to interphase extracts depleted of endogenous $p90^{Rsk}$ failed to establish any CSF activity (Bhatt and Ferrell 1999).

The spindle assembly checkpoint is an evolutionarily conserved surveillance mechanism that ensures chromosomes do not segregate until they are properly aligned and attached to the microtubules of the spindle. The core component is the *a*naphase-*p*romoting *c*omplex or *c*yclosome (APC/C), an E3 ubiquitin ligase that, when associated with its co-activator Cdc20 (also called Fizzy/$p55^{CDC}$), polyubiquitinates substrates such as cyclin B and securing earmarking them for immediate proteolysis through the proteasome (Eytan et al. 2006; Harper et al. 2002) (Fig. 9.3). All these components are present in *Xenopus* oocytes and function during oocyte maturation to sustain CSF arrest but, unlike the case of the spindle

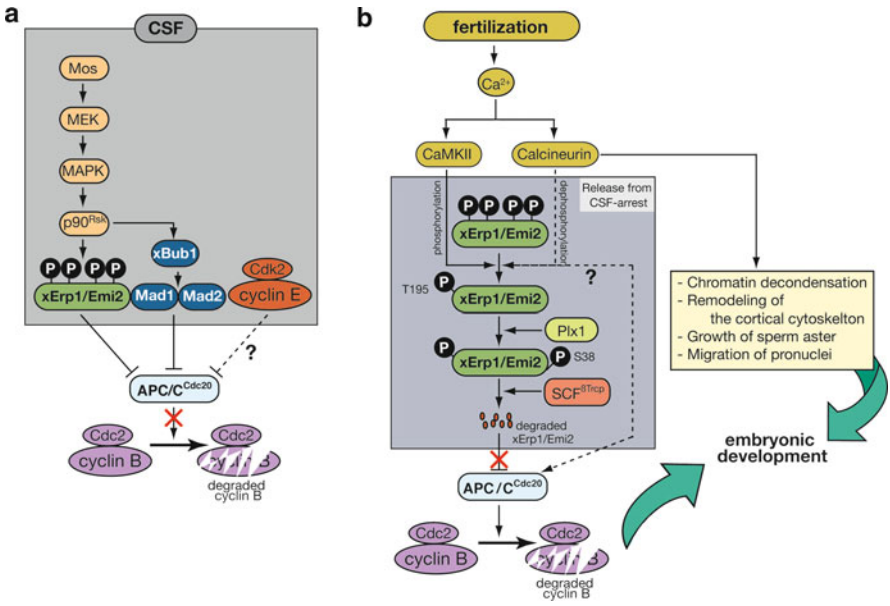

Fig. 9.3 Schematic of CSF arrest and release in *Xenopus* oocytes. (**a**) In meiosis II, the Mos-$p90^{Rsk}$ pathway promotes CSF arrest by increasing xErp1/Emi2, an inhibitor of APC/C^{Cdc20}, stability through a mechanism that includes phosphorylation of the inhibitor in at least four distinct sites: Ser335, Thr336, Ser342, and Ser344. Inhibition of APC/C^{Cdc20} activity results in suppression of cyclin B degradation and arrest at metaphase II. In addition, a xBub1-dependent mechanism that is dependent on MAPK activation promotes CSF arrest. Alternatively, PG-mediated synthesis of cyclin E and Cdk2 in meiosis II is sufficient to sustain APC/C^{Cdc20} inhibition by an unknown pathway that is independent of MAPK signaling. (**b**) Upon fertilization, Ca^{2+} surge activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calcineurin. Calcineurin dephosphorylates all sites targeted by $p90^{Rsk}$ during CSF arrest, whereas CaMKII and later Plx1 phosphorylate xErp1/Emi2 in Thr195 and Ser38, respectively. As a result, the $SCF^{\beta Trcp}$ ubiquitin ligase mediates xErp1/Emi2 degradation, releasing the APC/C^{Cdc20} from inhibition and reassuming progression. In an alternative pathway, calcineurin dephosphorylates a number of substrates implicated in chromatin decondensation, remodeling of cytoskeleton, growth of sperm aster, and pronuclei migration all of which promotes progression. *Dashed lines* indicate other hypothesized points of regulation

assembly checkpoint, there is no spindle disruption occurring during metaphase II arrest (for review see Tunquist and Maller 2003, Fig. 9.3). Early experiments have shown that inhibition of the APC/C is sufficient to prevent CSF release and maintain a metaphase-arrested spindle morphology simply by using antibodies against the xFzy protein (Taieb et al. 2001; Lorca et al. 1998). Remarkably, neither anti-xFzy antibodies nor antisense oligonucleotides directed against xFzy prevented calmodulin-dependent protein kinase II (CaMKII) from inducing metaphase–anaphase transition in *Xenopus* extracts and this event was associated with the suppression of cyclin B degradation (Taieb et al. 2001; Lorca et al. 1998).

More recently, the establishment of CSF activity has been linked to checkpoint proteins Bub1, Mad1, and Mad2 and placed upstream of APC/C for inhibition in

a manner that is independent of spindle dynamics (Schwab et al. 2001; Sharp-Baker and Chen 2001; Chen 2002; Tunquist et al. 2002) (Fig. 9.3a). Studies show *Xenopus* Bub1 (xBub1) is phosphorylated during oocyte maturation, an event that correlates with the intrinsic MAPK activity and that was later proven by using the MEK inhibitor U0126 (Schwab et al. 2001). Thus, this finding adds to the role of the MAPK pathway as an inhibitor of APC/C after meiosis I and for *de novo* synthesis of cyclin B in entry to meiosis II, by establishing MAPK to be upstream of xBub1 (Gross et al. 2000; Taieb et al. 2001; Schwab et al. 2001). Interestingly, microinjection of a constitutive active form of $p90^{Rsk}$ rescues the phosphorylation of xBub1 in the presence of MEK inhibitor and was able to directly phosphorylate xBub1 *in vitro* (Schwab et al. 2001; Tunquist et al. 2002) supporting a model in which the MEK/MAPK/$p90^{Rsk}$/xBub1 signal targets the spindle assembly checkpoint proteins such as Mad1/2, inhibits APC/C, and prevents the metaphase to anaphase transition.

Another activity involved in establishing CSF arrest pertains to cyclin E/Cdk2 (Tunquist et al. 2002) (Fig. 9.3a). Addition of cyclin E/Cdk2 to *Xenopus* egg extracts caused metaphase arrest even in the absence of Mos and was able to inhibit cyclin B degradation between meiosis I and II when injected in meiosis I oocytes depleted of cyclin E/Cdk2 activity but with an intact and active MAPK pathway (Tunquist et al. 2002). Importantly, once CSF arrest has been established, both the Mos/MEK1/MAPK/$p90^{Rsk}$/xBub1 pathway and cyclin E/Cdk2 become dispensable; thus, a new activity is needed for maintenance of CSF arrest. From this perspective, evidence points towards members of the family of *e*arly *m*itotic *i*nhibitor (Emi)/*E*mi-*r*elated *p*rotein (Erp) molecules (for review see Schmidt et al. 2006) that have recently emerged as prime players in promoting APC/C inhibition (Liu and Maller 2005; Rauh et al. 2005; Tung et al. 2005; Hansen et al. 2006; Inoue et al. 2007; Nishiyama et al. 2007a) (Fig. 9.3a). *Xenopus* Emi1, a homolog of the *Drosophila* Rca1 gene, is present at low, but steady, levels throughout oocyte maturation in CSF-arrested eggs and after fertilization through the long first interphase (Reimann and Jackson 2002). Later, and during the first mitotic cycle of the zygote, Emi1 levels follow cycles of synthesis and degradation that accompany cell cycle transitions in early *Xenopus* embryo (Reimann et al. 2001a). Emi1 regulates mitosis by physically interacting with and inhibiting the APC/C activator Cdc20/xFzy, suggesting a direct role in controlling CSF arrest (Reimann et al. 2001a). Consistent with this, immunodepletion of Emi1 from CSF extracts causes unscheduled CSF release even in the absence of calcium; addition of recombinant Emi1 protein to CSF extracts prevents CaMKII-mediated release, and microinjection of Emi1 into a blastomere causes arrest of cells with high Cdc2 activity (Reimann and Jackson 2002; Reimann et al. 2001a, b). However, these findings were later challenged by Kishimoto's group when additional studies proved that the level of Emi1 present in CSF-arrested cells was too low to participate in the regulation of APC/C activity (Ohsumi et al. 2004) and that Emi1-mediated M-phase arrest remains unaltered by calcium, all of which suggest a form of arrest that is distinct from CSF arrest. In support of this model, addition of Emi1 to cyclin extracts stabilizes both cyclin A and cyclin B, preventing exit from mitosis by a mechanism that appears to be different from that which is mediated by CSF

activity in unfertilized eggs where the sole accumulation of cyclin B results in a meiotic metaphase-arrested spindle. Later characterization of a *Xenopus E*mi1-*r*elated *p*rotein, named xErp1/Emi2, shed light on this apparent conflicting data. The xErp1/Emi2 protein contains a modular domain for Cdc20/xFzy binding that closely resembles the one characterized in Emi1 (Schmidt et al. 2005). Inactivation of xErp1/Emi2 led to premature APC/C activation and CSF release, whereas addition of recombinant xErp1/Emi2 to *Xenopus* egg extracts prevented release even in the presence of calcium (Schmidt et al. 2005). In addition, findings showed that some of the antibodies directed against Emi1 were found to co-immunoprecipitate xErp1/Emi2, suggesting that most of the initial observations could be explained in the context of xErp1/Emi2 depletion.

The current mode of regulation of meiotic metaphase arrest in *Xenopus* eggs involves multiple upstream pathways, some of which have been proved to converge in xErp1/Emi2. For example, Plx1 is required for APC/C activation at the metaphase–anaphase transition, whereas increased free calcium is responsible for eliciting a CaMKII-mediated signal that not only activates APC/C but also terminates CSF arrest in cell-free extracts. Further studies placed Plx1 as a necessary player in the calcium-induced CSF release signaling pathway (Liu and Maller 2005). Overexpression of either Plx1 or CaMKII has proven sufficient to trigger CSF release, whereas depletion of Plx1 in egg extracts blocks release even under conditions of increasing calcium. Importantly, Plx1 overexpression is sufficient to activate CaMKII by a mechanism that is calcium independent (Liu and Maller 2005). The proposed model involves a dual regulation of xErp1/Emi2 by both kinases in which CaMKII "primes" the inhibitor for phosphorylation, generating a docking site for Plx1, which subsequently phosphorylates xErp1/Emi2 making the inhibitor a target for ubiquitin-mediated degradation by the SCF pathway (Schmidt et al. 2005; Liu and Maller 2005). According to its role in CSF arrest release, xErp1/Emi2 is rapidly degraded shortly after calcium addition to CSF extracts and this event depends on Plx1 activity (Tung et al. 2005; Schmidt et al. 2005). Detailed mechanistic studies on Plx1 and CaMKII phosphorylation of xErp1/Emi2 are discussed in detail in the section below.

Overall, it seems reasonable to expect that a more complex pattern of regulation will emerge in coming years mainly due to the complexity of the crosstalk pathways operating to establish and maintain metaphase II arrest, the large number of players, the various control mechanisms, the hierarchy of its components, and the dynamic nature of its regulation.

9.1.4 Fertilization

A number of structural changes on the surface of the oocyte unfold for ~6 h, which follow progesterone release of G_2-arrest in stage VI oocytes until cells are fully mature and maintained in metaphase II. Some of these changes dramatically affect transport through the membrane, oocyte electric potential, properties of the cortex,

and morphology of the cortical endoplasmic reticulum, which closely correlates with the development of responsiveness to ionophores during maturation (Finidori-Lepicard et al. 1981; Charbonneau and Grey 1984; Robinson 1979; Kado and Baud 1981).

In *Xenopus*, the extracellular matrix surrounding the oocyte makes its first appearance in stage II to reach its full thickness by stage V and continues to remodel until two distinct layers, the perivitelline space glycocalyx and the overlaying envelope, are clearly distinguished in meiotically immature stage VI oocytes (Dumont 1972). After maturation, oocytes are liberated from the coelomic cavity and thus the oocyte extracellular matrix is referred to as the coelomic envelope (CE) (Gerton and Hedrick 1986). CE has a very peculiar structure that has been morphologically described by the early work of Grey et al. (1977). CE consists of a 1-μm-thick layer of fibrils arranged in bundles or fascicles with the innermost aspect of the CE being contiguous with the perivitelline space, whereas the exterior part contains some randomly spaced channels in a structure that is incapable of being penetrated by sperm. The remodeling of CE to a sperm-receptive vitalline envelope occurs as the oocyte passes through the pars recta portion of the oviduct and some of the envelope's glycoproteins are processed by specific proteolytic enzymes emerging as a new structure surrounding the oviposited egg (Gerton and Hedrick 1986).

The process of fertilization in the vertebrate egg begins with the fusion of the sperm with the egg to cause a transient mobilization of calcium that originates from the sperm entry point and spreads throughout the cell (Kubota et al. 1987) (Fig. 9.3b). Polyspermy events are unlikely to occur in eggs due to the release of intracellular calcium and the hardening of the zona pellucida that results from the breakdown of cortical granules in activated eggs (for review see Runft et al. 2002). In addition, mature eggs microinjected with either a calcium ionophore (i.e., A23187) or an inorganic calcium salt result in activated oocytes, whereas similar experiments using calcium-chelating agents (*i.e.*, EGTA and BAPTA) prevented sperm-induced egg activation (Tunquist and Maller 2003; Tokmakov et al. 2010). The increase in concentration of cytosolic calcium inactivates both CSF and MPF, allowing the egg to escape from its metaphase II arrest and progress through anaphase II and cytokinesis to enter interphase (for review see Tunquist and Maller 2003; Tokmakov et al. 2010).

Calcium release from intracellular stores results from the sequential activation of the Src family of kinases, phospholipase C (PLCγ), and the 1,4,5-inositoltriphosphate (IP3) receptor located in the endoplasmic reticulum (Saunders et al. 2002). PLC enzyme hydrolyzes phosphatidylinositol 4,5-biphosphate to IP3 and *sn*-1,2-diacylglycerol (DG) branching the signaling pathway; thus, IP3 binds its receptors in the endoplasmic reticulum gating the calcium channel and mobilizing calcium toward the cytosol, while DG activates PKC. Evidences suggest the latest association might be responsible for many of the morphological and cellular effects observed in fertilized eggs, including cortical granule exocytosis, cortical contraction, DNA decondensation, and formation of the nuclear envelope (Bement and Capco 1990; Grandin and Charbonneau 1991).

At fertilization, the increase in intracellular levels of calcium is approximately fourfold, from an initial value of 200–400 nM to roughly 1.2–1.4 μM (Busa and Nuccitelli 1985). Calcium wave travels across the egg from the sperm-binding site and is believed to be responsible for several biochemical events happening in eggs after activation (Bement and Capco 1990; Kline 1988; Grandin and Charbonneau 1992). For example, the calcium wave triggers activation of CaMKII, which is believed to mediate cyclin B degradation, inactivation of MPF, dephosphorylation in Ser3 of Mos, and subsequent degradation of Mos (Nixon et al. 2002). The action of calcium on cyclin stability was first demonstrated in *Xenopus* and later found to be conserved among species (Lorca et al. 1993, 1994). Accordingly, addition of CaMKII to CSF-arrested extracts promotes cyclin B degradation, MPF inactivation, and CSF-release, whereas an inhibitor of the kinase prevented exit from metaphase (Lorca et al. 1993) (Fig. 9.3b). CaMKII is also responsible for the destruction of securin by polyubiquitination, which, in turn, frees separase to act on the kleisin component of cohesin (Nasmyth and Haering 2005). Moreover, CaMKII directly targets the APC/C inhibitor xErp1/Emi2 for phosphorylation leading to the recruitment of the Plx1 that marks xErp1/Emi2 for degradation by phosphorylating a signal region that is recognized by the $SCF^{\beta Trcp}$ (Hansen et al. 2006). In addition, some evidence indicates that CaMKII activation might also play a role in Cdc25 activation (Hutchins et al. 2003). More recently, two independent groups discovered that shortly after calcium release, the calcium/calmodulin-dependent protein phosphatase calcineurin is rapidly and transiently activated in a CaMKII-independent fashion and that its inhibition prevents cyclin B degradation, dephosphorylation of various substrates posttranslationally modified during M-phase, migration of pronuclei, and cytoskeleton reorganization to mention a few (Nishiyama et al. 2007b; Mochida and Hunt 2007) (Fig. 9.3b). Thus, it seems likely that at least two signals triggered by a calcium spike, CaMKII and calcineurin, act together to break the meiotic arrest.

9.1.5 Mitotic Cycles

The development of multicellular organisms is a relatively slow process of changes that begins with a single, fertilized egg, or zygote, which divides mitotically to generate cellular diversity (named differentiation) in an orderly manner (morphogenesis). To achieve this, cells need to proceed through tightly regulated cycles of cell division (cleavages) that occur rapidly and wherein the volume of zygote cytoplasm generates numerous smaller cells (blastomeres), a process that is accompanied by cell-fate specification.

Following fertilization and after CSF release, eggs enter S-phase where chromosomes decondense, DNA replication begins, and the nuclear envelope assembles. As in other vertebrate and invertebrate species, the first mitotic cycle is exceptionally long compared to the following 12 embryonic cycles. The increased length of the first mitotic cycle (~90 min) is largely due to the time in which DNA replication

begins (29 min after fertilization), the length of the duplication time (23 min), the moment at which the nuclear envelop breaks down (~69 min after fertilization), and the existence of a 17-min G_2-phase that is uniquely present during the first mitotic cycle. The second, much shorter cycle, begins 34 min after completion of the first round of DNA replication and consists only of alternating S- and M-phases without any transcriptional activity or intervening gap phases. This abbreviated cell cycle lasts for about 30 min and continues synchronously in the animal, equatorial, and vegetal hemispheres 11 times until gap phases are reintroduced into the cell cycle at the *m*id*b*lastula *t*ransition (MBT, cycle 13), when zygotic transcription begins. Shortly after the MBT, cells lose their synchrony, the cell cycle lengthens, cells start to differentiate, and developmental transitions occur (Newport and Kirschner 1982a, b; Kimelman et al. 1987; Howe et al. 1995; Gerhart 1980; Graham and Morgan 1966).

Cell-free extracts of *Xenopus* eggs have been successfully used to determine the interplay among molecular components that drive meiotic exit, mitotic transitions, DNA replication, spindle assembly, and apoptotic processes. For example, CSF-arrested extracts have played a pivotal role in studying fertilization/activation-signaling processes following calcium mobilization and during the first mitotic cycle. As mentioned before, inactivation of MPF is due to the specific degradation of the cyclin B, but not the Cdc2, that occurs shortly after calcium release allowing progression through M-phase. Using *Xenopus* egg extracts, Nishiyama et al. and Chesnel et al. demonstrated the existence of nonproteolytic activity of the 26S proteasome capable of dissociating both subunits of MPF by a mechanism that is both independent of dephosphorylation of Thr161 in Cdc2 and proteolysis, thus reinforcing the concept that cyclin B dissociation from Cdc2 is the decisive step leading to MPF inactivation and that dissociation precedes cyclin B degradation (Nishiyama et al. 2000; Chesnel et al. 2006, 2007). Another well-established event that follows fertilization relates to the downregulation of MAPK activity, a process that is essential for embryos to enter the first mitotic cycle and that is conserved in the animal kingdom (Abrieu et al. 1997) (Fig. 9.1). Downregulation of MAPK activity is not required for calcium-dependent degradation of mitotic cyclins after fertilization and, indeed, its inactivation is slightly delayed when compared with detected MPF. Of note is that MAPK activity remains largely undetectable during the 11 forthcoming mitotic cycles until gap phases are newly established after the MBT and a peak in enzyme activity is newly detected (Fig. 9.1), while its phosphorylation is detectable following peaks of MPF at least in the first and the second cell cycle (Chesnel et al. 2005).

Extensive research in *Xenopus* egg extracts has led to the identification of factors involved in origin licensing, initiation, and replication; all of which are steps that are evolutionarily conserved from yeast to humans. In preparation for DNA replication in the S-phase, the *pre-r*eplication *c*omplex (pre-RC) is assembled from late mitosis to early G_1, a period in the cell cycle where the kinase Cdk2 is inactive (Nguyen et al. 2001). Licensing requires the orderly loading of the *o*rigin *r*ecognition *c*omplex *1–6* (Orc1–6), which in *Xenopus* remains bound to DNA throughout interphase (Carpenter et al. 1996), the *m*ini*c*hromosome *m*aintenance proteins

Mcm2 to Mcm7 (Mcm2–7, Chong et al. 1995; Thommes et al. 1997), which contain helicase activity, the *c*ell *d*ivision *c*ycle protein *6* (*C*dc6) and the *C*dc10-*d*ependent *t*ranscript (Cdt1). Although various Mcm complexes are able to bind the ori, only the Mcm2–7 (including Mcm2, 3, 4, 5, 6, and 7) is functional (Prokhorova and Blow 2000). Subsequently, a nuclear envelope forms and critical replication factors are imported. For a detailed analysis of S-phase regulation in *Xenopus*, see the accompanying chapter by Fisher (2011).

In order for licensed origins to initiate DNA replication, the S-phase-promoting factor (SPF), defined as the Cdk activity required for DNA replication, needs to be assembled. This involves the recruitment of both the Ser/Thr kinase Cdc7, whose major physiological substrate is Mcm2–7, and the Cdk2 kinase whose target substrates for DNA replication have been thoroughly reviewed by Fisher (2011). At the G_1/S boundary, the pre-RC becomes active by the recruitment of Cut5, active Cdc7, which is regulated by Dbf and expressed throughout embryo development, and Drf1, which is only present in pre-MBT cycles, and the action of cyclin E/Cdk2, Mcm10, Cdc45, and GINS chromatin loading to form the *pre-i*nitiation *c*omplex (pre-IC). Interestingly, the Mcm2–7 complexes are polyfunctional during the DNA replication process and are required for the formation of the pre-RC, pre-IC, origin unwinding, and elongation. In addition, Mcms have also been involved in transcriptional activation, in response to DNA damage and in chromatin remodeling (for review see Forsburg 2004). Additional studies have established a role for the histone acetyltransferase XHbo1 in origin activation by acting upon histone H4 before pre-RC formation and on Orc2, Mcm2, Cdc6, geminin, and itself, which prevents chromatin binding of the Mcm2–7 complex (Iizuka et al. 2006).

Once the pre-IC complex is assembled, DNA starts to unwind and *r*eplication *p*rotein *A* (RPA) associates and stabilizes single-stranded DNA allowing the binding of DNA polymerase α-Primase (for review see Walter and Newport 2000 and references within). The cyclin A/Cdk2 complex is present during the S-phase of the cell cycle in *Xenopus* embryos and whereas its precise role during DNA replication is not completely understood, it is believed that, like in mammalian cells, its Cdk activity does not influence the assembly of initiation complexes but acts in a later stage prior to DNA elongation (Fotedar et al. 1996). The *r*eplication *f*actor *C* (RF-C), a heteropentameric complex similar in structure to clamp loaders, possesses an ATPase activity that is needed for loading the sliding clamp *p*roliferating *c*ell *n*uclear *a*ntigen (PCNA) onto the DNA after which RF-C is dissociated. PCNA contacts the replicative polymerases pol δ, a phosphoprotein that physically interacts with cyclin/Cdks, and pol ε, an important sensor for UV damage and DNA replication during S-phase. Thus, the RF-C seems to be implicated in the last step of replication initiation and the various DNA repair processes (for review see Hubscher et al. 2002 and references within).

A number of redundant mechanisms exist to ensure that DNA replication is limited to one per cell cycle (Blow and Laskey 1988). In higher eukaryotes, the presence of a small multifunctional protein first identified in *Xenopus*, geminin (McGarry and Kirschner 1998), can act as both a promoter and an inhibitor of initiation of DNA replication. In mammalian cells, geminin is kept low (or even absent)

during G_1-phase and accumulates during S-, G_2-, and M-phases to disappear at the time of metaphase–anaphase transition by a mechanism that is dependent on its ubiquitination and is mediated by the APC. When the APC is inactivated at the end of G_1, geminin reaccumulates and titrates Cdt1 out of the system by keeping Cdt1 sequestered from S-phase throughout late mitosis. Thus, geminin's inhibitory role relates to its ability to prevent Cdt1 from loading the Mcm complex onto prereplication complexes in late S-, G_2-, and M-phases (McGarry and Kirschner 1998). In *Xenopus* egg extracts, the ratio between geminin and Cdt1 levels determines the amount of complex formed, the assembly of pre-RC, and whether the origins are licensed or not (Lutzmann et al. 2006). Although the promoter and inhibitory functions of geminin seem to conflict, both can be explained in the context of temporal separation for origin licensing versus DNA replication during the cell cycle, with pre-RC formation occurring during late M- and early G_1-phase, while pre-RC inhibition is observed in late S- to mid M-phase.

At present, there are three types of cyclin/Cdk complexes described in *Xenopus* early embryos: (a) cyclin B/Cdc2, (b) cyclin A/Cdc2, although a cyclin A/Cdk2 complex is detected later in development, and (c) cyclin E (also called E1)/Cdk2 (Fig. 9.4). As we previously mentioned when describing MPF, cyclin B-type molecules associate with their Cdc2 counterpart when transitioning into mitosis,

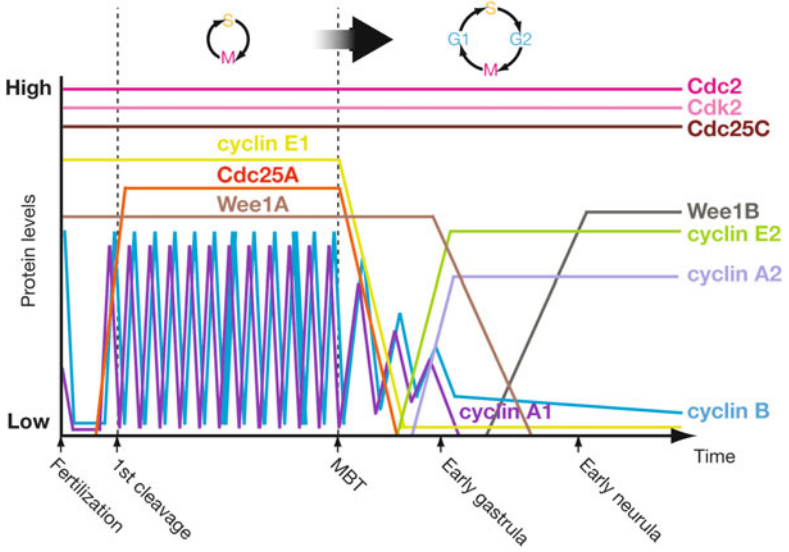

Fig. 9.4 Cell cycle remodeling at the *Xenopus* MBT. Embryos progress through the first 12 cleavage cycles altering phases of DNA synthesis and division until gap phases are established after MBT and consequently the cell cycle lengthens. Before the MBT, transitions are driven by oscillatory levels of cyclins A1 and B, whereas cyclin E1 level remains constant. Cyclins associate with their Cdc2/Cdk2 counterpart and the activity of the complex correlates with cell cycle transitions. Unlike Cdc25A whose level drops at MBT, Cdc25C remains constant throughout early development. The zygotic forms of cyclins A and E, A2 and E2, and the Wee1B kinase are detected shortly after the MBT

whereas other cyclin/Cdk complexes are involved in progression through S-phase of the embryonic cycle. The need of Cdk2 activity for progression through S-phase was first uncovered by using both a dominant negative form of Cdk2 (van den Heuvel and Harlow 1993) and microinjection of anti-Cdk2 antibodies in human fibroblasts (Pagano et al. 1993). Experiments using baculovirus-expressed cyclin/Cdk complexes in *Xenopus* extracts showed that cyclin E/Cdk2 and cyclin A/Cdc2 possess S-phase-promoting activity and are therefore able to progress DNA replication in extracts of activated *Xenopus* eggs. Indeed, cyclin E/Cdk2 is sufficient to enter into S-phase but is unable to promote nuclear envelope disassembly or full chromosome condensation; hence, the additional increasing SPF activity detected in cyclin A/Cdc2 during S-phase progression is needed to complete the process (Strausfeld et al. 1996). A role for cyclin E/Cdk2, but not cyclin A/Cdk2, in S-phase progression has been established across species from mammals to *Drosophila* (Knoblich et al. 1994; Knoblich and Lehner 1993; Girard et al. 1991). Further studies established that a very low level of Cdk activity is sufficient for DNA replication in *Xenopus* egg extracts; however, precisely because of its low level, small variations in Cdk activity greatly compromise replication efficiency and limit pre-RC to pre-IC transition (Krasinska et al. 2008). Although certain preference for cyclin E/Cdk2 has been established, DNA replication and origin firing do not seem to have an absolute requirement for one specific cyclin/Cdk complex in *Xenopus* egg extracts (Krasinska et al. 2008). A compelling analysis of the role of Cdks in S-phase regulation is presented by Fisher (2011).

It has become clear that the events that define the various phases of the cell cycle are driven by distinct forms of cyclin/Cdk complexes that are present in a timely manner in the early embryo. In frog eggs, the regulation of either Cdc2 or Cdk2 does not involve oscillations at the protein level as their amounts appear to be constant (~600 and ~60 nM for Cdc2 and Cdk2, respectively, Kobayashi et al. 1991a, b) during the different phases of the cell cycle in both somatic and early embryonic *Xenopus* cells (Strausfeld et al. 1996; Draetta et al. 1989; Draetta and Beach 1988) (Fig. 9.4). Interestingly, cyclin E1 levels remain constant and at a concentration of ~30–60 nM during the early stages of development and until MBT when it is replaced by a zygotic form of the protein, cyclin E2, that remains functional throughout development (Gotoh et al. 2007). Gabrielli et al. established that unlike in the case of Cdc2, in which there is a large molar excess of the kinase in the egg, most of, if not all, Cdk2 is complexed with cyclin E1 in pre-MBT embryos with relatively low levels of free Cdk2. In addition, the cyclin E1/Cdk2 complex, but neither of its components alone, possesses histone H1 kinase activity (Gabrielli et al. 1992). Although the complex level remains constant, its associated Cdk2 kinase activity cycles twice for each oscillation of MPF in agreement with a role of cyclin E in both S-phase and mitosis (Hartley et al. 1996).

In contrast, cyclins A and B are degraded at the end of each mitotic phase and therefore their levels oscillate within the cell cycle. Two forms of cyclin A are present in early *Xenopus* embryos, A1 and A2. In pre-MBT embryos, cyclin A1 is complexed with Cdc2, whereas cyclin A2 remains almost undetectable before MBT. By the time embryos reach stages 10–12, a switch occurs and cyclin A1

and E1 levels fall rapidly, whereas cyclin A2 levels rise to form a new complex with Cdk2 as seen in somatic cells (Howe et al. 1995; Rempel et al. 1995). Remarkably, these events roughly occur at the same time as key developmental processes and correlate with a complete remodeling of the embryonic cell cycle that resembles a somatic cycle.

The regulation of Cdc2 activity in early embryonic cycles results from understanding the interplay among various cell cycle regulators. As was the case during the meiotic cycles, the Cdc25 phosphatases and the Myt1 and Wee1 kinases play a central role in controlling MPF activity in early *Xenopus* embryogenesis. The Cdc25C phosphatase levels remain relatively constant throughout embryogenesis and alternate between a highly phosphorylated and a less phosphorylated form that correlates with high and low levels of cyclin B/Cdc2 kinase activity, respectively (Hartley et al. 1996) (Fig. 9.4). A maternal form of Cdc25A is translated at the beginning of the second cycle but disappears at about MBT (Hartley et al. 1996; Kim et al. 1999; Izumi and Maller 1995). Another level of regulation of cyclin B/Cdc2 function comes from controlling the activity and expression of Wee-like kinases that act on MPF in early embryos (Fig. 9.4). Leise and Muller determined that Myt1 is expressed throughout embryogenesis, whereas the maternal Wee1 kinase, initially translated in meiosis II, remains stable in pregastrula embryos until it is degraded and replaced by a zygotic isoform (named Wee1B/Wee2) (Leise and Mueller 2002; Okamoto et al. 2002). After exiting mitosis, cyclins A and B, but not E, are degraded and the S-phase-promoting factor triggers initiation of DNA replication in a process that, unlike others mediated by cyclin D/Cdk4, is independent of protein synthesis. During S-phase in *Xenopus* embryos with normal levels of protein synthesis and degradation, there is only an ~3-nM level of detectable cyclin A/Cdc2 kinase that contributes to SPF activity (Kobayashi et al. 1991a, b) and that it is speculated to help trigger the initiation of replicons that have not been already fired. As the cell cycle progresses, cyclins A and B levels are resynthesized and accumulate, and the MPF is required for a new entry into mitosis.

In most species, progression through the cell cycle results from an additional level of regulation that comes from the action of specific inhibitors that target either the Cdk kinase or the cyclin/Cdk complex. To date, only two closely related Cdk inhibitors, $p27^{Xic1}$ and $p28^{Kix1}$ (cyclin-dependent *k*inase *i*nhibitor from *Xenopus*), have been well characterized in *Xenopus* (Su et al. 1995; Shou and Dunphy 1996). The $p27^{Xic1}$ and $p28^{Kix1}$ inhibitors share over 90% identity and are probably derived from a single allele due to the pseudotetraploidy of *Xenopus laevis*. $p27^{Xic1}$ shares 44% identity (73% similarity) with human $p27^{Kip1}$ and 40% identity (54% similarity) with human $p21^{Cip1}$ in the conserved N-terminus (residues 30–91). In addition, $p27^{Xic1}$ shares stretches of homologous sequence in the C-terminus with mammalian $p27^{Kip1}$ and $p57^{Kip2}$, particularly in a defined QT domain containing various cyclin/Cdk phosphorylation sites. Interestingly, $p27^{Xic1}$ also possesses a short sequence within its C-terminus that resembles a PCNA-binding site defined in human $p21^{Cip1}$ (Su et al. 1995). The overall modular architecture of $p27^{Xic1}$ suggests that this inhibitor may be a primordial form of CKI that in mammals has diverged into the more specialized Kip1/Kip2 and Cip1 families.

The $p27^{Xic1}$ protein inhibits *Xenopus* cyclin E/Cdk2 more potently than cyclin A/Cdk2 and cyclin B/Cdc2 complexes *in vitro*, and is able to block both single-stranded and nuclear DNA synthesis in egg extracts (Su et al. 1995). The $p27^{Xic1}$ inhibitor is degraded in a nuclear- and Cdc34-dependent process by the ubiquitin–proteasome pathway in *Xenopus* egg extracts (Yew and Kirschner 1997; Swanson et al. 2000; Chuang and Yew 2001). The $p27^{Xic1}$ nuclear localization is independently mediated by binding to cyclin E/Cdk2 and by nuclear localization sequences within its C-terminus (Chuang and Yew 2001). Unlike $p27^{Kip1}$, binding of $p27^{Xic1}$ to cyclin E/Cdk2 is dispensable for $p27^{Xic1}$ ubiquitination and degradation (Chuang and Yew 2001). Interestingly, a C-terminal region (residues 180–183) of $p27^{Xic1}$ seems to define a motif essential for recognition by the ubiquitin-conjugation machinery, or, for binding an alternate protein required for degradation (You et al. 2002). Chuang et al. have recently shown that the 50 amino acid residues located at the C-terminus of $p27^{Xic1}$ are critical for its proteolysis. This is in addition to the motif's role in nuclear transport, and phosphorylation of $p27^{Xic1}$ is not critical for its nuclear ubiquitination and degradation (Chuang et al. 2005). Other studies indicate that degradation of $p27^{Xic1}$ is dependent on initiation of DNA replication in *Xenopus* egg extracts, and it requires not only the assembly of prereplication complexes on chromatin but also the origin-activating kinases Cdk2 and Cdc7, as well as the initiation protein Cdc45 (You et al. 2002). The requirement for Cdk2 and Cdc7 may be indirect in that loading of Cdc45 onto chromatin during initiation requires the activity of both these kinases (Mimura and Takisawa 1998). Other studies also indicate that $p27^{Xic1}$ degradation is absolutely dependent on its binding to PCNA in both *Xenopus* egg and gastrulation-stage extracts (Chuang and Yew 2005). Importantly, $p27^{Xic1}$ proteolysis requires the ability of PCNA to be loaded onto primed DNA by the RF-C suggesting that $p27^{Xic1}$ is targeted for ubiquitination and degradation through its interaction with PCNA at a site of initiation (Chuang and Yew 2005).

9.2 Concluding Remarks

Xenopus has been increasingly used for the direct investigation of mechanisms important in disease processes. For example, analysis of gene function in this system has contributed to the elucidation of the molecular properties and functional roles of genes that play analogous roles in more complex organisms and are involved in human diseases, such as neurodegenerative disorders, cystic fibrosis, heart disease, and various cancers. Cell proliferation depends on a timely integrated signaling network that results from the presence of positive growth signals that influence the expression, stability, and activity of cell cycle components. Unscheduled and uncontrolled cell proliferation in tissues result in chronic and progressive conditions that ultimately lead to severe diseases. We currently know that many of the processes governing the genesis and progression of various pathologies relate to the aberrant regulation of cyclins, cyclin-dependent kinases, and their inhibitor

proteins. The challenge that is now before the research community is to identify and understand the molecular anatomy of such pivotal regulation under normal physiology and disease pathology and to develop therapies that directly attack their points of convergence.

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Chapter 10
Control of DNA Replication by Cyclin-Dependent Kinases in Development

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Abstract Cyclin-dependent kinases (CDKs) are required for initiation of DNA replication in all eukaryotes, and appear to act at multiple levels to control replication origin firing, depending on the cell type and stage of development. In early development of many animals, both invertebrate and vertebrate, rapid cell cycling is coupled with transcriptional repression, and replication initiates at closely spaced replication origins with little or no sequence specificity. This organisation of DNA replication is modified during development as cell proliferation becomes more controlled and defined. In all eukaryotic cells, CDKs promote conversion of "licensed" pre-replication complexes (pre-RC) to active initiation complexes. In certain circumstances, CDKs may also control pre-RC formation, transcription of replication factor genes, chromatin remodelling, origin spacing, and organisation of replication origin clusters and replication foci within the nucleus. Although CDK1 and CDK2 have overlapping roles, there is a limit to their functional redundancy. Here, I review these findings and their implications for development.

10.1 Introduction

DNA replication, which commits a eukaryotic cell to dividing, can be considered a "passive driving force" for development, since it both creates a problem and provides a window of opportunity for doing something new. Due to cell division, even within an apparently simple organism with no development, such as yeast, not all cells are equal. In a colony of yeast cells, the cells in the middle of the colony are small and the cell cycle is arrested, due to nutrient starvation, whereas those at the edge of the colony are bigger and rapidly dividing. In this case, the size of the colony is limited by nutrient availability, and nutrient starvation provokes sexual reproduction among opposite mating types. Metazoans have solved the availability problem by centralising resources and despatching them to all cells, allowing them

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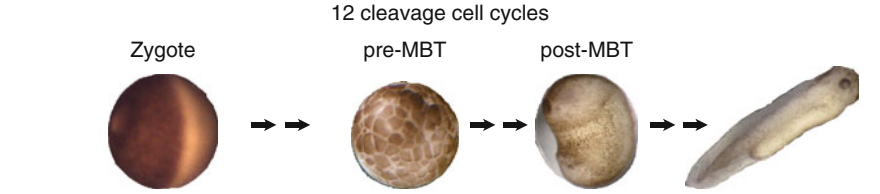
to become much bigger and uproot themselves in search of food, water and everything else they need. To achieve this level of development, thousands of genes are required, and this itself creates several other problems, already faced by simpler organisms, but which require even greater organisation in metazoans: firstly, how to store all the genetic information within the nucleus, which is achieved by packaging into chromatin; and then, how to replicate it efficiently and reliably, while at the same time transcribing those genes when and where they are needed. Because the latter is a difficult problem to solve and requires lots of energy, once the animal has developed, most cells don't bother. In an adult human being, only about 2% of the 10^{13} or so cells are dividing: reproductive cells, in males, and those cells that are needed to replace rapidly damaged cells to keep the organism alive, such as epithelial cells of the skin or intestine, and blood cells. During development, however, special mechanisms are required to coordinate cell proliferation with differentiation.

As well as creating problems, DNA replication provides a window of opportunity for epigenetic change, since, as the double helix is copied, the choice must be made of what to do with all the peripheral information encoded in chromatin determining if, when and where a gene should be transcribed. Because replication is coupled to chromatin assembly (Almouzni and Mechali 1988; Worcel et al. 1978) it provides a mechanism for controlling gene expression. In Xenopus oocytes, replication of injected single-stranded DNA is sufficient to repress basal transcription at the encoded promoter (Almouzni and Wolffe 1993). Inversely, in early mouse development, transcriptional activation of the zygotic genome occurs at the two cell stage, and requires DNA replication to relieve chromatin-mediated gene repression (Forlani et al. 1998). One way of achieving epigenetic change, during the course of DNA replication, would be to let parental and newly synthesised genes compete for limiting transcription "factors", in the general sense of the word. Over uncountable generations, selection for favourable outcomes of this competition might explain the programme of DNA replication in which transcribed genes are, in general, replicated early, and silent genes later on, within a single S-phase (Goldman et al. 1984) – a timing that appears to be mechanistically important (Zhang et al. 2002). But there may be a more mechanical reason why replication timing and transcription might be coupled. Whether a gene is to be replicated or transcribed, the DNA double helix first has to be unwound in order for the DNA or RNA helicase to copy the encoded information. Thus, DNA "unwinding factors" promoting transcription might also promote formation of replication origins, which would, on average, lead to proximal sequences being replicated earlier than distal ones. This would certainly be energetically economical, and again, over time, selection should lead to natural association of transcriptional and replication control elements. Indeed, almost all origins of replication are located close to transcriptional control regions and frequently contain transcription factor binding sites (see below). How unwinding at origins of replication is controlled, where it occurs in the genome, what the nature of the timing programme is, and what the consequences of this organisation are for the development of the organism, are all fundamental unresolved questions. In this chapter, I try to present a picture of what we know

about how cyclin-dependent protein kinases (CDKs), those universal cell cycle regulators, are involved in replication control, in the context of the development of *Xenopus laevis* (with frequent comparisons to other models). Xenopus is a wonderful model organism which has taught us much of what we know about transcriptional control, DNA replication, the cell cycle, development and nuclear reprogramming [for further reading on this subject, see the entertaining autobiographical account by Sir John Gurdon, winner of the 2009 Lasker prize for medical research, in which he correctly predicted that we should soon be able to reprogram somatic cell nuclei to totipotency by expressing the right combination of factors; (Gurdon 2006)].

10.2 Organisation of DNA Replication and Transcription in the Early Xenopus Embryo

Unlike mammalian development in utero, development of fertilised frog eggs must occur in the absence of any further provision from the mother. Eggs are therefore large, in order to provide material self-sufficiency until the animal has developed enough to feed itself, i.e. the tadpole stage. Early development is therefore organised very differently than in mammals, the first goal being to rapidly increase cell number (Fig. 10.1). For a start, it is quick, the entire cell cycle taking little more than 20 min for the first 12 cycles. To achieve this, replication origins are very closely spaced, from 5 to 25 kb apart, rather than the 50–300 kb seen in somatic cells (Blow et al. 2001; Hyrien and Mechali 1993), and are randomly positioned between different cells (Hyrien et al. 1995) and from one cell cycle to the next (Labit et al. 2008). Random positioning of origins is true also for early drosophila



Cell cycle length:	25 minutes	Hours
Replication origin spacing:	5-25 kb	50-300 kb
Replication origin positions:	non-specific, constrained	locus-specific, defined
Transcription:	silent	active
Linker histone expression:	B4, HMG1	H1, H1°

Fig. 10.1 Differences in organisation of DNA replication during development. In early Xenopus embryonic development, the cell cycles are rapid and synchronous, whereas after the mid-blastula transition they appear much more highly regulated. Some functional differences which have implications for replication control are highlighted

development (Shinomiya and Ina 1991), in which cells are cycling rapidly, suggesting that this may be a general rule, with mammals perhaps being an exception. Secondly, early development occurs in the absence of transcription (Brown and Littna 1966; Newport and Kirschner 1982b). This is due to a competition between transcription factor assembly and replication-coupled chromatin-mediated repression, in which chromatin wins (Kimelman et al. 1987; Prioleau et al. 1994). The exponential increase in DNA content during the rapid early cell cycles eventually titrates and alleviates this repression, as demonstrated by manipulating the nuclear-cytoplasmic ratio (Newport and Kirschner 1982a, b). In Xenopus, as in mice, DNA replication around the mid-blastula transition (MBT) appears to be required for relief of the repressed state (Fisher and Mechali 2003). Although much subsequent morphological development can occur in the complete absence of DNA replication, certain developmental abnormalities ensue, demonstrating that cell proliferation is, after all, required for correct development (Fisher and Mechali 2003; Harris and Hartenstein 1991; Rollins and Andrews 1991). Whereas all cells are dividing prior to the MBT, the mitotic index rapidly drops to about 30% at early gastrula stages to less than 10% by mid-gastrulation, and becomes regionalised (Saka and Smith 2001). The cell cycle (and, by inference, onset of DNA replication) is therefore controlled in a tissue-specific manner during development. However, that differentiation can occur in the absence of DNA replication suggests that global transcriptional gene activation at the MBT, dependent on replication, is then followed by progressive, cell type-specific gene repression which does not require replication. Transcriptional activation at the MBT also coincides with specific positioning of replication origins (Hyrien et al. 1995), which possibly reflects the influence of transcription factors on nucleosome positioning and thereby, the accessibility of DNA to replication factors. A recent study in somatic human cells found that most replication origins occur in GC-rich regions overlapping with transcriptional regulatory elements (Cadoret et al. 2008), especially those of the AP1 family of "immediate early" transcription factors. Significantly, origins mapped to evolutionarily conserved regions, suggesting that origin positioning is conserved across animal species. As such, origins of replication might be coordinated with transcription during later development. Whether this simply reflects a situation of "convenience", for example being energetically economical, or whether there are functional consequences of this origin positioning, are not known. Nevertheless, the absence of origin replication specificity in early development of Xenopus (and perhaps most animals other than mammals) is evidently associated with absence of transcription. The link between non-specific origins of DNA replication and transcriptional repression might be due to the necessity not only to replicate quickly, but perhaps also to maintain pluripotency of the dividing cells and prevent premature differentiation. Xenopus oocyte and egg extracts have an extraordinary capacity for nuclear reprogramming, and will even reprogram the nucleus within permeabolised cells. Such somatic cells introduced into oocytes are reset to a stem cell-like pattern of transcription in the absence of DNA replication (Byrne et al. 2003) whereas when introduced into egg extracts, all transcription is extinguished and DNA replication is activated (Alberio et al. 2005). However, not all nuclei

replicate with the same efficiency in egg extracts. Terminally differentiated chromatin, for example, from erythrocytes (which are nucleated in Xenopus) replicates much more slowly in Xenopus egg extracts, due to far fewer replication origins being activated. Passage through mitosis eliminates this pattern of replication origin spacing, and resets it to an early embryonic pattern (Lemaitre et al. 2005). Where do CDKs come in? Recent reports from our lab and others have found that in Xenopus egg extracts, CDKs control both the replication timing programme (Thomson et al. 2010) and replication origin spacing (Krasinska et al. 2008a). Taken together, these results suggest that CDK-mediated control of DNA replication is different between early embryos and somatic cells, which presumably reflects a different organisation of chromatin within the nucleus. However, although CDKs are required for initiation of DNA replication in all eukaryotes, their general mechanism of action at this stage is not clear.

10.3 Cyclin-Dependent Kinases and the Control of Initiation of DNA Replication

Initiation of DNA replication can be summarised as a series of sequential steps, in which DNA is first "licensed" to replicate by the formation of pre-replication complexes (pre-RC), which are then converted in a CDK-dependent manner to pre-initiation complexes (pre-IC) which unwind DNA, and DNA polymerase loading and elongation ensue (Walter and Newport 2000). However, replication of single stranded DNA in egg extracts does not require these steps and can occur in the absence of CDK activity (Blow and Laskey 1986; Blow and Nurse 1990) indicating that unwinding of the double helix at replication origins is a rate-limiting step in DNA replication, at which CDKs act. Pre-RCs are formed by loading of the MCM2-7 heterohexamer, which has only limited intrinsic helicase activity, onto origins containing the ORC1-6 complex, in a Cdt1 and Cdc6-dependent manner. To form a replicative helicase competent to unwind DNA processively requires association of the GINS (Go Ichi Ni San) complex and Cdc45 with MCM2-7 (Pacek et al. 2006); Fig. 10.2), mirroring the situation in yeast (Gambus et al. 2006). Several studies in yeast suggest how CDKs can control this process (Masumoto et al. 2002; Tanaka et al. 2007; Zegerman and Diffley 2007). Two components of DNA replication complexes, Sld2 and Sld3, which interact with the conserved replication proteins Cdc45 and Cut5/TopBP1/Dpb11, were identified as CDK substrates whose phosphorylation is essential for DNA replication. In the absence of CDK function, DNA replication could initiate, using yeast genetics to bypass the requirements for phosphorylation of Sld2 and Sld3, or with an activating mutation in the Sld3 interacting protein Cdc45. However, replication in these circumstances was inefficient, suggesting that whereas only two substrates might be indispensable, other substrates are also involved in promoting replication efficiency. The functions of Sld2 and Sld3 are not known, and there are no clear structural homologues of Sld2 and Sld3 in metazoans, although, given that other replication-origin complex

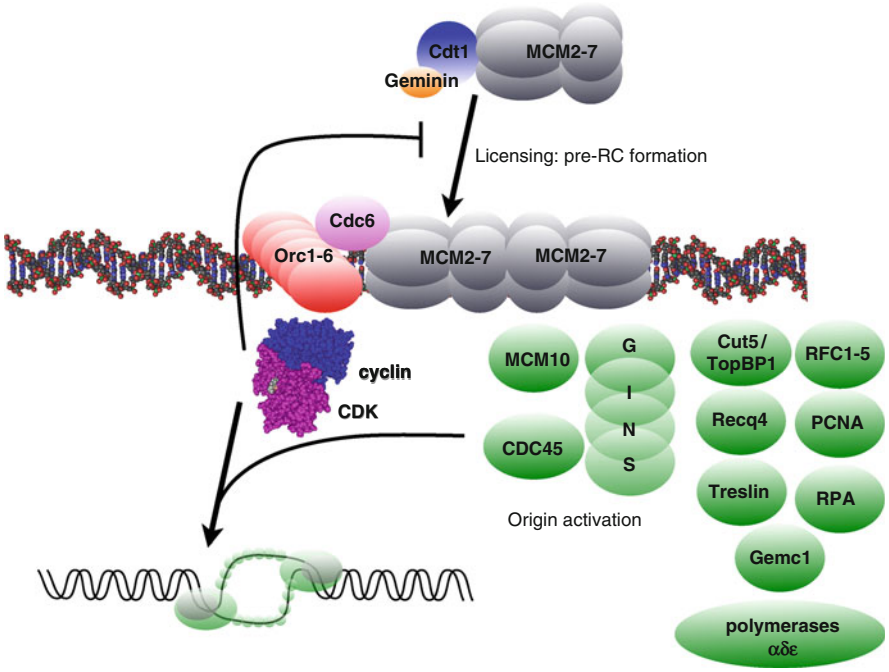


Fig. 10.2 The molecular organisation at replication origins and its control by CDKs. Replication origins are built in sequential steps: first, origins are licensed by the back-to-back loading of the double heterohexamer of MCM proteins around DNA, which requires the Orc complex, Cdc6, and a Cdt1-geminin complex. This step is inhibited by mitotic cyclin-dependent kinases. In a second step, which requires S-phase CDK activity, the pre-initiation complex (proteins in green) is loaded and DNA unwinding by the now processive helicase can occur

proteins are functionally conserved (Cdt1, Cdc6, MCM2-7, Cdc45, GINS, MCM10, Cut5/TopBP1/Dpb11) they almost certainly exist. The RecQ4 helicase, mutated in Rothmund-Thomson syndrome, and essential for DNA replication in Xenopus egg extracts, has a small region of limited sequence homology to Sld2, but it does not require CDK activity nor the Cut5 homologue to bind to chromatin (Matsuno et al. 2006; Sangrithi et al. 2005). It is, however, highly phosphorylated *in vivo*, and can be phosphorylated *in vitro* by CDKs. Recent papers identified in Xenopus two new putative CDK substrates required for pre-IC formation and DNA replication, Treslin and Gemc1. Treslin binds to Cut5/TopBP1/Dpb11 in a CDK-dependent manner, and is essential for replication in Xenopus and cultured human cells, as it allows Cdc45 recruitment to replication origins (Kumagai et al. 2010). Gemc1 is a protein containing Geminin-like coiled-coil domains, hence the name, and appears to serve a very similar function to that reported for Treslin: Cdc45 loading (Balestrini et al. 2010). Gemc1 directly binds Cut5/ TopBP1/Dpb11, Cdc45 and Cdk2-cyclin E, and, like Treslin, can be phosphorylated *in vitro* by the latter. The respective roles of Treslin and Gemc1 are so far up for grabs, but a recent bioinformatics paper reported that Treslin and Sld3 homologues almost certainly

share a common ancestor, and may be functional homologues (Sanchez-Pulido et al. 2010). It remains possible that in vertebrates, additional levels of control of replication by CDKs might be important, and additional substrates required, to govern activation of origin clusters and control the replication timing programme.

One well known substrate of CDKs in metazoans, whose phosphorylation is a pre-requisite for the G_1-S transition, is the retinoblastoma tumour suppressor, or pRb. The best known role of this protein, and its "pocket-protein" relatives p107 and p130, is to inactivate E2F-mediated transcription by formation of a direct repressive complex, in association with hbrm/BRG-1 (Trouche et al. 1997). Many E2F targets are required for DNA replication, such as Cdt1 (Yoshida and Inoue 2004), Cdc6, PCNA, RFC, polymerase alpha, MCMs 3, 5 and 6, RPA, ribonucleotide reductase, and so on (Ren et al. 2002). However, there is no transcription in Xenopus eggs or early embryos, and all components required for replication are already present. Nevertheless, pRb may have other functions repressive for DNA replication. In Xenopus egg extracts, addition of GST-pRb directly blocks DNA replication via a direct interaction with MCM7 which neutralises helicase activity (Pacek and Walter 2004; Sterner et al. 1998), and this repressive function can be alleviated by direct binding of Cyclin D1-CDK4 complexes (but not CDK2-cyclin E) (Gladden and Diehl 2003). The same Rb-MCM7 interaction, in somatic mammalian cells, is involved in TGF-beta1-induced late G1 arrest after pre-RCs have already formed, and may thus be a conserved and physiologically relevant mechanism to regulate DNA replication in response to cell signalling (Mukherjee et al.

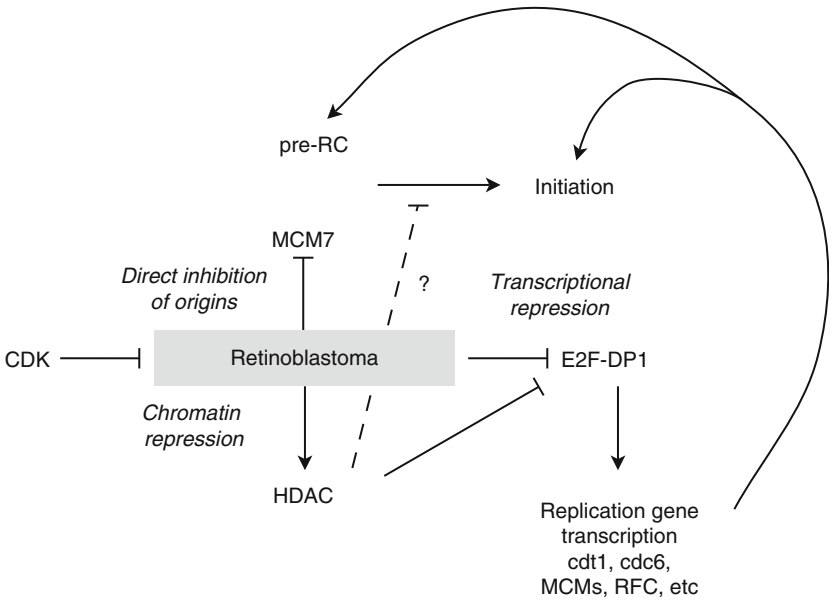


Fig. 10.3 Multiple potential roles for the Rb protein, a CDK substrate, in replication control. See main text for details

2010). Finally, Rb recruits histone deacetylases to chromatin (Magnaghi-Jaulin et al. 1998), providing a third potential mechanism of repression of DNA replication, either indirectly, by further inhibiting E2F-dependent transcription, or perhaps, by more directly repressing formation of replication origins. In metazoans, CDKs therefore probably control initiation of DNA replication not only by directly phosphorylating components of pre-ICs, but also by alleviating Rb-mediated chromatin repression (Fig. 10.3).

10.4 Chromatin and Replication Control by CDKs

The consequences of chromatin organisation for replication initiation are not well understood. Chromosomal DNA is highly organised, forming supercoils around nucleosomes, which are themselves compacted into higher-order structures. Post-translational modifications of core histones, including acetylation and phosphorylation, regulate nucleosome mobility and DNA condensation. We have found in Xenopus that histone acetylases and CDKs act at the same point to promote replication initiation, and their inhibition is synergistic in inhibiting DNA replication (Krasinska et al. 2008b). These and other results suggest that CDKs are likely to phosphorylate other substrates, possibly involved in chromatin remodelling to make DNA replication-competent. Indeed, histone acetyl-transferase activity peaks at the G1/S transition in somatic cells, due to CDK2-cyclin E-mediated stimulation of p300/CBP (Ait-Si-Ali et al. 1998), and anacardic acid, which blocks p300, prevents DNA replication in Xenopus egg extracts (Krasinska et al. 2008b; Lemaitre et al. 2005). Linker histone modifications might also control replication in a developmentally regulated manner. In somatic cells, phosphorylation of the linker histone H1, the archetypal CDK substrate, which binds linker DNA flanking the nucleosome core to stabilise higher-order chromatin structure (Wolffe 1997) is mediated by CDK2 during DNA replication, promoting chromosome decondensation (Alexandrow and Hamlin 2005). Whether or not CDK-mediated phosphorylation of histone H1 is required for replication is not known. Nevertheless, histone H1 is absent from chromatin in early development in Xenopus, with the embryonic histone H1 variant B4 substituting (Smith et al. 1988). Introduction of histone H1-containing somatic chromatin into an egg extract leads to replacement of H1 and the somatic H1 variant H1° by B4 and HMG1, which have similar functions (Dimitrov and Wolffe 1996; Nightingale et al. 1996). This may have functional consequences for DNA replication, since addition of recombinant H1 to an egg extract reduces replication origin firing (Lu et al. 1998). B4 does not contain any consensus sites for phosphorylation by CDKs, whereas H1 contains five. Possibly, histone H1 might titrate CDK activity; alternatively, by compacting chromatin, it might reduce accessibility of DNA to origin components. CDKs are also required for replication-coupled histone H2B and H4 gene transcription, which is essential for S-phase, by phosphorylation of the p220 NPAT transcriptional activator (Ma et al. 2000; Zhao et al. 2000).

10.5 Functional Redundancy of CDK-Cyclin Complexes, and its Limits

Which CDKs are involved in the initiation of DNA replication? In metazoans, CDK2 clearly plays an important role in regulating the G1 to S-phase transition, and was originally thought to be essential for DNA replication in Xenopus egg extracts (Fang and Newport 1991). The roles of CDK3, so far only described in mammals, are not clear, but while dispensable for the cell cycle in mice, in some circumstances it may regulate entry into S-phase via phosphorylation of pRb family proteins and promotion of E2F transcription (Hofmann and Livingston 1996; Ren and Rollins 2004). Mammalian CDK4 and CDK6 phosphorylate the retinoblastoma family of tumour suppressors, and can thus also control passage though the commitment point of serum-independence in G1, and exit from the cell cycle (Sherr 1995). In Xenopus, while CDK4 is expressed later in development (Goisset et al. 1998), it is not clear whether any CDK4 protein is present in early cell cycles, and a CDK6 homologue has not been identified. In vertebrates, many cell types can proliferate in the absence of CDK2, due to compensation by CDK1 (Aleem et al. 2005; Hochegger et al. 2007; Ortega et al. 2003; Tetsu and McCormick 2003). Indeed, it was recently found that in mice, embryonic cells can proliferate in the simultaneous absence of CDK2, CDK3, CDK4 and CDK6, only CDK1 having cell cycle functions that cannot be compensated for by other CDKs (Santamaria et al. 2007). These recent results in animal cells are reminiscent of our earlier studies in fission yeast, in which a single mitotic CDK-cyclin complex can promote both DNA replication and mitosis (Fisher and Nurse 1996). Even in Xenopus egg extracts, mitotic cyclin B can promote DNA replication in the absence of the S-phase promoting cyclin E, providing a nuclear-localisation signal is provided (Moore et al. 2003), suggesting that a CDK only has to be in the right place at the right time to phosphorylate its substrates, providing it has similar substrate specificity. The latter appears not to be a problem, which is not surprising given that CDK-consensus sites are extremely simple (in most cases, a solvent-accessible SP or TP sequence suffices). On the other hand, some cell types do require certain CDKs or cyclins in order to proliferate, and individual CDK knockout mice reveal many pathologies. For example, CDK2−/− mice are sterile, CDK4−/− mice are diabetic, and so on. This might reflect expression profiles of CDKs or cyclins within the particular tissue. For example, knockout of A-type cyclins in mice does not affect fibroblast proliferation, and in this case, cyclin E expression becomes upregulated throughout the cell cycle, but it *does* affect proliferation of embryonic stem cells and haematopoietic stem cells, which normally have high cyclin A expression (Kalaszczynska et al. 2009). Conversely, deletion of both E-type cyclins in mice causes embryonic lethality due to inability of placental trophoblast cells to endoreplicate (Geng et al. 2003), CDK2-cyclin A apparently unable to substitute, whereas CDK2 deletion has no effect on these cells, suggesting that differences between cyclin A and cyclin E are more important than differences between CDK1 and CDK2. Yet fundamentally, cyclin A and cyclin E can do the same things: in Xenopus egg

extracts, S-phase promoting activity can be provided by either cyclin A-CDK1 or cyclin E-CDK2, albeit with different efficiencies (Strausfeld et al. 1996). Therefore, cyclin A and cyclin E associated kinases are functionally redundant in some circumstances, but are required in certain specific contexts, probably in part due to developmentally-regulated or tissue-specific expression profiles. Differences in expression appear to explain why CDK1-cyclin E does not support normal meiosis in mice, because even expression of CDK2 from the CDK1 locus cannot replace the endogenous CDK2 gene for meiotic function (Satyanarayana et al. 2008). However, different CDK-cyclin complexes probably have different kinetic parameters (affinity for substrates and catalytic activity) – this is certainly true at least in yeast (Loog and Morgan 2005). Because knock-in of CDK2 into the CDK1 locus in mice cannot replace CDK1 function for the mitotic cycle (Satyanarayana et al. 2008) differences in kinetic parameters between CDK2 and CDK1 must also exist, and have important functional consequences. Therefore, functional redundancy has its limits.

By analogy with the situation in mammals, CDK2 might be expected to be dispensable per se for DNA replication in Xenopus, although it was originally found to be essential (Fang and Newport 1991). Possibly, in Xenopus, CDK2 might be required for rapid DNA replication occurring in egg extracts, reflecting the different organisation of DNA replication in the early embryo, compared to somatic cells. We therefore recently reinvestigated whether CDK1 and CDK2, and cyclin A and cyclin E, are redundant in Xenopus egg extracts, an early embryonic system, using single molecule DNA combing to investigate their respective influence on replication origin organisation in Xenopus egg extracts. We found that CDK1-cyclin A actually *is* involved in DNA replication even in the presence of CDK2-cyclin E; however, only very low CDK activities (of either CDK1 or CDK2) are sufficient to promote replication initiation, and there was an important difference between CDK1 and CDK2 efficiency in promoting replication origin firing (Krasinska et al. 2008a). CDK2-cyclin E is indeed rate-limiting for DNA replication in these circumstances, as depletion of either sub-unit reduced replication efficiency to around 30% of the control. However, the remaining 30% replication was no longer dependent on CDK2, but, rather, on CDK1. Depletion of either CDK1 or cyclin A, its main cyclin partner in extracts, slightly but reproducibly delayed DNA replication in the presence of CDK2, and activity of this complex was essential for DNA replication in the absence of CDK2. Surprisingly, however, at the level of individual replication origins, the effects of depleting CDK1 or cyclin A ostensibly appear similar to those of depleting CDK2 or cyclin E, in that the average inter-origin distance is approximately doubled to between 40 and 50 kb. This means that both CDK1 and CDK2 complexes stimulate firing of individual replication origins, and do not compensate for each other at the individual origin level. However, the main limitation of DNA combing is DNA breakage between replication origins, with the average fibre length being around 100 kb. Thus, many inter-origin distances cannot be measured – those between external origins on each fibre. When the number of initiation events per kb of DNA are calculated, irrespective of whether or not origins are present on combed fibres, it can be seen that CDK2 and cyclin E are much more important than CDK1 and cyclin A. In other words,

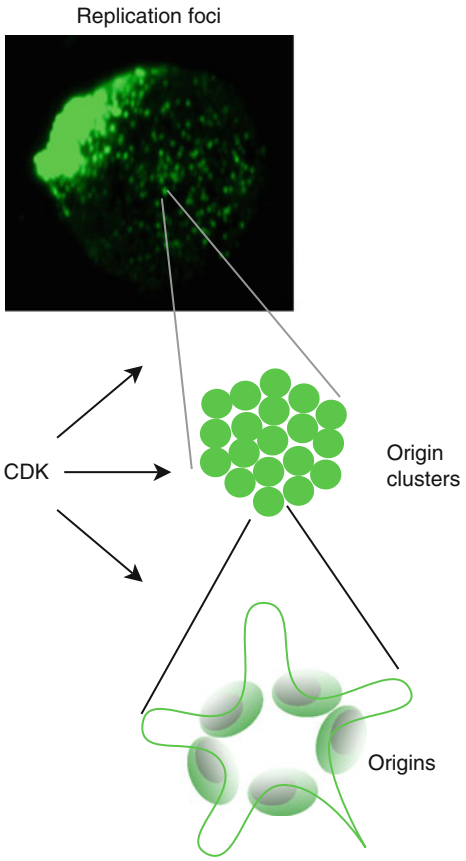


Fig. 10.4 Higher nuclear organisation in replication control. The organisation of replication in the nucleus is not yet well understood. In the generally accepted current model, origins of replication are clustered (bottom) and chromatin loops out from the origins. The origin clusters are probably also organised into "factories" (middle) which can be visualised microscopically as replication foci (top). CDKs have roles at these different levels of subnuclear structure, as explained in the main text

replication origins are clustered, and both CDK1 and CDK2 affect the numbers of replication origins within the cluster, whereas only CDK2 appears to be limiting for the number of clusters firing (Fig. 10.4). This probably has something to do, firstly, with how replication origins are organised within the nucleus, and secondly, with different efficiencies of CDK1 and CDK2 for phosphorylating whatever substrates are required for activation of origin clusters.

10.6 Replication Origin Organisation, Replication Timing and Control by CDKs

How origins are organised into clusters, and the relationship of clusters to replication foci, is something of a mystery. In Xenopus, replication foci, associated with large, megabase scale DNA regions, colocalise from one cell cycle to the next, even though individual replication origins do not (Labit et al. 2008). In somatic cells, the pattern of replication foci changes throughout S-phase, suggesting that early and late-replicating

DNA is associated with different sub-nuclear structures. In Xenopus, concomitant with changes in origin specification at the midblastula transition are changes in attachment of DNA to the nuclear matrix (Vassetzky et al. 2000), which, as with replication origins, changes from random to specific site attachment. This is too much of a coincidence for the origin positioning *not* to be related to chromatin organisation at the structural level. Although the nature of the nuclear matrix itself is debated, "anti-matrix" proponents writing off the visible skeleton seen on electron microscopy of matrix preparations as a precipitation artefact of the extraction conditions (see Pederson 2000), the inference is that the internal structure of the nucleus itself is different in early development prior to the midblastula transition. Indeed, the length of replicons in early and later development correlates with the "halo" radius, i.e. the length of chromatin loops between sites of attachment to the matrix (Lemaitre et al. 2005), suggesting that replication origins might be located at the base of the loops and associated with the matrix, whether or not the latter is soluble or filamentous. In early embryonic development, activation of origin clusters appears to be the rate-limiting step for the overall speed of DNA replication, and the inference from our results is that CDK1-cyclin A is simply less efficient at promoting this step than CDK2-cyclin E. By analogy with yeast, in which different cyclin-CDK1 combinations have different Michaelis constants (Km) for typical S-phase and M-phase substrates (Loog and Morgan 2005), reflecting differential affinity of the protein substrate for one or other cyclins, I suggest that the same is likely to be true for metazoan CDK-cyclin complexes. Potentially, therefore, early and late-replicating origins could be preferentially controlled by different CDK-cyclin complexes, reflecting their different organisation within the nucleus (Fig. 10.4). There has been some recent evidence for this in somatic cells, with CDK1-cyclin A controlling firing of late origins, even in the presence of CDK2 (Katsuno et al. 2009).

Although there is no obvious timing programme of origin firing in early Xenopus development, nevertheless, use of statistical methods demonstrates that clustering of origins occurs, that clusters fire at different times even within the rapid S-phase of Xenopus egg extracts, and that cluster firing and overall initiation rate is limited by the nuclear – cytoplasm ratio and a constitutively active S-phase checkpoint (Blow et al. 2001; Marheineke and Hyrien 2004). A more obvious timing programme can be reproduced in Xenopus egg extracts using somatic cell nuclei. In this case, the level of CDK activity in the extract controls both the number of replication foci and the fraction of DNA replicated early and late (Thomson et al. 2010). This again points to CDK requirements for replication being related to the structural organisation of DNA within the nucleus.

10.7 CDK Requirements for S-Phase Entry from a Quiescent State

Assuming the above statement to be true, one might infer that nuclear substructure is different when cells are already cycling (such as in early development in Xenopus), from when cells are in a quiescent state (most of the cells, most of the

rest of the time), because requirements for CDKs to replicate are different. Thus, cyclin E is dispensable for cell-cycling during the majority of early development in mice, but is required for cells to enter S-phase from a quiescent state (Geng et al. 2003). This requirement was proposed to be independent of the ability of cyclin E to activate its associated CDK, since cell cycle entry could be rescued using a mutant cyclin E, apparently incapable of activating CDK2 but which appears to link Cdt1 to the MCM helicase (Geng et al. 2007). Nevertheless, CDK requirements for initiating DNA replication appear to be vanishingly low (Krasinska et al. 2008a) and we can detect basal CDK2 activity against histone H1 in the complete absence of cyclins (CDK2 expressed and purified from in *E. coli*) providing it is phosphorylated by CAK (unpublished data). Thus, it cannot yet be ruled out that cyclin E promotes cell cycle entry from a quiescent state by activating a CDK to low levels attainable even by mutant cyclin E, or by cyclin-E dependent association of basal (i.e. cyclin-independent) CDK activity with the correct substrates, and this is not normally required once cells are happily cycling. Could this be because the nuclear structure is altered once cells become quiescent, and remodelling the nucleus to make DNA competent to replicate requires particular CDK activities?

Whether or not this is the case, other CDK-dependent mechanisms operate during cell cycle entry of quiescent somatic cells. In cycling mammalian cells, replication origins become licensed (coincident with MCM loading onto chromatin) at the end of mitosis (Okuno et al. 2001), whereas during cell cycle exit, at least one protein required for licensing, Cdc6, is targeted for destruction by APC/C, preventing inappropriate DNA replication. Stabilisation of Cdc6 requires CDK-mediated phosphorylation dependent on cyclin E (whose degradation is promoted by an alternative ubiquitin-ligase, SCF), and this is critical for forming pre-RCs and entering S-phase from quiescence (Mailand and Diffley 2005). Interestingly, if Cdc6 expression is restricted to G1, CDK activity becomes essential for pre-RC formation in cycling cells. The inference is that CDK activity is dispensable for pre-RC formation (indeed, it inhibits pre-RC formation) because pre-mitotically stabilised Cdc6 is sufficient for pre-RC formation post-mitosis.

10.8 Conclusions

In summary, therefore, CDKs probably control DNA replication at multiple steps, including transcription, pre-RC formation, the pre-RC to pre-IC transition, chromatin remodelling and regulation of higher order nuclear structure, depending on the cell-type and developmental context (Table 10.1). In a hypothetical ideal world, it should be possible to understand cell cycle control and the relative contributions of different regulators, from a knowledge of interaction kinetics of all different molecules. More realistically, we should at least be able to understand how different CDKs are involved in cell cycle control, why some appear essential in some circumstances but not others, and the consequences of using particular inhibitors, from analysis of kinetics of phosphorylation of their substrates in a physiological

Table 10.1 CDK substrates in DNA replication, and their cellular functions.

Physiological process	Substrates
Chromatin remodelling	HAT, Rb, Histone H1, others?
Transcription	Rb, NPAT
Pre-RC formation	Cdc6
Origin activation	Rb, Treslin, Recq4, Gemc1, others?
Origin cluster/replication foci activation	Unknown

context. To move towards this goal, it is obvious that we first need to know what the substrates are, determine their kinetics of phosphorylation by different CDKs, and study how CDK-mediated phosphorylation regulates their function in a physiological system. The emerging picture is that the increasing diversity of metazoan CDKs and cyclins undoubtedly reflects the selective advantage from having CDK-cyclin complexes with different affinities, which, while not essential in any given situation, may provide for flexibility in replicating DNA at different speeds and different times.

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Chapter 11
Greatwall Kinase, ARPP-19 and Protein Phosphatase 2A: Shifting the Mitosis Paradigm

Olivier Haccard and Catherine Jessus

Abstract Control of entry into mitosis has long been seen in terms of an explosive activation of cyclin-dependent kinase 1, the mitotic driver ensuring the phosphorylation of hundreds of proteins required for cell division. However, if these phosphorylations are maintained during M-phase, they must be removed when cells exit mitosis. It has been surmised that an "antimitotic" phosphatase must be inhibited to allow mitosis entry and activated for returning to interphase. This chapter discusses a series of recent works conducted on *Xenopus* egg extracts that provide the answers regarding the identity and the regulation of such a phosphatase. PP2A-B55δ is the major phosphatase controlling exit from mitosis; it is negatively regulated by the kinase Greatwall that phosphorylates the small protein ARPP-19 and converts it into a potent PP2A inhibitor. These findings provide a new element of paramount importance in the control of mitosis.

11.1 Introduction

Since the nineteenth century, it is established that cells reproduce by means of the cell cycle: *omni cellula e cellula*. This process underlies growth and development in all living organisms. After fertilization, patterns of mitotic divisions contribute cells to lineages destined to form first the embryo and then the mature organism; in the adult, mitotic activity of stem cells supports the regeneration and maintenance of tissues. Cell cycle is also central to heredity and evolution. Indeed, all living organisms on the earth originate by an unbroken series of divisions from an ancestral cell that appeared over a billion years ago (for hypotheses on early cell cycle emergence and evolution, see Adamala and Luisi 2011 and Dobrzyński et al. 2011). Understanding how the cell cycle operates is therefore a crucial problem in

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biology, if we are ever to understand embryo development or maintenance of tissues in an adult organism or why tumor cells divide in an unregulated fashion.

The cell cycle is the period during which events required for successful cell reproduction are completed. In its simplest form, it consists of a round of chromosomal DNA replication in S-phase followed by segregation of the replicated chromosomes into two daughter nuclei during M-phase or mitosis. The process of cell division has fascinated biologists since its discovery in the late 1870s (Flemming 1879, 1965). During mitosis, cells undergo dramatic ordered structural changes in the nucleus and cytoskeleton as centrosome separation, chromosome condensation, nuclear envelope breakdown, spindle formation, chromosome congression onto the metaphase plate, sister chromatids separation and spindle elongation, formation of a microtubule-based midbody, and separation of two daughter cells. This temporal sequence of events in mitosis was appreciated by histologists before the turn of the twentieth century. Starting from the 1970s, cell cycle studies entered a new era by investigating the biochemical controls of cell cycle progression. The first data evidenced that the M-phase state is characterized by the appearance of newly phosphorylated and hyperphosphorylated proteins (Maller and Smith 1985). Then, pioneer experiments combining genetic analysis of yeast and biochemical studies of clam, frog, and echinoderm eggs and oocytes led to the molecular identification of MPF (M-phase promoting factor), the key component that initiates and orchestrates mitosis. MPF is composed of the protein kinase catalytic subunit Cdc2 and its positive regulatory subunit, cyclin B (Dunphy et al. 1988; Gautier et al. 1988; Labbe et al. 1989; Gautier et al. 1990). Cdc2 was then renamed Cdk1 as the founder member of a conserved family of Ser/Thr protein kinases known as the Cyclin-dependent kinases (Cdks; for details on Cdks, see Gopinathan et al. 2011). The analysis of the regulation of the Cdk1-cyclin B complex led to the emergence of a unified view of the cell cycle (Murray and Kirschner 1989). The level of Cdk1 is constant during the cell cycle, but cyclin B accumulates during interphase. Cyclin B and Cdk1 associate to form a complex that lacks MPF activity due to inhibitory phosphorylations on Thr14 and Tyr15 of Cdk1 catalyzed by the Wee1/Myt1 kinases (Russell and Nurse 1987; Mueller et al. 1995). Entry into mitosis is triggered by the conversion of this complex into active MPF through the dephosphorylation of Cdk1 by the Cdc25 phosphatase. Once activated, MPF directly or indirectly induces the phosphorylation of numerous proteins implicated in mitosis progression (Nigg 2001). It eventually induces the ubiquitin-dependent proteolysis of cyclin B by the anaphase-promoting complex (APC), leading to the inactivation of MPF and the return to interphase (Evans et al. 1983; Murray et al. 1989; King et al. 1996).

We only have a fragmentary picture of the regulation of the mitotic phosphorylations occurring during mitosis progression. Hundreds of proteins get phosphorylated under MPF control, the primary kinase, whereas other kinases, such as Polo or Aurora, are themselves dependent on Cdk1 (Nigg 2001). Mitotic phosphoproteins are responsible for the key events of mitosis progression: chromosome condensation, nuclear envelope breakdown, centrosome separation, spindle assembly, chromosome attachment and congression, activation of APC. Importantly, this set of

mitotic phosphoproteins has to be converted into a hypophosphorylated state to pass from M-phase back to interphase and to be kept under this form until next mitosis. This observation raises the question about the regulation of phosphatases that remove the mitotic phosphates and of their identity and substrate specificity. Despite the well-documented role of protein kinases in mitosis, little is known regarding the control of Ser/Thr phosphatases in this process.

11.2 Regulated Activity of Protein Phosphatase 2A (PP2A) Is Required for Entry into and Exit from Mitosis

11.2.1 PP2A, a Regulator of Cdk1-Cyclin B Activity

Entry into mitotic prophase can be understood in terms of Cdk1-cyclin B activation that depends on Cdc25, the phosphatase responsible for Cdk1 dephosphorylation. During the G2 phase, Cdc25 activation is restrained by Ser287 phosphorylation (*Xenopus* numbering; Ser216 of human Cdc25C) that promotes binding of 14-3-3 proteins (Peng et al. 1997; Kumagai et al. 1998). Dephosphorylation of this residue is essential for Cdc25 activation and entry into M-phase. It is catalyzed by protein phosphatase 1 (PP1) (Margolis et al. 2003, 2006a, b), in agreement with the observation that inhibition of PP1 activity prevents entry into M-phase (Huchon et al. 1981).

In parallel to this dephosphorylation event, Cdc25 activation depends on an extensive phosphorylation on Ser and Thr residues located in the N-terminal region of this phosphatase (Izumi et al. 1992; Kumagai and Dunphy 1992), catalyzed by Cdk1, Polo, and perhaps other kinases (Izumi and Maller 1993; Kumagai and Dunphy 1996; Karaiskou et al. 1999; Jessus 2010). Several findings strongly suggest that PP2A is the phosphatase that counteracts the hyperphosphorylation required for Cdc25 activation. It is well-known that the algal toxins, okadaic acid and microcystin, that are powerful inhibitors of the two main phosphatases, PP1 and PP2A, are able to activate Cdk1 in *Xenopus* and starfish oocytes, in cultured cells, as well as in *Xenopus* egg extracts (Goris et al. 1989; Felix et al. 1990; Picard et al. 1991). The IC50 values of okadaic acid are an order of magnitude higher for PP1 than for PP2A (Bialojan and Takai 1988), theoretically allowing the drug to be used to distinguish between the roles of these two phosphatases. In cell-free systems derived from oocytes or eggs, okadaic acid triggers MPF activation at concentration not sufficient to inhibit PP1 (Felix et al. 1990; Karaiskou et al. 1998). Moreover, PP2A depletion or inhibition is sufficient to lead to Cdk1 activation, whereas PP1 level does not affect Cdk1 activation promoted by PP2A removal (Clarke et al. 1993; Maton et al. 2005). A specific isoform of PP2A (containing the regulatory B56δ subunit) was described to dephosphorylate the Thr138 site needed for activation of Cdc25. This reaction occurs during interphase and is shut down during M-phase (Margolis et al. 2006a, b). It is therefore quite clear that

PP2A is the major phosphatase responsible for the dephosphorylation of the Cdc25 activatory residues.

In contrast to mitosis entry, Cdk1 phosphorylation appears not to be primarily involved in mitotic exit. Cyclin B degradation, separating Cdk1 from its cyclin partner, is sufficient to inactivate Cdk1 independently of any essential phosphorylation (Chesnel et al. 2006, 2007). Therefore, whereas PP2A is clearly involved in mitosis entry by regulating Cdk1-cyclin B activity through Cdc25 phosphorylation level, it would be dispensable for inactivating MPF at mitosis exit.

11.2.2 PP2A, a Regulator of the Phosphorylation Level of Cdk1 Targets

Until recently, the control of entry into mitosis was seen in terms of a sudden increase in Cdk1 activity, as the master kinase that would overcome the basal level of phosphatase activities of the cell. From this point of view, regulating the phosphatase activities would not be essential for entering or exiting mitosis: once activated, Cdk1 overcomes the phosphatases activities, promoting mitotic phosphorylations; after Cdk1 inactivation by cyclin B degradation, the phosphatases would not be anymore counteracted by MPF, leading to housekeeping dephosphorylation of the mitotic substrates. Alternatively, it has been proposed that mitotic phosphorylations depend not only on the activation of Cdk1, but also on the inhibition of the antagonizing protein phosphatases. Hence, inhibition of the phosphatase activities targeting Cdk1 substrates would be important in allowing cells to enter mitosis, and conversely, their activation would be required for returning to interphase. There is indeed now well-established evidence to support this last view.

The bulk of Ser/Thr dephosphorylations are catalyzed by families of protein Ser/Thr phosphatases, the two most abundant being members of the PP1 and the PP2A family (Virshup and Shenolikar 2009). The complexity in the number of Ser/Thr phosphatases does not lie in the number of genes encoding catalytic subunits, but in their assembly as multimeric complexes containing only a small number of catalytic subunits combined with many regulatory subunits. This gives rise to hundreds of combinations (Virshup and Shenolikar 2009).

In *Aspergillus* and yeasts, PP1 has been proposed to be essential for mitotic exit, as PP1 mutants exhibit mitotic arrest phenotypes (Doonan and Morris 1989; Ohkura et al. 1989; Hisamoto et al. 1994). Using mitotic extracts derived from *Xenopus* eggs, it was recently proposed that PP1 would be activated at the exit of M-phase after Cdk1 inactivation and would dephosphorylate mitotic phosphoproteins for returning to interphase (Wu et al. 2009). The budding yeast dual specificity phosphatase Cdc14 was similarly shown to counteract the activity of Cdk1-cyclin B at the end of mitosis, thus playing an essential role in mitotic exit (Amon 2008). However, even though Cdc14 is present in a wide range of organisms from yeast to human, its deletion in human and avian somatic cell lines has

suggested functions that are quite different from those of yeast Cdc14 (Mocciaro et al. 2010).

Several lines of evidence derived from genetic, biochemical, and cellular approaches led to the conclusion that PP2A controls the massive protein dephosphorylation that occurs at mitotic exit. PP2A enzymes typically exist as heterotrimers comprising catalytic C-, scaffolding A-, and different regulatory B-type subunits (B55/B, B56/B', B72/B", each of them presenting several isoforms). The B-type subunits function as targeting and substrate-specificity factors (Janssens et al. 2008). Biochemical studies identified one isoform of PP2A as the major enzyme in vertebrate cell extracts that dephosphorylates physiological substrates of Cdk1 (Ferrigno et al. 1993; Che et al. 1998). In parallel, two *Drosophila* mutants, *aar* and *twins*, defective in the gene encoding the single fly B55 subunit of PP2A exhibit mitotic defects that are likely to result from the lack of dephosphorylation of mitotic substrates by PP2A (Mayerjaekel et al. 1993, 1994). Recent advances in understanding the critical role of PP2A in cell cycle progression came from biochemical studies of *Xenopus* egg extracts. These extracts are obtained by crushing *Xenopus* eggs in the absence of buffer and can undergo multiple rapid cell cycles, monitored either by the morphology of added nuclei or by assays of Cdk1 activity. These cell cycles are driven by the synthesis and degradation of endogenous cyclin, or, if protein synthesis is inhibited, by the addition of exogenous cyclin. This powerful system allows the fundamental cell cycle regulators to be studied in relative isolation (Murray 1991). Using such extracts, Mochida and Hunt recently discovered that the phosphatase calcineurin is required to release *Xenopus* eggs from the cell cycle arrest of the second meiotic metaphase (Mochida and Hunt 2007). In the course of this study, these authors discovered that a second wave of phosphatase activity, sensitive to okadaic acid and directed towards mitotic substrates of Cdk1-cyclin B, appears after the spike of calcineurin activity. In contrast to calcineurin, whose activation is limited to *meiotic* M-phase exit, the okadaic-sensitive phosphatase activity disappears when extracts enter the next M-phase and reappears at the end of mitosis (Mochida and Hunt 2007). It was therefore proposed that inhibition of the okadaic-sensitive phosphatase is important to allow M-phase entry while its activation would be required for a proper return to interphase by dephosphorylating mitotic targets of Cdk1-cyclin B. The high protein concentrations of PP1 and PP2A present in the *Xenopus* extracts (higher than the IC_{50} value for okadaic acid) do not allow the drug to be used to distinguish between the roles of PP1 and PP2A under these conditions. To identify the phosphatase, Mochida et al. (2009) adopted an immunodepletion technique consisting in removing one by one each catalytic subunit of PP1, PP2A, PP4, PP5, and PP6. Their results led to the conclusion that PP2A is the major phosphatase acting on Cdk1 phospho-substrates in interphase extracts (Mochida et al. 2009). To identify which form of PP2A holoenzyme could be responsible for this interphase phosphatase activity, the same immunodepletion strategy was used, but this time depleting each regulatory B-subunit. The results showed that PP2A associated with the B55δ subunit is the major enzyme controlling exit from mitosis through dephosphorylation of Cdk1 targets (Mochida et al. 2009). An independent study, also based on *Xenopus* egg

extracts, showed that immunodepletion of PP2A using antibodies against either the structural A-subunit or the B55δ subunit removes most of the phosphatase activity directed against Cdk sites, confirming the crucial role of the trimeric B55δ-associated form of PP2A in exiting mitosis (Castilho et al. 2009).

11.3 The Greatwall Kinase Is a Key Negative Regulator of PP2A

The next step was to understand how the activity of PP2A-B55δ could be regulated during the cell cycle, being high in interphase and suppressed during mitosis. The first clue arose from the work of Zhao et al. (2008), who proposed that the newly discovered Greatwall kinase is a phosphatase suppressor accounting for its role in promoting the activation of Cdk1-cyclin B.

Greatwall kinase was originally identified in a screen for *Drosophila* mutants defective in chromosome condensation (Yu et al. 2004). The *Greatwall* gene encodes an evolutionarily conserved protein kinase (also known as MAST-L) that belongs to the AGC family of Ser/Thr kinases (Hanks and Hunter 1995). The kinase domain of Greatwall is unusually split in two parts by a long stretch of amino acids separating subdomains VII and VIII (Fig. 11.1a). This domain structure is conserved from flies to mammals: the kinase domains of the fly and human Greatwall proteins share 59% overall amino acid sequence identity, the

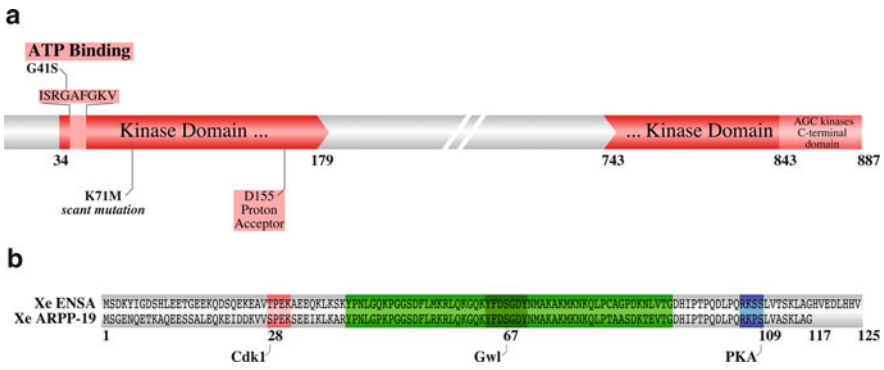

Fig. 11.1 Analysis of Greatwall, ARPP-19 and ENSA sequences. (**a**) Schematic representation of Greatwall protein kinase. The kinase domain is divided into two parts between sub-domains VII and VIII. Mutation of the Glycine 41 into a Serine in the ATP binding site kills the kinase activity. Mutation of the Lysine 71 into a Methionine (*Scant* mutation) was described to generate a hyperactive kinase in *Drosophila* (Archambault 2007) (**b**) ARPP-19 and ENSA. Alignment of *Xenopus laevis* ARPP-19 and ENSA protein sequences. The *red box* marks the Cdk1 phosphorylation recognition motif. The *blue box* marks the PKA phosphorylation recognition motif. In *green*: ARPP/ENSA block (IPB006760, http://blocks.fhcrc.org). The highest homology between species within this block is found in the *dark green box* that surrounds the Greatwall phosphorylation site (S67)

insertions between subdomains VII and VIII being less conserved (Sun et al. 2006; Voets and Wolthuis 2010).

Mutations in the *Drosophila Greatwall* gene cause improper chromosome condensation and delay cell cycle progression in larval neuroblasts (Yu et al. 2004; Archambault et al. 2007). Cells take much longer time to transit the period of chromosome condensation from late G2 through nuclear envelope breakdown. Mutant cells are also subsequently delayed at metaphase (Yu et al. 2004) and oocytes from females hemizygous for a mutant Greatwall isoform specific of the female germ line fail to arrest in metaphase I of meiosis (Archambault et al. 2007). These observations highlight the multiple roles of Greatwall in both mitotic and meiotic progression in *Drosophila*, suggesting that this kinase helps to activate cell cycle regulators that prepare interphase cells to enter mitosis.

Greatwall's mitotic function was further investigated in *Xenopus* egg extracts and during oocyte meiotic maturation. It was first shown that Greatwall is activated downstream of MPF and participates in the autoactivation loop that generates and maintains high MPF activity, by contributing to the activation of the Cdc25 phosphatase (Yu et al. 2006). It then became rapidly clear that Greatwall's major role was not restricted to help activating MPF as part of the autoregulatory loop (Zhao et al. 2008). Indeed, it was observed that several of the cell cycle effects of Greatwall were independent of MPF activity, as it is capable to promote and sustain mitotic phosphorylations even when MPF activity is undetectable. For instance, Greatwall can induce phosphorylations of Cdc25 in the absence of the kinase activities of Cdk1 and Polo. More generally, the effects of active Greatwall on cycling *Xenopus* extracts mimic in many respects those associated with addition of the phosphatase inhibitor, okadaic acid (Zhao et al. 2008). Therefore, this study proposed a model in which Greatwall negatively regulates PP2A, protecting the phosphorylations added under the control of MPF to many protein substrates, including components of the autoregulatory loop as Cdc25.

This model was soon validated by two independent studies, both of them exploiting cycling *Xenopus* egg extracts (Castilho et al. 2009; Vigneron et al. 2009). Greatwall depletion from M-phase extracts induces the activation of an okadaic-sensitive phosphatase specifically acting on Cdk1 phospho-sites as well as on the mitotic exit, even in the constant presence of active MPF. Conversely, addition of active Greatwall to interphase extracts inhibits the Cdk1-phosphosites phosphatase activity even in the absence of Cdk1-cyclin B activity. As already shown by Mochida et al. (2009), both studies confirmed that the phosphatase activity directed against Cdk1 substrates and inhibited by Greatwall corresponds to PP2A. Finally, in agreement with the findings of Mochida et al. (2009), Castilho et al. (2009) showed that the inability of Greatwall-depleted extracts to enter M-phase is corrected by the removal of PP2A-B55δ. The overall conclusion of these studies is that Greatwall controls mitosis through the suppression of PP2A-B55δ activity that is specifically directed against Cdk1 phospho-sites, with no requirement for MPF activity. A recent study extended this conclusion to human cells, by showing that human Greatwall is a critical and evolutionally conserved regulator of PP2A, controlling M-phase in HeLa cells (Burgess et al. 2010).

Mitotic entry, progression, and exit were all envisaged as entirely driven by Cdk1-cyclin B activity. These new results lead to a clear conclusion that the regulation of PP2A, and especially the PP2A-B55δ isoform, by the Greatwall kinase is of paramount importance for the regulation of entry into and exit from mitosis. It happens, of course, in parallel with the control of Cdk1-cyclin B. Entry into and progression through mitosis require PP2A downregulation achieved by Greatwall: first, it prevents the dephosphorylation of MPF autoregulatory loop components, as Cdc25 but also probably Wee1 and Myt1 kinases (Lorca et al. 2010), allowing the explosive activation of MPF; second, it protects the phospho-sites of hundreds of targets of MPF, needed for the cellular mitotic events and completion of the cell division. The exit from mitosis requires PP2A upregulation in order to dephosphorylate the broad range of Cdk1 mitotic substrates.

Nevertheless, several questions are still pending. A first one is related to the interdependency of MPF and Greatwall activation. Several observations suggest that during the mitotic entry, Greatwall suppresses PP2A-B55δ only after Greatwall itself becomes turned on by MPF (Yu et al. 2006; Zhao et al. 2008). However, the recently published results show that the removal of Greatwall from interphase extracts, where the kinase should be inactive, leads to an increase in PP2A activity (Castilho et al. 2009). One can envision that a low level of Greatwall activity could be present in interphase and that Greatwall might participate in triggering M-phase entry, as well as functioning downstream of MPF. Until we understand the precise mechanism allowing Greatwall activity to be turned on and off, it will be difficult to provide a coherent view on what really changes at the end of G2 phase, to flip the switch between Cdc25 and Wee1/Myt1 to allow the entry into mitosis. A recent study using in vitro *Xenopus* egg extracts proposes that Greatwall plays a critical role to promote mitotic entry during recovery from a DNA damage-induced arrest (Peng et al. 2010). The Greatwall/PP2A feedback loop would be actively shut down during a DNA damage response, opening the possibility that checkpoint kinases such as ATM/ATR or Chk1/Chk2 can directly phosphorylate and inactivate Greatwall. Conversely, the positive feedback loop comprising Greatwall and PP2A would play an active role in the reactivation of Cdk1-cyclin B during DNA damage recovery (Peng et al. 2010). Future studies are now required to understand if Greatwall regulation during the DNA damage response is an indirect consequence of Cdk1 activation and if its role in recovery is distinct from the role during normal cell cycle progression.

A second question is the possibility that Greatwall might also control phosphatases other than PP2A-B55δ. Multiple phosphatases are probably required to undo the effects of multiple mitotic protein kinases. PP2A-B56δ is required to dephosphorylate the activatory phospho-Thr138 residue of Cdc25 (Margolis et al. 2006a, b). PP1 has recently been proposed to be involved in the dephosphorylation of mitotic substrates (Wu et al. 2009), in agreement with its activity level that has been reported to be lower during M-phase than during interphase (Walker et al. 1992). Many scenarios involving cross-talks in the regulation of various phosphatases can be conceived, where they act in parallel and/or in tandem (i.e., regulating each other in cascade). An important goal of future investigations will be certainly

to clarify which phosphatases, besides the major PP2A-B55δ, are involved in M-phase regulation, and which ones are controlled by Greatwall.

The third is to know how Greatwall promotes PP2A-B55δ inactivation. Although a fraction of Greatwall was shown to coimmunoprecipitate with some PP2A (Vigneron et al. 2009), opening the possibility that the inhibition mechanism could be based on a direct interaction between both partners, direct phosphorylation of PP2A by this kinase has never been detected. Therefore, the model that Greatwall would work indirectly through other regulators of PP2A was favored.

11.4 Greatwall Phosphorylates ARPP-19/α-Endosulfine and Converts it into a Specific and Potent Inhibitor of PP2A that Is Essential for Mitosis

While we were finishing writing this review article, we were informed about new development of the Greatwall story. Tim Hunt and Thierry Lorca nicely communicated with us their results that appeared now in the journal "Science". To ascertain that Greatwall acts by phosphorylating and activating some as-yet unidentified PP2A inhibitor, their laboratories hunted for Greatwall substrates, using traditional biochemical approaches: *Xenopus* interphasic extracts were fractionated on chromatography columns, active Greatwall kinase was then incubated with fractions containing potential substrates under conditions where endogenous kinase activities are suppressed, and the major substrate was identified by mass spectrometry. Both laboratories found that one small heat-stable protein, cAMP-regulated phosphoprotein-19 (ARPP-19), a close relative of the small protein α-Endosulfine (ENSA), was the best substrate for Greatwall in *Xenopus* extracts (Gharbi-Ayachi et al. 2010; Mochida et al. 2010). This finding was quite unexpected, since the well-conserved protein ARPP-19 is a prominent PKA substrate in the striatum and was more generally proposed to mediate actions of PKA in a variety of cell types in diverse organisms (Dulubova et al. 2001). Interestingly, studies in *Drosophila* had pointed the implications of ENSA in M-phase (Drummond-Barbosa and Spradling 2004; Goshima et al. 2007; Von Stetina et al. 2008). ARPP-19 and ENSA are very close relatives (Fig. 11.1b), and both proteins were used in the studies of Hunt's and Lorca's laboratories. The two laboratories identified Ser67 as the unique residue phosphorylated by Greatwall. This site is distinct from the PKA targeted residue, which is Ser109, as well as from the Cdk consensus site, Ser28 of ARPP-19 and Thr28 of ENSA (Fig. 11.1b). In vitro, ARPP-19 and ENSA phosphorylated by Greatwall strongly inhibit the PP2A-B55δ trimeric phosphatase but not the monomeric catalytic subunit or the A-C dimer, whereas the dephosphorylated proteins are unable to inhibit any form of PP2A. Moreover, the mechanism of this inhibition involves a phosphorylation-dependent physical interaction between PP2A-B55δ and ARPP-19 or ENSA. The authors then turned to *Xenopus* egg extracts to analyze the phosphorylation and function of ARPP-19/ENSA during mitosis. Addition of

Greatwall-phosphorylated forms of both proteins in interphase extracts promotes rapid mitotic entry; additionally, it prevents mitotic exit of M-phase extracts, both effects being strictly dependent on the phosphorylation by Greatwall. Conversely, extracts depleted of ARPP-19 never enter M-phase, despite high levels of Cdk1 activity. Induction of mitosis can be restored by adding back ENSA or ARPP-19, but not by the addition of the versions mutated on the Greatwall site (S67A). Therefore, both studies clearly identify how PP2A phosphatase is regulated by Greatwall in M-phase: ARPP-19 is phosphorylated by Greatwall in mitosis converting this small protein into a potent and specific PP2A-B55δ inhibitor that acts by direct binding to the phosphatase. When dephosphorylated, it looses its capacity to bind and to inhibit PP2A (Gharbi-Ayachi et al. 2010; Mochida et al. 2010). This conclusion is reminiscent of previous observations showing that *Drosophila* oocytes mutated for ENSA have a prolonged prophase I and fail to progress to metaphase I. Despite high Cdk1 activity, these oocytes display little phosphorylation of M-phase substrates, consistent with a failure to inactivate protein phosphatases upon entry into meiotic M-phase (Von Stetina et al. 2008).

These studies raise the question of the respective role of ARPP-19 and ENSA: are they both physiological regulators of PP2A? Several observations point distinct characteristics for both proteins in the *Xenopus* system. First, ARPP-19, but not ENSA, was identified in the two biochemical screens designed to identify Greatwall substrates. Second, both proteins are expressed in *Xenopus* egg extracts, but at different concentrations, endogenous ARPP-19 being hardly detectable whereas ENSA is present at about 150–300 nM in egg extracts, therefore in large excess compared to PP2A-B55δ (50–70 nM). Third, only ARPP-19 seems to be phosphorylated during M-phase. Fourth, using specific antibodies against ARPP-19 or ENSA, Gharbi-Ayachi et al. (2010) show that only ARPP-19 depletion causes rapid exit of mitosis. Therefore, although ENSA and ARPP-19 exhibit the same biochemical properties in vitro, both being substrates of Greatwall, inhibitors of PP2A, and producing the same effects when added in egg extracts, only the endogenous ARPP-19 would be phosphorylated by Greatwall and would control mitosis by inhibiting PP2A-B55δ in vivo.

Finding that the main Greatwall substrate was a well-known PKA substrate was surprising. Vertebrate ARPP-19 family members contain a conserved consensus site for phosphorylation by PKA (Fig. 11.1b) and this site is efficiently phosphorylated both in vitro and in intact cells by PKA (Dulubova et al. 2001). The conservation of this protein family through evolution led to the proposal that it subserves an important cellular function that is regulated by PKA, a conclusion now challenged by the findings of Mochida et al. (2010) and Gharbi-Ayachi et al. (2010). The involvement of ARPP-19 in mitosis control is clearly dependent on its phosphorylation by Greatwall on a Ser residue located in the middle of a sequence (FDSGDY) that is highly conserved within all available genomes, hence representing the signature of the ENSA family through evolution (Fig. 11.1b). On the basis of the *Xenopus* egg extracts experimental model, the PKA site of ARPP-19 and ENSA is dispensable for their cell cycle function. The interesting possibility that PKA phosphorylation of ARPP-19 serves to transduce the information carried by

extracellular signals deserves a closer look. *Xenopus* egg extracts are cycling through self-autonomous mechanisms and do not allow to easily address this question. Cellular models where M-phase entry depends on extracellular signal, such as oocyte meiotic maturation that is under cAMP control in vertebrates, deserve such investigations. It would be also worth investigating if ARPP-19 and ENSA phosphorylation by Cdk1 could participate to their regulation and cell cycle function, as both of them have a conserved Cdk consensus site (Ser28 of ARPP-19 and Thr28 of ENSA) that is in vitro phosphorylated by Cdk1 (Mochida et al. 2010).

The question of the control of MPF activation at the mitotic entry by the Greatwall/ARPP-19/PP2A system also came back in the work of Hunt's and Lorca's laboratories. Interestingly, Moshida and colleagues convincingly show that depletion of ARPP-19/ENSA in egg extracts block M-phase entry despite high Cdk1 kinase activity (Mochida et al. 2010). This implies that the main effect of PP2A-B55δ is to antagonize Cdk1 phosphorylation of downstream target proteins rather on the control of MPF activity itself. Therefore, the PP2A isoform that is required to launch MPF activation and entry into M-phase through the regulation of the Cdc25 and Myt1/Wee1 couple would not involve the specific PP2A-B55δ trimer complex and the Greatwall/ARPP-19 system, a suggestion in agreement with previous observations showing that Greatwall activation depends on MPF activity (Yu et al. 2006; Zhao et al. 2008). The contribution of Greatwall/ARPP-19/PP2A to MPF activation, therefore, remains an open and important question.

11.5 Conclusion

All these results now converge to a new vision of the molecular control of mitosis. Until now, the Cdk1-cyclin B complex was considered as the unique mitotic engine. Cdk1-cyclin B now looses this monopoly position with the discovery that PP2A counterbalances its action and that PP2A regulation is as essential as the one of MPF to orchestrate properly M-phase fulfilment. The powerful mitotic kinase, MPF, found its counterpart, the antimitotic phosphatase PP2A-B55δ. Figure 11.2 summarizes this renewed model of M-phase. What happens at the end of G2 phase to flip the balance between Cdc25 and Wee1/Myt1 to allow the sharp activation of MPF is still not understood. Even though PP2A plays a role in this switch, this step would not be under the control of Greatwall/ARPP-19/PP2A-B55δ. Greatwall requires activation by MPF and once turned on, it phosphorylates ARPP-19, which in turn binds and inactivates PP2A-B55δ. This is essential to protect MPF mitotic substrates from dephosphorylation. It simultaneously influences the autoregulatory loop enhancing MPF activity by protecting Cdc25 and Wee1/Myt1 from dephosphorylation. It is now clear that the inhibition of PP2A by Greatwall and ARPP-19 is as critically important for M-phase accomplishment than MPF activation. The return to interphase is brought about by Cdk1 inactivation, caused by cyclin B degradation, and PP2A activation that actively dephosphorylates MPF mitotic substrates (Fig. 11.2). For the moment, there are no molecular insights into

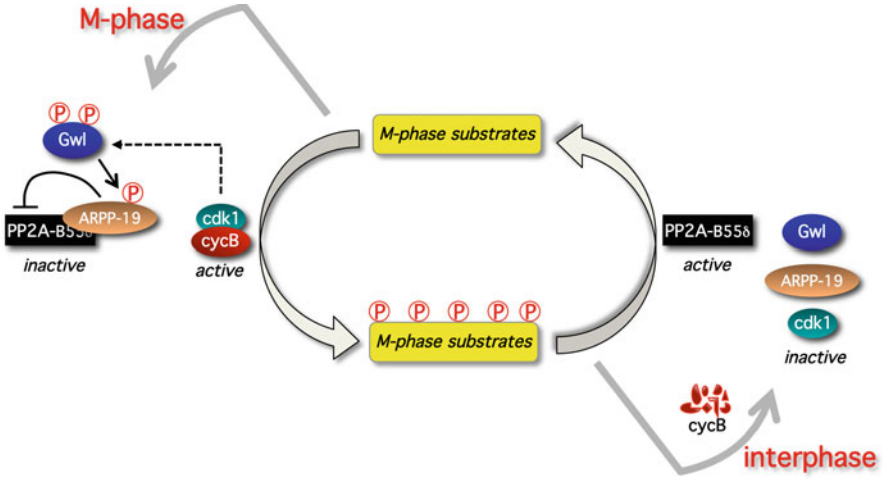

Fig. 11.2 Mitosis is governed by two opposing enzymes, Cdk1 kinase and PP2A-B55δ phosphatase. Cdk1-cyclin B induces the activation of Greatwall that phosphorylates ARPP-19. Phosphorylated ARPP-19 binds and inhibits PP2A-B55δ. The inhibition of this essential antimitotic phosphatase protects MPF mitotic substrates from dephosphorylation. This allows the maintenance of the mitotic state. The return to interphase is brought about by Cdk1 inactivation, achieved through degradation of cyclins, and by PP2A-B55δ activation, induced by dephosphorylation of Greatwall and ARPP-19

the manner of how Greatwall and ARPP-19 are dephosphorylated, presumably by protein phosphatases other than PP2A-B55δ, allowing the exit from M-phase. Understanding how Greatwall activity is regulated will enlighten our understanding of the entry into and exit from mitosis.

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Chapter 12
The Role of RanGTP Gradient in Vertebrate Oocyte Maturation

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Abstract The maturation of vertebrate oocyte into haploid gamete, the egg, consists of two specialized asymmetric cell divisions with no intervening S-phase. Ran GTPase has an essential role in relaying the active role of chromosomes in their own segregation by the meiotic process. In addition to its conserved role as a key regulator of macromolecular transport between nucleus and cytoplasm, Ran has important functions during cell division, including in mitotic spindle assembly and in the assembly of nuclear envelope at the exit from mitosis. The cellular functions of Ran are mediated by RanGTP interactions with nuclear transport receptors (NTRs) related to importin β and depend on the existence of chromosome-centered RanGTP gradient. Live imaging with FRET biosensors indeed revealed the existence of RanGTP gradient throughout mouse oocyte maturation. NTR-dependent transport of cell cycle regulators including cyclin B1, Wee2, and Cdc25B between the oocyte cytoplasm and germinal vesicle (GV) is required for normal resumption of meiosis. After GVBD in mouse oocytes, RanGTP gradient is required for timely meiosis I (MI) spindle assembly and provides long-range signal directing egg cortex differentiation. However, RanGTP gradient is not required for MI spindle migration and may be dispensable for MI spindle function in chromosome segregation. In contrast, MII spindle assembly and function in maturing mouse and *Xenopus laevis* eggs depend on RanGTP gradient, similar to *X. laevis* MII-derived egg extracts.

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12.1 Introduction

In sexually reproducing species, haploid gametes are formed through the process of meiosis, which involves two specialized cell divisions following single DNA replication. Many species-specific variations exist as well as differences between male and female meiosis in sexually dimorphic organisms. In mammals, meiosis starts at the onset of puberty in males, while in females, meiosis starts during fetal life when the entire pool of primary oocytes is formed. To survive, primary oocytes must recruit somatic granulosa cells to form primordial follicles enclosing the oocytes. In adult females, hormonal signals activate transformation of a subset of follicles into primary follicles (Binelli and Murphy 2010), inducing dramatic growth of oocytes as they stockpile cytoplasmic and nuclear components for the first embryonic cell cycles. Fully grown oocyte is a large spherical cell (~1.2 mm diameter in *Xenopus laevis*, Brown et al. 2007; 70–76 μm in mouse, Eppig and O'Brien 1996), which contains an unusually large nucleus called germinal vesicle (GV) and remains arrested at the dictyate stage of the first meiotic prophase under control of cAMP-dependent protein kinase A, PKA (Tunquist and Maller 2003).

Hormonal signals to follicular cells trigger signaling cascade whose conserved endpoint is decrease of cAMP levels below threshold sufficient to maintain meiotic arrest (Solc et al. 2010; Sun et al. 2009; Tunquist and Maller 2003). As in all dividing cells, meiotic divisions are primarily governed by M-phase promoting factor (MPF, also known as maturation promotic factor) consisting of cyclin-dependent kinase 1 (CDK1, cdc2 in yeast) bound to its activator cyclin B (Brunet and Maro 2005; Tunquist and Maller 2003). As discussed in Sect. 12.3.1, regulated bidirectional transport of MPF and its regulators between GV and oocyte cytoplasm has a critical role in controlling the prophase I arrest and resumption of meiosis.

GV breakdown (GVBD) is the first major morphological change marking the resumption of meiosis. Following GVBD, the metaphase I spindle forms, and when all chromosome bivalents have established stable microtubule–kinetochore interactions, anaphase I occurs during which one set of homologous chromosomes is ejected to a small polar body (PB). Following anaphase I, oocytes enter directly into meiosis II without an intervening S-phase. A spindle is formed around a replicated haploid chromosome set and meiosis arrests for the second time when metaphase II is reached. Fertilization triggers resumption and completion of meiosis II.

Studies of oocyte maturation in a variety of animal models have yielded many important insights into cell cycle regulation, signal transduction, cytoskeleton dynamics, nuclear reprogramming, and others. One of such discoveries started with the observation of a long-range effect of chromatin on the stabilization of microtubules (MTs) in *X. laevis* (African clawed frog) eggs and in native extracts prepared from them (Dogterom et al. 1996; Karsenti et al. 1984). Later, studies in *X. laevis* egg extracts revealed that the GTP-bound form of *Ra*s-related *n*uclear GTPase Ran induces MT nucleation and assembly of spindle-like structures (Carazo-Salas et al. 1999; Kalab et al. 1999; Ohba et al. 1999; Wilde and Zheng 1999), leading to the proposition that RanGTP gradient emanating from chromosomes underlies their

ability to control MTs over distance (Carazo-Salas et al. 1999; Kalab et al. 1999). Experiments in *X. laevis* egg extract revealed that in mitotic cytoplasm, RanGTP activates essential spindle assembly factors (SAFs) by releasing them from inhibitory complexes with importin β and importin α (Gruss et al. 2001; Nachury et al. 2001; Wiese et al. 2001). This suggested that the distance effect of chromosomes on MTs is mediated by a diffusion-limited gradient of cargos released from importins by RanGTP gradient formed on the chromatin in reaction with RCC1. The existence of such a regulatory cascade of gradients was revealed by imaging with Förster resonance energy transfer (FRET) biosensors (Kalab and Heald 2008; Kalab et al. 2002, 2006). Although in *X. laevis* egg extracts the role of RanGTP gradient in spindle assembly is dominant, it soon became evident that this is not a general rule in all vertebrate cell types (Arnaoutov and Dasso 2003; Kalab et al. 2006) and that RanGTP gradient functions in parallel to, and may be experimentally bypassed with, kinetochore-dependent signaling mechanisms even in *X. laevis* egg extracts (Maresca et al. 2009; O'Connell et al. 2009).

Here, we review the role of Ran during vertebrate oocyte maturation, focusing on its function in prophase I arrest, in the assembly of meiotic spindles, and in the signaling of chromosomes to oocyte cortex. Although the contribution of Ran-regulated pathways to mitotic vs. meiotic cells is different (Dumont et al. 2007b; Kalab and Heald 2008; Kalab et al. 2006; Schuh and Ellenberg 2007), many of the underlying mechanisms are conserved. Therefore, we start with the introduction to current models of Ran function, which are based on studies in a variety of experimental systems, including *X. laevis* egg extracts and somatic tissue culture cells.

12.2 Mechanisms of Ran-Regulated Cellular Functions

12.2.1 RanGTP Gradient and its Role in Transport Between Nucleus and Cytoplasm

Due to the separation of mRNA synthesis in the nucleus and protein translation in the cytoplasm, transport between nucleus and cytoplasm is critical for virtually all cellular functions in eukaryotes. The gateways between nucleus and cytoplasm are channels inside nuclear pore complexes (NPCs), which are large protein structures spanning the nuclear envelope (NE). Unstructured phenylalanine (F)- and glycine (G)-rich hydrophobic domains of nucleoporins (Nups; components of NPC) surrounding the NPC channel form interlocking protein hydrogel that serves as a selective permeability barrier controlling the passage between nucleus and cytoplasm (Frey and Gorlich 2009; Frey et al. 2006; Weis 2007). Only molecules smaller than passive exclusion limit of NPCs (2.6 nm in HeLa cells; Mohr et al. 2009) freely diffuse through NPC channels. The passage of larger molecules requires their loading onto transport receptors capable of binding to and passing through the

FG-domain barrier. With the exception of most mRNA nuclear export, the transport of most other cargos (proteins, tRNA, microRNA, and a subset of transcripts) through NPCs is thought to depend on Ran-regulated nuclear transport receptors (NTRs) of the importin β family, also called karyopherins or Kaps (Pemberton and Paschal 2005). The ability of NTRs to concentrate cargos on either side of the NPC channel depends on coupling of the NTR cargo loading and unloading reactions to concentration gradient of RanGTP across NE (Hetzer and Wente 2009; Lowe et al. 2010; Weis 2007). With the exception of some mRNAs exported in complexes with adaptor proteins and exportin 1, the bulk of mRNA nuclear export is mediated by heterodimer Nxf1/Nxt1 (Mex67/MTR2 in yeast) and is not dependent on RanGTP gradient (Carmody and Wente 2009).

Due to the concentration of RCC1 in nuclei and cytoplasmic localization of RanGAP, nuclear Ran is mostly GTP-bound, while cytoplasm contains mostly RanGDP. NTRs functioning as nuclear import receptors, importins, load their nuclear localization signal (NLS)-containing cargos in the cytoplasm. In the nucleus, RanGTP binding to importins induces release of the NLS cargo. Nuclear export signal (NES) cargos are stably loaded on exportins in heterotrimeric complexes with RanGTP. In cytoplasm, nuclear export complexes are destabilized by RanGAP-catalyzed GTP hydrolysis on Ran. The gradient of RanGTP across NE, therefore, provides direction to both nuclear export and import reactions, and the energy required for the transport is expended on the maintenance of RanGTP gradient (Pemberton and Paschal 2005; Weis 2003). Nuclear Ran is less mobile than cytoplasmic (Abu-Arish et al. 2009), partially contributing to the higher concentration of Ran in the nucleus that primarily depends on nuclear import of RanGDP by NTF2 (Ribbeck et al. 1998; Smith et al. 1998) and possibly on Ran binding to nuclear factor Mog1 (Kelley and Paschal 2007).

The nuclear localization of RCC1 depends on its regulated binding to chromatin (Hitakomate et al. 2010; Makde et al. 2010) and on its own import mediated by importin β with importin α3 (Quensel et al. 2004). RanGAP is excluded from the nuclei and its SUMO-conjugated form concentrates on cytoplasmic filaments of NPCs where it is bound to nucleoporin RanBP2/Nup358, together with Ubc9, the E2 enzyme of SUMO pathway (Matunis et al. 1996, 1998; Saitoh et al. 1998; Zhang et al. 2002). The complex of RanBP2, RanGTP-SUMO, and Ubc9, called RRSU (Wozniak et al. 2010), remains intact in mitosis and supports mitotic spindle function.

Because RanGTP bound to NTRs is protected from RanGAP, dissociation factors containing RanGTP-binding domain (RBD) are required to present RanGTP to RanGAP-catalyzed GTP hydrolysis on Ran. In human cells, such function is carried by small cytoplasmic single RBD-containing RanBP1 protein and by RanBP2/Nup358 that contains four RBDs (Bischoff and Gorlich 1997; Yaseen and Blobel 1999). Interestingly, the RanGTP-dissociation activity of RBDs is enhanced by importin α (Bischoff and Gorlich 1997), possibly compensating for the low abundance of RBDs. The levels of RanBP1 are regulated, with a peak in mitosis followed by late anaphase/telophase degradation that is required for post-mitotic functions of Ran (Ciciarello et al. 2010).

Ran-regulated transport across NPC is an impressively efficient process providing for simultaneous bidirectional translocation of estimated ~1,000 molecules per second through each NPC (Ribbeck and Gorlich 2001). Based on 27% rate of nuclear proteins in yeast genome (Kumar et al. 2002) and number or coding genes in human genome (Clamp et al. 2007), nuclear import in human cells may have to handle at least ~6,000 different protein cargos. How Ran-regulated NTRs achieve correct nuclear and cytoplasmic localization of so many cargos is a challenging question, considering that only about 20 NTRs exist in the human genome (Bednenko et al. 2003). Of those, at least nine are importins (importin β, importin β2 (transportin), importin β3, transportin SR, importins 4, 7, 8, 9, and 11), six are exportins (exportins 1, 5, 6, 7, exportin T, and CAS/Cse1), two carry cargos in both directions (importin 13 and exportin 4; Bono et al. 2010; Gontan et al. 2009), and some remain uncharacterized.

Studies of the particularly abundant and versatile carrier of NLS cargos importin β (KPNB1, ~95 kDa) provided important insights into the molecular mechanism of nuclear import. The structure of free importin β, which resembles letter S twisted in three dimensions, offers surfaces for different cargos and Nups and dramatically changes conformation in response to their loading and in response to the binding of RanGTP (Lott et al. 2010; Stewart 2006; Zachariae and Grubmuller 2008). Some cargos bind to importin β directly and many other through adaptor proteins including importins α (seven isoforms in human genome; Kelley et al. 2010) and Snurportin 1 (Huber et al. 1998). The enhancement of direct loading of Rae1 on importin β by Nup98 (Blower et al. 2005) showed that Nups can also serve as importin β adaptors. Finally, histones are loaded on importin β–importin 7 heterodimer (Jakel et al. 1999), suggesting that combinations of NTRs expand the spectrum of cargo recognition.

Importins α (~60 kDa) contain a C-terminal Armadillo repeat domain where the NLS cargos are loaded and a flexible N-terminal importin β-binding domain (IBB). The IBB domain also contains NLS sequence and can, therefore, efficiently bind to importin α C-terminus and compete with NLS cargo loading. Binding of IBB to importin β relieves this autoinhibition, stabilizing the import complex (Goldfarb et al. 2004). In the nucleus, RanGTP binding to importin β dissociates IBB and promotes cargo unloading, which is further accelerated by RanGTP-dependent binding of importin α to its nuclear export factor CAS/Cse1 (Stewart 2006). Some NLS cargos are preferably imported to nuclei by a specific importin α/β complex, such as RCC1 by importin α3/importin β complex (Quensel et al. 2004). However, many NLS cargos are efficiently transported by a subset of importins α and some by any of them (Huenniger et al. 2010). Unlike other importins α, importin α1 mRNA levels display strong cell cycle periodicity in HeLa and are highest in mitosis (Whitfield et al. 2002), suggesting its specialized role as a mitotic regulator. Interestingly, importin α1 is also the preferred nuclear importer of dedifferentiation transcription factors OCT3/4, and its overexpression is sufficient to maintain the dedifferentiated state of mouse embryonic stem cells (Yasuhara et al. 2007). Whether importin α1 has a specialized role in vertebrate meiosis or embryonic development is not known. Such a role is certainly played by recently

discovered importin α7 (KPNA7), which is specifically expressed in mouse and bovine oocytes and early embryos (Hu et al. 2010; Tejomurtula et al. 2009). In mouse oocytes, importin α7 localizes to meiotic spindles, but its major function appears to be in the regulation of chromatin state and embryonic gene expression. Deletion of importin α7 in mouse induced partial fetal death, eliminating mostly female offspring, and was associated with defects in histone H3 methylation and abnormal zygotic mRNA expression, including strong downregulation of OCT3/4 transcriptional targets DPPA2 and DPPA4 (Hu et al. 2010).

The major nuclear export receptor is exportin 1. Exportin 1 forms a C-shaped loop (Petosa et al. 2004) carrying NES cargo on its lateral surface. As a dedicated nuclear exporter of profilin–actin complex (Stuven et al. 2003), exportin 6 has a special relevance in meiotic *X. laevis* oocytes. Because exportin 6 is absent in oocytes, actin accumulates in GV, and fills it with a dense branched actin filament network, providing a crucial mechanical support to this large nucleus that contains somatic G2-level DNA content in a volume ~100,000× larger than typical somatic cell (Bohnsack et al. 2006).

12.2.2 Mitotic Functions of Ran

After the disassembly of NE, the dynamic interactions of nuclear transport system (NTS) components take place in a single compartment formed by the fusion of nuclear and cytoplasmic contents. Due to the continuing RCC1 binding to chromatin, free RanGTP is released at the surface of mitotic chromosomes. RanGTP diffusing into cytoplasm either is nonproductively converted to RanGDP in a reaction with RanGAP or interacts with its many ligands, the most abundant of which are NTRs. Each of such interactions induces formation or disassembly of NTR–cargo complexes, depending on whether the NTR is exportin or importin. The products of such reactions diffuse through the cytoplasm until their conversion to yet another molecular form (Bastiaens et al. 2006; Caudron et al. 2005; Dehmelt and Bastiaens 2010; Kalab and Heald 2008; Soh et al. 2010). A cascade of spatially overlapping gradients regulated by RanGTP thus marks the position of mitotic chromosomes. The extent of such gradients is determined by the diffusion constant of a given molecular form and by the sum of the rates of its dissipation (Caudron et al. 2005; Soh et al. 2010). Some of the NTR cargos function as SAFs whose activity and/or localization is affected by their RanGTP-regulated binding to NTRs. In this manner, RanGTP gradient relays the role of chromosomes in spatial and temporal regulation of many mitotic events.

12.2.2.1 Activity Gradients and Recruitment to Structures: Two Mechanisms of Mitotic Ran Function

The mitotic regulation of SAFs by Ran occurs by at least two mechanisms. First, RanGTP gradient releases SAFs from complexes with importins, triggering a

diffusion-limited volume of activated SAFs surrounding chromosomes. Within such volume, a critical activation threshold of SAF is reached, which locally outweighs the opposing inhibitory activities present in the cytoplasm. As a result, SAF-regulated MT nucleation, stabilization, and organization into bipolar spindle structures are all spatially biased toward mitotic chromosomes. Most Ran-regulated mitotic functions regulated by importins fall within this category.

In the second mechanisms, RanGTP recruits SAFs to preexisting structures within the mitotic spindle apparatus, thus overcoming the low spatial resolution of diffusion-limited gradients. Such mechanism is involved in RanGTP and exportin 1-dependent recruitment of RRSU complex to kinetochores discussed in Sect. 12.2.3.2. RanGTP-dependent unloading from importins promotes the binding of chromokinesin Kid to spindle MTs, while the binding of importin α and importin β is required for loading of Kid on mitotic chromosomes (Tahara et al. 2008). The two mechanisms of SAF regulation by Ran are, therefore, not exclusive with each other.

12.2.2.2 Short- and Long-Range Effect of RanGTP Gradient

Quantitative imaging of FRET sensors in HeLa cells revealed that RanGTP gradient induces locally only small local increase (~15%) of active SAFs around metaphase chromosomes, rather than operating as an on/off switch at that level, as it could be expected. However, even smaller RanGTP-induced increase of free importin β cargos was sufficient to activate assembly of MTs in *X. laevis* egg extracts (Kalab et al. 2006). This suggested that RanGTP gradient drives spindle assembly by locally inducing a fractional break of symmetry between mitotic spindle assembly activators and inhibitors (Kalab and Heald 2008; Kalab et al. 2006). According to this model, the rapid activation of spindle assembly at a short range from chromosomes is promoted by cooperation between RanGTP-activated SAFs, by formation of multifunctional complexes of SAFs, and by their concentration on nascent spindle MTs. In virtually all types of dividing cells, their major MT organizing centers (MTOCs) are localized close to chromosomes in G2–M transition and therefore within the peak of the RanGTP gradient. That is the case of centrosomes in somatic cells as well as of acentriolar MTOCs in oocytes (Schuh and Ellenberg 2007), and of the MTOCs activated at kinetochores (Tulu et al. 2006). In this manner, RanGTP gradient and major cellular MTOCs in spindle assembly are poised to act synergistically. Finally, in meiotic *X. laevis* egg extracts, the sharp cytoplasmic response to RanGTP gradient involves MT-driven MT generation, which is promoted by the recruitment of noncentrosomal MTOCs to nascent MTs (Clausen and Ribbeck 2007), a process involving augmin (Goshima and Kimura 2010).

Experiments in *X. laevis* egg extracts showed that chromatin-centered RanGTP gradient stabilizes MTs with about the same strength relatively far to the cytoplasm (20–25 μm) where the stabilizing effect sharply drops off (Athale et al. 2008). Computational modeling suggested that this mode of regulation could

be reasonably explained by coupling of RanGTP gradient to a gradient of phosphorylation activity promoting MT stabilization. Indeed, a kinase with properties fitting such model, Cdk11, was identified as RanGTP- and importin β-regulated SAF (Athale et al. 2008; Yokoyama et al. 2008). However, the coupling of Cdk11 activity to RanGTP gradient remains hypothetical, and the proposed mathematical model did not fit the measured data equally well as a simple on/off model (Athale et al. 2008), indicating that other mechanisms may be at play. The short- and long-range signaling by RanGTP gradient is particularly relevant to large cells such as oocytes.

12.2.2.3 Visualization of RanGTP Gradient by FRET

The understanding of the reaction-diffusion processes underlying the mitotic role of RanGTP gradient was advanced by computational models that incorporated known kinetic parameters and concentrations of Ran, its regulators, and NTRs (Caudron et al. 2005; Gorlich et al. 2003; Kalab et al. 2006; Ribbeck and Gorlich 2001; Smith et al. 2002). Importantly, such mathematical models reasonably agreed with results of experiments where the spatial distribution of several RanGTP-regulated molecular species was visualized with FRET sensors (Caudron et al. 2005; Kalab et al. 2002, 2006).

A FRET sensor called YRC was composed of donor–acceptor fluorescent protein (FP) pair, ECFP and EYFP flanking RBD domain from *Saccharomyces cerevisiae* homologue of RanBP1 (Kalab et al. 2002). When YRC binds to RanGTP, its RBD domain extends and FRET efficiency decreases. YRC reported on the gradient of RBD–RanGTP complex around chromatin in mitotic *X. laevis* egg extracts (Kalab et al. 2002), which, as expected based on computational modeling (Caudron et al. 2005), decayed at a short distance from mitotic chromatin (~17 μm in *X. laevis* egg extracts) (unpublished, based on Kalab et al. 2002). Sensor for *Ran*-regulated importin β car*go*s, called Rango, contains IBB domain of importin α or of Snurportin 1 flanked by donor–acceptor FPs, such as ECFP and EYFP (Kalab et al. 2002) or CyPet-YPet (O'Connell et al. 2009). Because IBB domain is not structured in free form and extends to >5 nm when bound to importin β (Cingolani et al. 1999; Mitrousis et al. 2008), Rango displayed low FRET when mixed with importin β and high FRET in free form. Thus, high FRET signal of Rango indirectly reported on RanGTP-induced release of importin β cargos (Kalab et al. 2002, 2006; Kalab and Soderholm 2010). The behavior of Rango sensors presumably mimics behavior of endogenous cargos carried by importin α-like adaptors of importin β. Consistent with computational Ran models, the gradient of free Rango (importin β cargos) detected in *X. laevis* egg extracts was wider than YRC-RanGTP gradient (~25 μm, Kalab et al. 2002, 2006). Experiments with dominant-negative Ran and importin β mutants demonstrated that RanGTP-RBD gradient detected by YRC acts upstream of the importin β cargo gradient detected by Rango and that both depended on the activity of RCC1 and both were required for spindle assembly and maintenance in *X. laevis* egg extracts (Kalab et al. 2002). Finally, a bimolecular sensor composed of Alexa

488-Ran as a donor and Cy3-importin β as an acceptor detected a broad gradient of RanGTP–importin β complex in *X. laevis* egg extracts (Caudron et al. 2005). The long range of this gradient (~30–35 μm) was again consistent with Ran system models (Caudron et al. 2005) and demonstrated that RanGTP gradient influences meiotic cytoplasm over distances relevant to large vertebrate oocytes.

Given the technical challenges of FRET techniques, the use of antibodies specifically recognizing GTP-bound form of Ran appears as a useful alternative. Indeed, such antibodies were used for detecting pools of RanGTP at centrosomes (Keryer et al. 2003), kinetochores (Torosantucci et al. 2008), and in cilia (Dishinger et al. 2010). However, it is not clear whether such antibodies can distinguish between RanGTP bound to NTRs or other ligands from free RanGTP. In addition, the true location of free and, therefore, highly mobile form of RanGTP may not be reliably captured by cell fixation, further limiting the interpretation of such results.

12.2.2.4 Mitotic Regulation of Ran and of its GTP/GDP Nucleotide Cycle

In mitotic cells, Ran is phosphorylated on Serine 135 and phosphomimetic S135D Ran induced defects of mitotic spindle pole (Feng et al. 2006), suggesting a specific function of phospho-S135 Ran in mitotic spindle assembly. Some evidence implicated polo-like kinase 1 (PLK-1) as the Ran-specific kinase (Feng et al. 2006; Jang et al. 2004). However, Ran does not have PLK-1 phosphorylation consensus site and was only inefficiently phosphorylated by PLK-1 in vitro (Bompard et al. 2010; Jang et al. 2004). Instead, the kinase responsible for mitotic S135 Ran phosphorylation was identified as p21-activated kinase 4 (PAK4) (Bompard et al. 2010). The S135-phospho RanGTP induces MT assembly in *X. laevis* egg extracts, indicating that the phosphorylation does not affect binding to NTRs. However, the binding of S135-phospho Ran to RCC1 was impeded, and the phosphorylated Ran was also resistant to RanGAP1-mediated GTP hydrolysis. As a result, the nucleotide-binding state of Ran is stabilized upon S135 phosphorylation, transferring the control of Ran from its core regulators to the dynamics of PAK4 kinase and cytoplasmic phosphatases. Because active PAK4 is concentrated at mitotic chromosomes, much of the phospho-S135 Ran is likely GTP-bound. Remarkably, phospho-S135 Ran colocalizes with PAK4 at centrosomes and at cytoplasmic foci around metaphase plate and in the spindle midzone and midbody during anaphase–telophase transition. This is suggesting that RanGTP stabilized by phosphorylation carries specific functions at such locations, consistent with reports on the function of centrosomal pool of Ran (Keryer et al. 2003), RanBP1 (Di Fiore et al. 2003), and RanGTP–exportin–nucleophosmin complex (Wang et al. 2005). The existence of the PAK4-dependent phosphorylation of Ran in *X. laevis* egg extracts and in HeLa cells (Bompard et al. 2010) suggests that PAK4-mediated regulation of Ran is common to mitotic and meiotic cell divisions in vertebrates.

It could be expected that RCC1 is stably anchored to chromatin in mitotic cells to support the formation of RanGTP gradient. Instead, the binding of RCC1 is dynamic and regulated by several mechanisms. Mutational and live imaging studies

suggested that the guanine nucleotide exchange reaction on Ran is coordinated with RCC1-Ran binding to chromatin. In this model, RanGDP binding to RCC1 promotes temporary docking of their complex on chromatin, while RanGTP–RCC1 complex quickly dissociates from chromatin and disassembles, releasing free RanGTP in a thin cytoplasmic volume surrounding the chromosomes (Li et al. 2003). Several mechanisms controlling the binding of RCC1 to chromatin, and thus the formation of RanGTP gradient, converge on the N-terminal flexible tail of RCC1, which is extending from the donut-shaped β-propeller domain of RCC1 (Renault et al. 2001). The N-terminal tail is capable of anchoring RCC1 to chromatin by at least two mechanisms. First, RCC1 directly binds to double-stranded DNA on the chromatin through an N-terminal α-methylated motif, which is a result of posttranslational modification carried by N-terminal RCC1 methyltransferase (NRMT) (Chen et al. 2007; Schaner Tooley et al. 2010). The NRMT-dependent modification of RCC1 involves removal of the N-terminal methionine and trimethylation of the group on serine or proline residues on the newly exposed N-terminus (Chen et al. 2007). The second mechanism involves the binding of RCC1 N-terminal tail to histones H2A and H2B on the nucleosome (Hitakomate et al. 2010; Hutchins et al. 2004; Nemergut et al. 2001).

The histone binding motif of RCC1 overlaps with NLS for importin α3 and contains phosphorylation sites targeted by CDK1 kinase. The binding of importin α3/importin β complex inhibits RCC1 interaction with the chromatin, while CDK1 phosphorylation inhibits the NLS. Phosphorylation by mitotic CDK kinase, therefore, promotes RCC1 interaction with the chromatin by prohibiting importin binding to RCC1 (Hutchins et al. 2004; Li and Zheng 2004). This suggested a compelling model in which the CDK1-dependent entry to mitosis promotes the formation of a robust mitotic RanGTP gradient (Hutchins et al. 2004; Li and Zheng 2004). However, in human cells there are at least three tissue-specific RCC1 splicing variants (RCC1α, RCC1β, and RCC1γ), which differ exactly in the region targeted by importin α3 and CDK1 (Hood and Clarke 2007). The preferred substrate of CDK1 is RCC1γ isoform whose binding to chromatin is promoted by phosphorylation directly, rather than through preventing importin α3 binding (Hood and Clarke 2007). Interestingly, RCC1γ isoform is prevalent in extracts of human ovaries (Hood and Clarke 2007), suggesting that this phosphorylation-sensitive isoform may be prevalent in oocytes.

A more recent study showed that rather than by mitotic CDK1 kinase, RCC1 is preferably phosphorylated on its N-terminal tail sites by proapoptotic MST1 and MST2 kinases, which in turn are activated by binding to tumor suppressor protein RASSF1A (Dallol et al. 2009). Moreover, RanGTP directly binds to RASSF1A and prevents RCC1 phosphorylation by MST1/2, potentially providing a negative feedback. The depletion of RASSF1A caused dramatic relocation of RCC1 to spindle poles in mitotic cells, concomitant with enhanced spindle pole MT asters (Dallol et al. 2009). Interestingly, MST2 is expressed in porcine oocytes and its concentration possibly slightly increases during meiosis (Pelech et al. 2008), showing that a key player of this remarkable and potentially very important pathway is present on the meiotic scene.

The crystal structure of *Drosophila melanogaster* RCC1 bound to the *Xenopus* nucleosome particle (Makde et al. 2010) added an unexpected twist to the models of RCC1 regulations. The structure showed that RCC1 β-propeller core binds to the nucleosome via its edge, such that two symmetrically positioned RCC1 molecules on the nucleosome resemble pedals on the front wheel of a tricycle (Makde et al. 2010). Moreover, the "switchback" loops of the β-propeller core (England et al. 2010) interact with both the histone and the DNA component of the nucleosome (Makde et al. 2010). This configuration is strikingly different from the previous hypothetical models where RCC1 binds to histones and to Ran with the opposite sides of its β-propeller core (Hitakomate et al. 2010; Li et al. 2003). The challenge will now be to reconcile the "tricycle wheel" structure with the evidence that binding to nucleosomes increases RCC1 exchange activity on Ran (Nemergut et al. 2001) and to explain how Ran interacts with chromatin both in the absence and in the presence of RCC1 (Bilbao-Cortes et al. 2002). A plausible scenario involves the idea that RCC1 and/or Ran undergo significant conformational changes when bound to the nucleosome, such that Ran binds to histone H3 via its C-terminus and RCC1 pivots along its edge toward the nucleosome (England et al. 2010; Makde et al. 2010). Either scenario would be compatible with N-terminal tail function in supporting RCC1–nucleosome interaction.

12.2.2.5 RanGTP Gradient Function at the Exit from Mitosis

In *X. laevis* egg extracts, the levels of RCC1 bound to chromatin strongly increase in M-phase and fall off sharply at the onset of anaphase. Moreover, RCC1 levels control the activity of mitotic spindle assembly checkpoint, implying the role of RanGTP in the process (Arnaoutov and Dasso 2003). Consistent with the evidence for the role of Ran in postmitotic MT dynamics observed in *D. melanogaster* embryos (Silverman-Gavrila and Wilde 2006), RanGTP is required for the role of MT binding protein ISWI in stabilization of MTs in the anaphase spindle and thus for chromosome segregation (Yokoyama et al. 2009). The role of Ran in NE reformation and assembly of NPCs was reviewed recently (Hetzer and Wente 2009; Kutay and Hetzer 2008).

Similar to the regulation of the major mitotic kinases by APC-dependent degradation of cyclins, the exit from mitosis is marked by Cdh1/APC-dependent ubiquitylation and proteasome degradation of TPX2 (Stewart and Fang 2005) and by less well-understood mechanism clearing RanBP1 from cells (Ciciarello et al. 2010; Guarguaglini et al. 2000). In HeLa cells, Cdc20 and APC-dependent degradation of at least four Ran-regulated SAFs (Bard1, Hmmr, hepatoma upregulated protein [HURP], and NuSAP) requires RanGTP-dependent release of those SAFs from importin β (Song and Rape 2010). Interestingly, the protein levels of TPX2 in maturing mouse oocytes are controlled by Cdh1- and APC/C-dependent degradation (Brunet et al. 2008). Because Cdh1-dependent APC/C degradation of mitotic substrates is also regulated by RanGTP and importins (M. Rape, personal communication), it is possible that the maintenance of low TPX2 levels during MI spindle

depends on sufficient RanGTP levels. It is then conceivable that the recovery of MI spindle assembly in oocytes treated with RanT24N (GTP-binding deficient Ran mutant, which binds to and inhibits RCC1) (Dumont et al. 2007b; Schuh and Ellenberg 2007) could have been in part supported by the increased stability of TPX2.

12.2.3 Ran-Regulated Mitotic and Meiotic Spindle Assembly Pathways

X. laevis egg extracts are by far the most commonly used meiotic system where the functions of Ran were investigated. Typically, extracts are prepared from dejellied ovulated eggs arrested in meiosis II by cytostatic factor (CSF), a Mos-, Emi1- and Emi2-dependent inhibitor of anaphase promoting complex (APC) (Liu et al. 2007; Wu and Kornbluth 2008). The CSF egg extracts reconstitute meiotic spindle assembly around the added source of chromatin, such as demembranated sperm nuclei, and exit to interphase upon addition of calcium. Interphase egg extracts recapitulate the NE and NPC assembly around added chromatinized DNA templates and many other functions of somatic cell nuclei including DNA replication and nuclear transport. The possibility to initiate experiments in either phase of the cell cycle enabled the discovery of nuclear transport-independent role of Ran in mitotic spindle assembly (Carazo-Salas et al. 1999; Kalab et al. 1999; Ohba et al. 1999; Wilde and Zheng 1999). Because the *X. laevis* egg extracts originate from MII eggs, it is expected that the role of Ran in other maturing vertebrate oocytes should be similar. Indeed, many similarities were observed, as well as significant differences pointing to the specialization of the meiosis I vs. meiosis II spindle assembly.

In contrast to CSF *X. laevis* egg extracts that are essentially devoid of MTs when not supplemented by DNA, MTs spontaneously formed in nucleus-free halves (Brunet et al. 1998) or enucleated cytoplasts (Yang et al. 2007) of maturing mouse oocytes, forming radial, bipolar, or multipolar MT arrays. These results demonstrated that meiotic spindle assembly in mouse oocytes is driven by self-organization of MT arrays into bipolar structures (Brunet et al. 1999), similar to spindle assembly in other systems (Karsenti and Vernos 2001). Because the enucleation or bisection of oocytes (Brunet et al. 1999; Yang et al. 2007) could not remove all Ran and RCC1, RanGTP likely supported the observed MT self-organization. It is possible that *X. laevis* egg extracts are depleted of components responsible for spontaneous MT formation.

Despite such potential limitation, most Ran-regulated SAFs were originally discovered in meiotic *X. laevis* egg extracts, and in many cases, their Ran-regulated function was confirmed in studies employing somatic tissue culture cells (Clarke 2005; Kalab and Heald 2008). The function of few of such SAFs in meiotic spindle assembly was confirmed in maturing mouse oocytes (Brunet et al. 2008; Illingworth et al. 2010; Saskova et al. 2008; Schuh and Ellenberg 2007), although the role of Ran and NTRs in their regulation in this context has not been examined.

12.2.3.1 Importin-Regulated SAFs

Only a small subset of importins has known mitotic functions in regulating SAFs. Most SAFs are regulated by importin β and importin α (Kalab and Heald 2008). Together with importin β, Importin 7 binds to and regulates HURP (Koffa et al. 2006; Sillje et al. 2006; Wong and Fang 2006). Although transportin was identified as a potential SAF regulator as well (Lau et al. 2009), the evidence for its specific interaction with mitotic SAFs is inconclusive.

Among the SAFs regulated by RanGTP and importin α/importin β complex, a particularly prominent role is played by TPX2, which functions as nucleator of mitotic MTs (Gruss et al. 2001; Gruss and Vernos 2004). The RanGTP-activated binding of N-terminal TPX2 to catalytic domain of Aurora A protects the kinase from phosphatases and locks Aurora A in active state (Bayliss et al. 2003; Eyers et al. 2003; Gruss et al. 2002). Aurora A-bound TPX2 can simultaneously bind to MTs and mediate Aurora A localization at spindle poles (Kufer et al. 2002), where TPX2 accumulates in a dynein- and Eg5-dependent mechanism (Ma et al. 2010). Another RanGTP-regulated mechanism required for TPX2 accumulation on spindle poles requires ubiquitin E3 ligase activity of BRCA1/BARD1 heterodimer (Joukov et al. 2006), which balances the activity of XRHAMM, a TPX2- and γ-tubulin-interacting protein with a role in spindle pole formation in *X. laevis* egg extracts (Groen et al. 2004). Interestingly, BRCA1/BARD1 forms a complex with XRHAMM, TPX2, and NuMa, all proteins localized at the spindle pole (Joukov et al. 2006).

The RanGTP- and TPX2-dependent activation of Aurora A escalates the control of spindle assembly to Aurora A-dependent phosphorylation cascade (Tsai et al. 2003), which promotes the transition from astral to bipolar stage of spindle assembly. This concept was dramatically illustrated in experiment where synthetic beads coated with Aurora A were added to *X. laevis* CSF egg extracts together with RanGTP and induced bipolar spindles carrying single Aurora A bead on each pole (Tsai and Zheng 2005). In CSF egg extracts, Aurora A activates MT nucleation and promotes binding and activation of SAFs at spindle poles (Sardon et al. 2008). Aurora A mediates phosphorylation of SAFs such as HURP (Wong et al. 2008; Yu et al. 2005), NDEL1 (Mori et al. 2007), TACC3/maskin (Kinoshita et al. 2005; LeRoy et al. 2007), Eg5 (Giet et al. 1999), and MCAK (Zhang et al. 2008a).

In mouse oocytes, TPX2 levels are low at GVBD and gradually increase during meiosis, being controlled by the balance of synthesis and Cdh1/APC-dependent degradation (Brunet et al. 2008). Despite the limiting levels of TPX2, Aurora A became activated even before GVBD, as evidenced by the presence of its phosphorylated form on MTOCs prior to GVBD (Saskova et al. 2008). The rise of TPX2 correlated with increasing phosphorylation of TACC3/maskin during MI–MII transition, while TPX2 depletion caused inhibition of TACC3/maskin phosphorylation and severe defects in spindle pole integrity and chromosome congression (Brunet et al. 2008). Together with the evidence that N-terminus of TPX2 is required for MI spindle assembly (Brunet et al. 2008) and that Aurora A

localizes on MI spindle pole (Saskova et al. 2008), this data suggests that TPX2-dependent activation of Aurora A triggers phosphorylation of TACC3/maskin, which is then required for MI spindle assembly. However, the role of RanGTP gradient in the activation of this pathway in oocytes, if any, is not known. The slow rise of TPX2 levels during MI suggests that its "purpose" is to stall the RanGTP–importin α/β–TPX2–Aurora A pathway and to prevent its premature activation, ensuring the physiologically important timing of the MI spindle assembly.

The SAFs directly binding to importin β include HURP, Rae1, Crb1-CLP1, and NuSAP (Kalab and Heald 2008). Notably, HURP is a RanGTP- and importin β-regulated MT-binding protein stabilizing kinetochore MTs (Sillje et al. 2006; Wong and Fang 2006), which in *X. laevis* egg extracts forms a large complex containing MT polymerase XMAP215, MT motor protein Eg5, TPX2, and Aurora A (Koffa et al. 2006). Thus, a single Ran-regulated SAF can deliver to nascent spindle MTs several activities involved in their stabilization and reorganization to spindle structural subassemblies ranging from linear, radial, to bipolar MT structures.

The role of RNA in mitotic spindle assembly emerged from studies of nucleoporin Rae1 (Ribonucleic acid exporter 1), which was identified as a SAF binding to the N-terminus of importin β in RanGTP-regulated manner (Blower et al. 2005). Rae1 is composed of WD40 repeats domain, which often functions as scaffold for the assembly of multimolecular complexes (Stirnimann et al. 2010). Remarkably, several Rae1 complexes function in mitotic spindle assembly. Together with Nup98, Rae1 functions in many steps of mRNA export from the nucleus (Ren et al. 2010). In *X. laevis* egg extracts, complex of Rae1 with ribonucleoprotein (RNP) particles induced formation of MTs in a mechanism that was inhibited by importin β (where Nup98 apparently served as an adaptor for importin β binding to Rae1), activated by RanGTP, required intact RNA and did not require protein translation (Blower et al. 2005), suggesting structural role of RNA in spindle assembly. The conserved role of Rae1 in spindle assembly was supported by defects of mitotic spindle pole in HeLa cells depleted of Rae1 by RNAi (Blower et al. 2005) and in plant cells depleted of Rae1 homologue (Lee et al. 2009). In addition to its interaction with RNPs, Rae1 also binds NuMa at the spindle poles and its interaction with cohesin subunit 1 (SMC1) may be required for dynein-dependent mitotic spindle pole assembly (Wong 2010; Wong and Blobel 2008). A structural role of mRNA in other cytoskeletal elements is discussed by Dobrzyński et al. (2011).

12.2.3.2 Exportin-Regulated Mitotic Functions

At the onset of mitosis in somatic cells, a fraction of exportin 1 is loaded on kinetochores and recruits RRSU complex (Arnaoutov et al. 2005; Arnaoutov and Dasso 2005) (see Sect. 12.2.1 and 12.2.2.1). The localization of RRSU on kinetochores is required for normal kinetochore fiber formation and for chromosome alignment and segregation (Arnaoutov et al. 2005; Joseph et al. 2002; Salina et al. 2003). An earlier study suggested that the recruitment of exportin 1 and RRSU

requires previous loading of Nup106–170 complex to kinetochores (Zuccolo et al. 2007). However, only mild and variable effects of Nup106/107 depletion on RRSU loading on kinetochores were observed in a more recent report, which demonstrated that, instead, the kinetochore Nup107/160 is required for the recruitment of chromosome passenger complex (CPC) consisting of INCENP, borealin, survivin, and Aurora B (Platani et al. 2009). Consistent with this result, proteomic analysis of mitotic chromosomes revealed that RRSU binding to chromosomes requires kinetochore protein Ska3/Rama1 whose depletion, on the other hand, had no effect on the recruitment of Nup107/160 (Ohta et al. 2010). The mechanism of exportin 1-dependent recruitment of RRSU to Ska3/Rama1 remains to be determined.

In *X. laevis* egg extracts where the formation of RanGTP gradient was prevented by the addition of dominant-negative RanT24N (mostly nucleotide-free form of Ran that strongly binds to and inhibits RCC1), bipolar spindles were formed around beads coated with antibodies to INCENP, which locally concentrated active Aurora B (Maresca et al. 2009). This effect, which also required the presence of RanQ69L (GTP hydrolysis-deficient Ran mutant, locked in GTP-bound state), demonstrated that even in *X. laevis* egg extracts, RanGTP gradient has a partially redundant role in spindle assembly, which is paralleled by the gradient of Aurora B-dependent phosphorylation surrounding chromosomes. The ability of Aurora B to direct spindle formation in this system depends on inhibitory phosphorylation of at least two cytoplasmic MT destabilizing activities: Stathmin/Op18, a MT destabilizing protein, and MCAK, a motor protein with MT depolymerizing activity (Maresca et al. 2009). In addition, Aurora B-mediated phosphorylation of kinetochore constituents, which is spatially graded across the kinetochore, locally inhibits their interaction with MTs, allowing for the correction of improper MT attachment (Liu et al. 2010; Welburn et al. 2010).

Whether the aurora B kinase gradient is indeed fully independent of Ran or requires RanGTP for its activation is an interesting question, which is not completely understood. In human somatic cells, survivin, which can drive CPC accumulation, is apparently loaded on kinetochores in exportin-1-dependent manner (Knauer et al. 2006), suggesting potential requirement for RanGTP. However, in mitotic *X. laevis* egg extracts, CPC binds to chromatin in RanGTP-independent manner via Dasra (Kelly et al. 2007). In detail, Ran-regulated SAFs were discussed in recent reviews (Clarke 2005; Goodman and Zheng 2006; Kalab and Heald 2008; Wozniak et al. 2010).

12.3 Ran Functions in Vertebrate Oocyte Maturation

12.3.1 The Role of Nuclear Transport in GVBD

Maintenance of prophase I arrest depends on low levels of cyclin B1 (Holt et al. 2010) and on prevailing inhibitory phosphorylation of Thr14 and Tyr15 on CDK1, which depends on the balance of activities of the Wee1/Myt1 kinases and Cdc25

phosphatases (Solc et al. 2010). A signaling cascade triggered by MPF activation involves powerful positive feedbacks, including phosphorylation-dependent inactivation of APC/Cdh1 (Holt et al. 2010), and thus causes, in physiological context, an irreversible cell cycle transition. A critical role in the orderly transition to M-phase is played by NTR-dependent transport of MPF and its regulators between nucleus and cytoplasm (Gavet and Pines 2010a, b; Solc et al. 2010).

Cyclin B1 (presumably bound to CDK1) is mainly cytoplasmic in GV-stage mouse oocytes due to its prevailing exportin 1-dependent nuclear export (Marangos and Carroll 2004; Reis et al. 2006). Nuclear import of cyclin B1 is mediated by its direct loading on importin β (Moore et al. 1999; Takizawa et al. 1999). Experiments in permeabilized somatic tissue culture cells indicated that the importin β-dependent accumulation of cyclin B1 does not require Ran (Takizawa et al. 1999), employing a mechanism that remains not well understood. The low levels of cyclin B1 in the GV are further assured by faster APC/Cdh1-dependent degradation of cyclin B1 in the GV than in the oocyte cytoplasm (Holt et al. 2010). Just before M-phase in somatic cells and in oocytes, CDK1/cyclin B1 rapidly translocates to the nucleus. In somatic cells, the activity of CDK1/cyclin B1 drives its own nuclear import, apparently through modification of functional properties of NPC complexes (Gavet and Pines 2010a, b). In maturing starfish oocytes, the passive exclusion limit of NPC channels increased in two distinct steps prior to GVBD, concomitant with release of peripheral nucleoporins from NPCs (Lenart and Ellenberg 2003; Lenart et al. 2003). Phosphorylation of Nups and lamins by mitotic kinase, namely, MPF but also Aurora A and PLK1, is a major driving force of NE breakdown during the entry to M-phase in many cell types examined. However, mechanisms dependent on active reshaping of ER membranes and lipid synthesis are involved, as well as dynein-mediated tearing forces exerted by MTs in some species, such as in maturing starfish oocytes (reviewed in Hetzer 2010).

Mouse genome encodes for three isoforms of Cdc25 phosphatases that are differently required in somatic vs. germ cells. While Cdc25c-deficient mice are viable and fertile (Chen et al. 2001), Cdc25b-deficient mice are viable and sterile because of the inability of oocytes to release from prophase I arrest and resume meiosis (Lincoln et al. 2002). Finally, knockdown and overexpression experiments in mouse oocytes also demonstrated that Cdc25a, which is exclusively nuclear until GVBD, has a nonredundant role during meiosis resumption (Lee et al. 2008; Solc et al. 2008). In contrast, Cdc25b is cytoplasmic until shortly before GVBD when it is imported to the nucleus (Solc et al. 2008). In prophase I-arrested oocytes, PKA-mediated phosphorylation of Ser321 in Cdc25b induces binding of 14-3-3β protein, which then blocks an adjacent NLS in Cdc25b (Pirino et al. 2009; Zhang et al. 2008b), thus allowing exportin 1-dependent nuclear export to prevail (Uchida et al. 2004a, b). Decrease of PKA activity prior to GVBD releases Cdc25b from 14-3-3 binding and Cdc25b is imported to the nucleus (Oh et al. 2010; Pirino et al. 2009; Zhang et al. 2008b) where it can act on CDK1/cyclin B1. By an unknown mechanism, activated CDK1 then promotes nuclear export of Wee2, concomitant with a marked decrease of Wee2 activity. The NES in Wee2 overlaps with its own

catalytic domain, suggesting that exportin binding may contribute to Wee2 inhibition (Oh et al. 2010). Wee2 cooperates with Myt1 kinase that remains both cytoplasmic and nuclear during meiosis resumption (Oh et al. 2010). The nuclear export of Wee2 takes place at the time when NPCs do not allow passive diffusion of 70 kDa dextran (Oh et al. 2010), and the same applies for the nuclear entry of Cdc25b (Petr Šolc and Jan Motlík, unpublished). These observations suggest that the decreased selectivity of NPCs prior to GVBD is not likely to be involved and that nuclear–cytoplasmic translocation of Cdc25b, Wee2, and CDK1/Cyclin B1 all involve energy- and carrier-dependent transport mechanisms.

The critical role of Ran- and NTR-regulated nuclear transport in GVBD is supported by the observation that microinjection of dominant-negative importin β (45–462) blocked meiosis resumption in starfish oocytes (Lenart et al. 2003). This truncated importin β mutant lacks N-terminal sequence required for RanGTP binding and also C-terminal domain, which is required for binding of importin α and blocks nuclear transport mediated by multiple NTRs due to its strong irreversible binding to NPCs (Kutay et al. 1997).

However, although expression of RanT24N or RanQ69L in mouse oocytes inhibited Ran-regulated gradient of importin β cargos (Dumont et al. 2007b), these treatments had no effect on the timing of GVBD (Dumont et al. 2007b; Schuh and Ellenberg 2007). Similarly, the entry to mitosis was not inhibited in HeLa cells expressing RanT24N or RanL43E (GTP hydrolysis-resistant form of Ran, similar to RanQ69L) (Wollman et al. 2005). Finally, experiments with tsBN2 tissue culture cell line carrying temperature-sensitive mutation of RCC1 demonstrated that loss of RCC1 function caused abrupt entry to mitosis in somatic cells, overriding interphase replication checkpoint, rather than preventing the entry to M-phase (Nishijima et al. 2000; Nishimoto et al. 1985). One interpretation of this data could be that RanGTP gradient-regulated nuclear transport is dispensable for entry to mitosis or for meiosis resumption. However, it is significant that the G2–M transition involves a regulated balance of nuclear export and import and that the dominant Ran mutants, or RCC1 inhibition, have different effects on both sides of this balance (Moore 2001). Thus, depletion of RanGTP production induced by inhibition of RCC1 (in RanT24N expressing cells or in tsBN2 cells at 40.5°C) would rapidly inhibit nuclear export and have a less immediate and severe effect on nuclear import, thus causing premature nuclear accumulation of continuously shuttling cyclin B1 and Cdc25B. Consistently, in tsBN2 cells exposed to nonpermissive temperature nuclei accumulate Cdc25c, which is normally cytoplasmic just before precocious entry to mitosis (Seki et al. 1992). On the other hand, RanQ69L expression would be expected to inhibit nuclear import by preventing importins to bind their cargos, possibly causing premature exclusion of Wee2 from the nucleus. Finally, experiments based on RCC1 inhibition are complicated by the possible RanGTP generation in a mechanism involving importin β and RanBP1 acting as GDP-to-GTP exchange factor for Ran (Lonhienne et al. 2009). In summary, the evidence for nuclear transport signal-dependent accumulation of MPF machinery prior to GVBD against concentration gradient argues that RanGTP

gradient-regulated NTRs have an important role in the maintenance of GV arrest and in the resumption of meiosis. However, many important facets of this process are yet to be examined.

12.3.2 Ran Function in the Assembly and Function of Meiotic Spindles In Vivo

Female gametogenesis in animals involves degradation of centrioles prior to the arrest of primary oocytes at the diakinesis stage of the first meiotic prophase (Manandhar et al. 2005). Without centrioles, the replication capacity of the major MTOC in the oocyte is lost, as well as its autonomy as a regulatory center. These changes help to prevent parthenogenetic activation of zygotic development and support normal transition from meiotic to zygotic control of cell divisions (Manandhar et al. 2005). Meiotic spindle assembly in maturing vertebrate oocytes is therefore an acentrosomal process. Instead of centrosomes, GV-stage mouse oocytes are equipped with MTOCs containing many components characteristic to centrosomes, such as pericentrin and γ-tubulin (Calarco-Gillam et al. 1983; Carabatsos et al. 2000; Gueth-Hallonet et al. 1993; Szollosi et al. 1972).

An important insight into the assembly of acentrosomal meiotic spindle was obtained with high-resolution 3D time-lapse imaging of GFP-tubulin in maturing mouse oocytes (Schuh and Ellenberg 2007). This analysis revealed that MI spindle assembly was driven by MT organization in about 80 MTOCs dispersed in the cytoplasm of the maturing oocytes, a much larger number than previously estimated. Before GVBD, the MTOCs were connected by a dense array of MTs, as they were congressing toward GV. As it would be expected if nuclear factors are involved in MT dynamics, GVBD coincided with a massive ~35-fold increase of MTs emanating from the MTOCs. Injection of oocytes with RanT24N protein inhibited this burst of MT growth, demonstrating that RanGTP is indeed the key nuclear factor that propels the rapid onset of MI spindle assembly (Schuh and Ellenberg 2007). Interestingly, imaging of MT + end tips labeled with EB3-GFP showed that no new MTs nucleated directly at the chromatin after GVBD (Schuh and Ellenberg 2007), in contrast with a model proposing that high RanGTP level at the peak of its gradient serves to promote MT nucleation, while lower RanGTP threshold at cell periphery stabilizes MTs (Caudron et al. 2005). On the other hand, this observation was in line with the evidence that at GVBD, mouse oocytes contain very low levels of TPX2 (Brunet et al. 2008), the major RanGTP-regulated MT nucleator.

Direct evidence for RanGTP gradient in maturing mouse oocytes was provided by live imaging of Rango FRET sensor (see Sect. 12.2.2.3) expressed from injected mRNA (Dumont et al. 2007b). As expected, high Rango FRET signal was detected in GV prior to the resumption of meiosis, consistent with the presence of a steep RanGTP gradient across the NE of the GV. Upon GVBD, a broad gradient of FRET signal surrounded the condensed chromosomes and followed their migration

toward cortex throughout meiosis I. The gradient marked the position of both sets of chromosomes produced in MI anaphase and surrounded chromosomes of the MII spindle located at the egg cortex (Dumont et al. 2007b). Consistent with the role of Ran-GTP in promoting MTs, the expression of RanQ69L in the oocytes induced formation of cytoplasmic MT asters that later became incorporated in MI spindle whose size was in average larger than in control oocytes (Dumont et al. 2007b). On the other hand, inhibition of RCC1 by the injection of RanT24N protein (Schuh and Ellenberg 2007) or mRNA encoding for RanT24N (Dumont et al. 2007b) failed to inhibit MI spindle assembly, in contrast to severe disruption of spindle assembly in *X. laevis* egg extracts induced by similar treatment (Kalab et al. 1999, 2002). Instead, the MI spindle assembly was delayed by 2–3 h but eventually reached bipolar stage (Dumont et al. 2007b; Schuh and Ellenberg 2007), although the resulting spindles contained only ~50% MTs compared to controls (Schuh and Ellenberg 2007). Remarkably, even such partially depleted MI spindles were in principle functional, as evidenced by the successful segregation of 20 bivalent chromosomes (Dumont et al. 2007b). Because statistical significance of this finding was not reported (Dumont et al. 2007b) and MI suffers ~8% segregation errors even in oocytes from untreated young mouse females (Duncan et al. 2009), such a conclusion deserves to be reexamined. Moreover, in a physiological context, it is likely that a 2–3 h delay of MI spindle assembly would have an effect on fertilization success.

Reliance of spindle assembly on RCC1-generated RanGTP gradient observed in *X. laevis* egg extracts is more an exception rather than a rule. In *Caenorhabditis elegans* embryos depleted of RCC1 by dsRNA treatment, bipolar spindle assembly was only delayed, although spindles then failed to segregate chromosomes properly. In contrast, bipolar spindle assembly failed in *C. elegans* embryos depleted of RanGAP, RanBP2, importin α (IMA2), or importin β (Askjaer et al. 2002). Bipolar spindles assembled in the absence of active RCC1 in tsBN2 hamster cell line carrying temperature-sensitive RCC1 mutation, although chromosome segregation was severely impaired (Arnaoutov and Dasso 2005). Finally, inhibition of RCC1 in prometaphase HeLa cells by microinjection of RanT24N did not prevent formation of bipolar spindles (Kalab et al. 2006). In many cell types, the assembly of bipolar spindle, therefore, does not absolutely require RCC1-generated RanGTP gradient, although the normal kinetics of the assembly and/or the function of such spindles in chromosome segregation may be impaired.

That the contribution of Ran to vertebrate oocyte maturation is indeed essential and at the same time different between MI and MII was most dramatically displayed by the failure of MII spindle assembly induced by both RanT24N and RanQ69L in mouse oocytes (Dumont et al. 2007b). In most mouse RanT24N or RanQ69L expressing oocytes, MI spindles recovered from problems described above but following an apparently normal MI anaphase, the MII spindle assembly was severely disrupted. In half of RanQ69L-treated and 78% RanT24N-treated oocytes, numerous MT asters appeared in MII cytoplasm and connected to bipolar MII spindles, disrupting their symmetry (Dumont et al. 2007b). The ectopic MT aster formation induced by both Ran mutants in MII mouse was reminiscent of

phenotypes induced by the depletion of DOC1R (Terret et al. 2003) and MISS1 (Lefebvre et al. 2002), both of which are meiotic spindle MT-binding proteins and MAPK kinase substrates. It is interesting to note that MISS1 contains a predicted bipartite NLS sequence, which neighbors its MAPK phosphorylation site (Lefebvre et al. 2002) and a predicted C-terminal NES (Petr Kaláb, unpublished; la Cour et al. 2004). The mechanism of DOC1R or MISS1 function in meiotic spindle assembly is not known. If MISS1 is required to suppress ectopic MT aster formation in MII oocytes, MISS1 sequestration in a complex with either exportin (which would be promoted in RanQ69L-treated oocytes) or importin (consequence of RanT24N treatment) would be expected to produce the observed phenotypes. The MEK1/2-MAPK (Erk 1/2) pathway has an important role in the assembly of meiotic spindles in a variety of maturing vertebrate oocytes (Gordo et al. 2001; Sun et al. 2008; Verlhac et al. 1993, 1996, 2000). Remarkably, inhibition of MEK1/2 activation by U0126 induced formation of multiple small spindle-like structures (Sun et al. 2008) similar to the effect of RanGTP in *X. laevis* egg extracts. The interplay between MAPK and Ran-regulated functions in meiotic spindle assembly deserves further investigation.

In mouse oocytes RCC1 concentration increased about twofold during meiosis, and a much more dramatic RCC1 increase was observed in maturing *X. laevis* oocytes, indicating increasing requirement for RanGTP gradient (Dumont et al. 2007b). Nevertheless, similar to inhibiting RCC1 with RanT24N in mouse oocytes, inhibition of RCC1 synthesis had virtually no effect on MI spindle assembly and first PB extrusion but caused a dramatic disruption of MII spindles, although without inducing ectopic MT asters (Dumont et al. 2007b). This result is consistent with the sensitivity of MII-derived *X. laevis* egg extracts to RCC1 depletion or inhibition and suggests that the requirement for RCC1 function is a conserved feature of meiosis II spindle assembly in vertebrate oocytes. Apart from supporting the much more rapid MII spindle assembly, another conspicuous difference from MI is that the RanGTP gradient-dependent processes in MII egg must provide correct positioning of two genomes after fertilization.

12.3.3 Ran Function in Asymmetric Meiotic Cell Divisions

The asymmetry of oocyte meiotic divisions ensures that only a limited fraction of the fully grown oocyte is lost through ejection of polar bodies, while most remain available for the first rounds of embryonic cell divisions following fertilization. The partitioning of the cellular contents between polar bodies and oocytes is dictated by the MI and MII cell division planes and, therefore, by the position, size, and orientation of the meiotic spindles. In vertebrate oocytes, the MI spindle assembly starts at a slightly asymmetric position, due to the off-center location of GV (reviewed in Azoury et al. 2009). Next, the MI spindle migrates toward the nearest cell cortex, heading along its long axis with one pole leading the way. In *X. laevis* oocytes, the spindle migration clearly requires MTs because MI spindle must be first assembled before a basket-like MT structure draws it toward cortex. In mouse

oocytes, MI chromosomes can reach the oocyte cortex even without MTs (Verlhac et al. 2000), but this mode of translocation is probably not physiologically relevant (Verlhac and Dumont 2008). Instead, imaging of actin dynamics with Utrophin-GFP sensor revealed that spindle MTs are pulling on actin filaments through myosin as the MI spindle reaches the cortex along its long axes (Schuh and Ellenberg 2008). The requirement for a specific form of dynamic actin filaments in this process was shown by the dependence of MI spindle migration on Formin-2, which is an oocyte-specific nucleator of long unbranched actin (Dumont et al. 2007a; Leader and Leder 2000; Leader et al. 2002).

Normal first PB extrusion observed in RanT24N-treated mouse oocytes showed that RanGTP gradient was not required for MI spindle migration (Dumont et al. 2007b; Schuh and Ellenberg 2007). Previously, the asymmetric position of MI and MII spindles in oocytes was shown to precisely coincide with differentiation of surrounding egg cortex into a zone which is devoid of microvilli and cortical granules and is surrounded by a ring of myosin II (Longo and Chen 1985; Maro et al. 1986). Elegant experiments involving microinjection of MII mouse oocytes with chromatinized beads in lieu of chromosomes demonstrated that RanGTP gradient is the required and sufficient signal emanating from meiotic chromosomes which directs the cortical zone specification (Deng et al. 2007). In those experiments, cortical actin patch surrounded by Myosin II ring formed in MII cortex area overlaying injected chromatin beads. In a dose-dependent manner, inhibition of RCC1 by coinjected RanT24N abolished the cortical patch and Myosin II ring formation. These experiments also suggested that RanGTP gradient acts upstream of myosin II activation, possibly locally activating MAPK kinase which was enriched around chromatin beads (Deng et al. 2007).

12.4 Conclusions and Perspectives

The guanine nucleotide cycle on Ran is a central node in an interaction network of many nuclear and cytoplasmic constituents. Above we discussed examples showing that the cellular roles of Ran, whether in meiotic cells or other cell types, cannot be well understood outside of the context of this network and its spatial and temporal dynamics. This network, which we call Ran-regulated NTS, consists of Ran, regulators of GTP/GDP cycle on Ran and of RanGDP nuclear import (RCC1, RanGAP, Mog1, RanBP1, RanBP2, and NTF2), NTRs and NTR adaptors (~30 in humans), and of Nups (~30 in humans) (Fried and Kutay 2003; Hetzer 2010; Pemberton and Paschal 2005; Terry et al. 2007). Given such complexity, the understanding of Ran functions during oocyte maturation would benefit from the application of computational system analysis of NTS, which has already provided important insights into the mitotic role of RanGTP gradient (Bastiaens et al. 2006; Caudron et al. 2005; Kalab and Pralle 2008; Kalab et al. 2006). Because of the practical and ethical challenges of studying vertebrate oocyte maturation, it is significant that the relevant experimental toolbox is becoming more efficient, such

as with the advances in quantitative live cell imaging with fluorescent biosensors and also with the development of specific cell permeable small molecule inhibitors of NTS components, such as importin β (Hintersteiner et al. 2010; Soderholm et al. 2011).

The recombination and random assortment of parental chromosomes into haploid gametes during meiosis is the key mechanism generating genetic diversity within species and the major evolutionary purpose of meiosis. However, many steps of meiosis in vertebrate females suffer surprisingly high rate of errors. In humans, it is estimated that between 5 and 25% of all zygotes in humans are aneuploid, whereby with the exception of 47,XXY and chromosome 2 trisomies, most of the defects are of maternal origin (Hassold and Hunt 2001). Particularly given the rising average age of mothers at childbirth (Martin et al. 2007), it is of a great concern that the rate of aneuploid oocytes dramatically increases in mothers reaching the end of their reproductive age. While the risk of embryonic trisomy is about 2–3% in women in their twenties, it is more than 30% at the age of 40 (Hassold and Hunt 2009). Most of the maternal meiotic defects originate in MI and studies in mouse, and *D. melanogaster* models indicated that loss of sister chromatid cohesion (SCC) in MI is the leading cause of such defects (Hassold and Hunt 2001, 2009). In aging mouse model, the key factor driving the loss of SCC in MI is age-induced depletion of meiotic cohesin subunit Rec8 and that of Sgo1 (shugoshin), which serves to protect cohesin (Chiang et al. 2010; Lister et al. 2010). Remarkably, the expression of another cohesin subunit SMC1β in a mouse knockout model was only required before the dictyate stage of meiosis to prevent the loss of SCC (Revenkova et al. 2010), suggesting that the entire pool of cohesins available to maintain SCC is loaded on chromosomes prenatally and is not replenished by new synthesis throughout the reproductive age. Although it is rather likely that depletion of cohesins is also the major age-related cause of MI defects in human females, it is not expected that a single factor could account for all observed types of meiotic aneuploidy (Hassold and Hunt 2009). Is age-related deterioration of NTS one of the factors contributing to meiotic aneuploidy?

Cell senescence induced by various factors, including advanced age, is associated with significant changes of composition and function of NTS. These include dramatic but selective decrease of many but not all components of NTS (Kim et al. 2010) and decreased efficiency of Ran-regulated nuclear transport (Busch et al. 2009). In addition, low synthesis and turnover of scaffold Nups, such as members of Nup106/170 complex, lead to deterioration of the molecular gating function of NPCs (D'Angelo et al. 2009). However, in contrast to human fibroblast where Ran mRNA decreased during replication-induced senescence (Kim et al. 2010), Ran mRNA levels actually increased in oocytes from aging mouse females, together with concentration of TPX2, and no other significant changes of NTS components were noted (Pan et al. 2008). This suggests that although primary oocytes remain arrested in prophase for a very long time (up to 4–5 decades in humans), the composition of NTS in fully grown oocytes from aging mothers is unlike in senescent cells because much of the nuclear and cytoplasmic content of growing oocyte is synthesized anew following follicle activation. On the other hand, oocytes

and eggs derived from aging mouse females displayed reduced levels of BRCA1 (Pan et al. 2008), which functions as a SAF downstream of Ran (Joukov et al. 2006) and whose function is required for normal assembly and function of meiotic spindles in maturing mouse oocytes (Pan et al. 2008). It is therefore possible that maternal age-related imbalanced expression of some components of NTS (such as Ran) and its downstream targets (TPX2 and BRCA1) indeed contributes to increased meiotic aneuploidy.

A fascinating aspect of the role of Ran in meiotic spindle assembly is that most of the so far identified SAFs regulated by Ran in *X. laevis* egg extracts are known for their either proven or suspected role in cancer (Kalab and Heald 2008). It is also remarkable that RanGTP gradient upregulation caused transformation of NIH3T3 cells apparently through deregulating mRNA cytoplasmic decapping, which is directly linked to global control of protein synthesis (Ly et al. 2010). Whether cancer cells are "addicted" to Ran function (Xia et al. 2008) through the enhanced contribution of Ran to mitotic spindle assembly and/or through the interphase role of RanGTP gradient in mRNA decapping (Ly et al. 2010) is not clear. Studies of Ran function in meiotic and early embryonic cell cycles may provide important insights into such questions.

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Chapter 13
Cell Cycle Control of Germ Cell Differentiation

Cassy M. Spiller and Peter Koopman

Abstract The germ cell lineage is our lifelong reservoir of reproductive stem cells and our mechanism for transmitting genes to future generations. These highly specialised cells are specified early during development and then migrate to the embryonic gonads where sex differentiation occurs. Germ cell sex differentiation is directed by the somatic gonadal environment and is characterised by two distinct cell cycle states that are maintained until after birth. In the mouse, XY germ cells in a testis cease mitotic proliferation and enter G_1/G_0 arrest from 12.5 dpc, while XX germ cells in an ovary enter prophase I of meiosis from 13.5 dpc. This chapter discusses the factors known to control proliferation and survival of germ cells during their journey of specification to sex differentiation during development.

13.1 Introduction

The germ cell lineage is our lifelong reservoir of reproductive stem cells and represent "immortal" cells, linking our genetic information to the past, present and future generations. Specification of the germ cell lineage occurs early during embryo development and is followed by proliferation and migration to the developing gonads during gestation. During this journey, the XX and XY germ cells are identical in morphology and behaviour. Once in the gonad, the surrounding somatic cells direct differentiation of germ cells into oogonia or spermatogonia.

While the ultimate purpose for every germ cell is to undergo the unique cell cycle division of meiosis, this transition occurs at different developmental timepoints for oocytes and spermatogonia. In the ovary, oogonia proceed through the

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early stages of meiosis I during gestation, progress further during ovulation, but only complete the process at fertilisation. In the testis, spermatogonia enter meiosis at puberty, a process that then continues in self-renewing waves for life.

Sex-specific differentiation that occurs during gestation is characterised by two distinct cell cycle states. In the ovary, germ cell entry into meiosis from 13.5 days *postcoitum* (dpc) signals oogonial commitment, while entry into G_1/G_0 arrest from 12.5 dpc signals commitment to the spermatogonia fate. Cell death also plays an important role in germ cell development and differentiation, thought to ensure fidelity of cells destined to contribute to a new organism.

This chapter covers the life and death of germ cells from their specification to sex differentiation, which occurs over 9 days of gestation in the mouse embryo. Because information surrounding the foetal germ cell cycle is only recently emerging, factors expressed by both the somatic and germ cells, which are known to control proliferation and survival, are discussed. Since most of our understanding of this process has arisen from studies of the mouse model, the work described in this chapter pertains to this system. It should be noted that although the sequence of germ cell differentiation events is largely identical across different mammalian species, the timing of these events differs significantly.

13.2 Specification of the Germ Cell Lineage

The germ cell lineage is specified within the dynamic, rapidly dividing environment of the gastrulating embryo. Between 5.5 and 7.5 dpc paracrine signals originating from the extra-embryonic ectoderm (ExE) and visceral endoderm (VE) set aside a small number of cells within the proximal ectoderm to become primordial germ cell (PGC) precursors (Fig. 13.1).

Secretion of bone morphogenetic protein (BMP) signalling members BMP2, BMP4 and BMP8 from the ExE and VE has been detected as early as 5.5 dpc and is known to induce the activation of Smad1/5/8 transcription factors in the PGC precursor population, which number just 6 cells at 6.25 dpc (Lawson et al. 1999; Ying et al. 2000; Ying and Zhao 2001). From about 7.25 dpc, in response to the BMP signalling, the expression of interferon-inducible gene 3 (*Ifitm3*/*fragilis*), PR domain-containing 1 (*Prdm1*/*Blimp1*) and developmental pluripotency-associated 3 (*Dppa3*/*Stella*/*PGC7*) characterises the restricted PGC population that now numbers approximately 40 cells (Ying and Zhao 2001; Saitou et al. 2002; Ohinata et al. 2005; Vincent et al. 2005).

Consistent with an important role in PGC specification, the mouse models of $Bmp4^{-/-}$ and $Bmp8b^{-/-}$ display significantly reduced numbers of PGCs at embryonic stages 8.5–11.5 dpc (Lawson et al. 1999; Ying et al. 2000). Loss of only one *Bmp4* allele sees a 50% reduction in PGC numbers (Lawson et al. 1999), and the heterozygous deletion of both *Bmp4* and *Bmp8b* reveals no additive effect of these factors (Ying et al. 2000). Although these factors were originally thought to affect PGC proliferation, regression analysis of germ cell numbers over time indicated

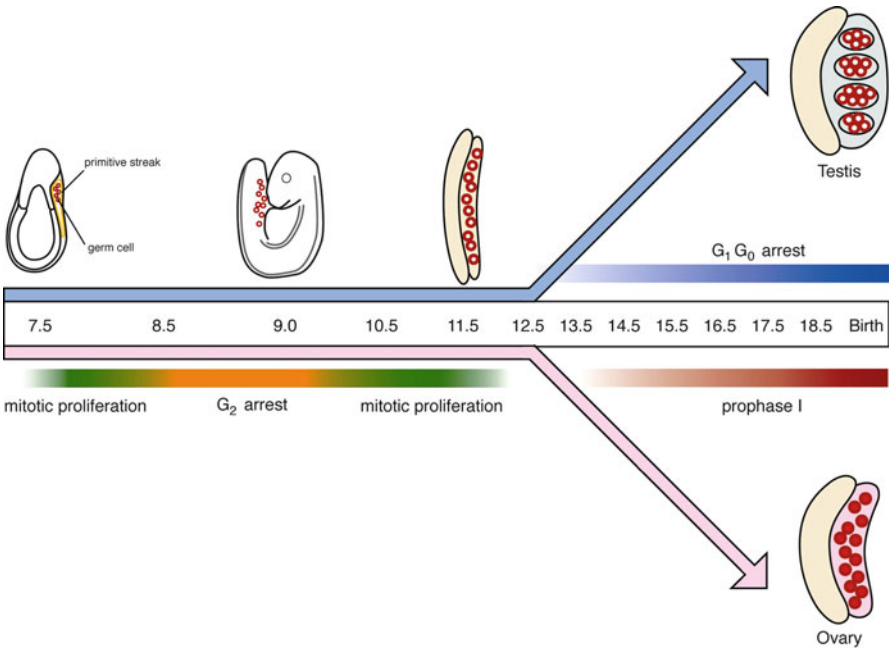

Fig. 13.1 Developmental timeline of germ cell proliferation. Germ cells are specified within the primitive streak by 7.5 dpc and start proliferating. During migration through the hindgut germ cells enter transient G_2 arrest before resuming proliferation and colonising the genital ridge from 10.5 to 11.5 dpc. In a testis, germ cells enter G_1/G_0 arrest from 12.5 dpc, while in an ovary, germ cells enter prophase I of meiosis from 13.5 dpc. These sex-specific cell cycle states are maintained until after birth

that *Bmp4/8b* deletion disrupts correct germ cell specification only, leading to reduced total numbers (Ying et al. 2000).

In keeping with BMP activation of Smad5, deletion of *Smad5* resulted in lower numbers of germ cells specified by 8.5 dpc. Again, the reduced size of the PGC founding population and a proportional increase in PGC numbers during migration indicated that *Smad5* is important for BMP induction of PGC specification rather than proliferation. *Smad5* is known to be expressed in the urogenital system at 12.5 dpc, but because *Smad5* homozygous deletion results in embryonic lethality between 9.5 and 11.5 dpc, a role in later germ cell proliferation has not been examined (Chang and Matzuk 2001). With a null phenotype analogous to that of *Smad5* loss of function, *Smad1*$^{-/-}$ mutants also display a drastically reduced PGC founding population at 8.5 dpc (Tremblay et al. 2001).

The newly specified PGC population is transcriptionally active, expressing multiple pluripotency and PGC-specific markers, while suppressing somatic lineage genes. *Blimp1* is known to be an important suppressor of somatic gene expression (Ohinata et al. 2005; Hayashi et al. 2007). Genes specifically upregulated or maintained within the PGC population include pluripotency marker

octamer-3/4 (*Oct3/4* and *Pou5f1*) (Scholer et al. 1990), which is also expressed in the inner cell mass of the developing embryo but becomes restricted to the germline after 7.5 dpc (Ohbo et al. 2003; Ohmura et al. 2004). Additionally, SRY-box containing gene 2 (*Sox2*) (Ohinata et al. 2005; Yabuta et al. 2006) and nanog homeobox (*Nanog*) (Yamaguchi et al. 2005) are pluripotency markers expressed by the PGCs. Other unique PGC genes include *Nanos3* (Tsuda et al. 2003), *Prdm14* (Yabuta et al. 2006), *Pmrt5*, stage-specific embryonic antigen-1 (*Ssea1*) (Sakurai et al. 1995; Saitou et al. 2002), cell adhesion molecule E-cadherin (Okamura et al. 2003) and tissue non-specific alkaline phosphatase (Tnap) (Chiquoine 1954; Ginsburg et al. 1990).

13.3 Germ Cell Migration and Proliferation

Once the PGCs have been specified and start proliferating, they become motile in response to directional cues from the surrounding somatic cells. The PGCs migrate from their original location within the posterior primitive streak to the endoderm from 7.5 dpc (Anderson et al. 2000). Migration continues anteriorly through the hindgut tissue from 8 to 9.5 dpc, before bilateral migration into the newly forming gonads from 10.5 to 11.5 dpc (at this stage referred to as genital ridges as they have not yet differentiated into testes or ovaries; Fig. 13.1) (Lawson and Hage 1994; Molyneaux et al. 2001) (for a comprehensive review on PGC migration, see Richardson and Lehmann 2010).

13.3.1 Signals for Migration

Because PGCs isolated before migration (8.5 dpc) do not acquire active locomotion in culture, it is thought that a signal is required to trigger PGC migration out of the primitive streak. (Godin et al. 1990; Godin and Wylie 1991). Motile PGCs are distinguishable from the original founding population by cellular morphology, which includes polarisation and extension of cytoplasmic protrusions at the onset of migration (Anderson et al. 2000). Once migration has begun, stromal derived factor 1 (SDF), an attractant expressed by the genital ridges and mesenchyme, directs PGC migration to the developing gonads. In its absence, PGC migration does not occur. The SDF receptor, GPCR chemokine receptor 4 (CXCR4), is expressed by the PGC population during their journey, and deletion of CXCR4 also affects both PGC migration and survival (Ara et al. 2003; Molyneaux et al. 2003).

Although PGC migration is predominantly an active process, initial incorporation into the hindgut is passive (Clark and Eddy 1975). In *Sox17* mutants that display defective hindgut expansion, PGCs remain scattered and fail to colonise the genital ridges (Hara et al. 2009). Germ cell–germ cell interactions are also important, and by 10.5 dpc the PGCs are linked by long processes (Gomperts et al. 1994). E-cadherin expression is important for this association: in its absence, PGCs are lost

to extra-gonadal locations (Bendel-Stenzel et al. 2000; Di Carlo and De Felici 2000). A second adhesion molecule, β1 integrin, is also essential for successful PGC migration and is thought to facilitate germ cell–extracellular matrix interactions (Anderson et al. 1999).

13.3.2 Proliferation During Migration

During the journey from the primitive streak to the genital ridges, the PGCs proliferate rapidly. At around 8–8.5 dpc, the total PGC pool numbers approximately 100, which increases to around 350 by 9–9.5 dpc once they have reached the hindgut epithelium. At the time of genital ridge colonisation (10.5 dpc), approximately 1,000 PGCs are present (Clark and Eddy 1975; Tam and Snow 1981). This proliferation requires a cell cycle time of about 16 h, and DNA content analysis reveals a clear G1 peak, a broad S phase and a less prominent G_2 peak, indicative of rapidly dividing cells (Seki et al. 2007). Between 8.0 and 9.0–9.25 dpc, PGC numbers increase at a slower rate and this coincides with extensive chromatin remodelling as parental imprints are erased (Surani 2001; Li 2002; McLaren 2003; Seki et al. 2007). During this period of reduced proliferation, as little as 15% of the PGC population are positive for BrdU incorporation, and analysis of DNA content revealed that, between 7.25 and 8.75 dpc, over 60% were in G_2 phase of the cell cycle. Furthermore, around 80% of PGCs in this period are positive for cytoplasmic expression of the G_2 phase cyclin, CyclinB1. By 10.5 dpc, DNA content analysis revealed a rapidly cycling PGC population once more, with a cell cycle distribution of G_1 (29.7%), S (34.4%) and G_2 (35.4%), respectively (Seki et al. 2007).

From these analyses, we now know that PGCs begin rapid proliferation following their specification, but undergo transient arrest in G_2 phase of the cell cycle from 7.25 to 8.75 dpc. Once they have reached the genital ridges by 10.5 dpc, PGCs have re-entered the cell cycle and increased in number. While the cause of transient PGC cell cycle arrest remains undefined, a functional association with the coincident epigenetic modifications is a likely explanation. Further studies surrounding the cause and effect of these two phenomena will shed light on this possibility.

13.3.3 Factors that Control PGC Proliferation and Survival During Migration

During migration to the genital ridges, PGC proliferation and survival are dependent on many factors expressed by, or secreted from, both germ and somatic cells (Table 13.1). Most of our knowledge surrounding proliferation control has been gleaned from in vitro culture systems and naturally occurring and artificial genetic mutations. In these analyses, it is often difficult to distinguish between effects on PGC proliferation and altered cell survival. Growth factors that stimulate PGC

Table 13.1 Factors that modulate PGC proliferation and/or survival during development

Germ cell developmental stage	Gene/protein	Information	Effect(s)	Reference(s)
Migrating PGCs	SDF1	Stromal derived growth factor-1, C-X-C-motif ligand 12 (CXCL12); intercrine family chemokine	Migration and survival	Ara et al. (2003)
	CXCR4	C-X-C motif receptor 4; alpha-chemokine receptor for SDF1	Migration and survival	Molyneaux et al. (2003)
	IL4	Interleukin-4; cytokine	Migration and survival	Cooke et al. (1996)
	FGF-2	Fibroblast growth factor 2, basic fibroblast growth factor (bFGF); cytokine	Proliferation	Matsui et al. (1992), Resnick et al. (1992)
	FGF-4	Fibroblast growth factor 4; cytokine	Proliferation	Kawase et al. (1994)
	FGF-8	Fibroblast growth factor 8; cytokine	Proliferation	Kawase et al. (1994)
	TNF-α	Tumour necrosis factor-α; cytokine	Proliferation	Kawase et al. (1994)
	LIF	Leukaemia inhibitory factor; interleukin-6 class cytokine	Survival	Pesce et al. (1993)
	Gp130	Receptor for LIF; IL-6 family cytokine receptor	Survival	Matsui et al. (1991), Hara et al. (1998)
	SCF	Stem cell factor Kit ligand (KITL), steel factor (SF); growth factor	Migration, proliferation and survival	Godin et al. (1990), Dolci et al. (1991), Matsui et al. (1991), Buehr et al. (1993), Pesce et al. (1993)
	C-KIT	Kit oncogene; KITL tyrosine kinase receptor	Migration, proliferation and survival	
	Dead-end	Dead-end homologue 1 (zebrafish) (Dnd), Teratoma mutation (*Ter*); RNA-binding factor	Survival	Noguchi and Noguchi (1985), Cook et al. (2009)
	Fanca	Falconi anaemia, complementation group A; nuclear envelope component	Proliferation	Wong et al. (2003)
	Fancc	Falconi anaemia, complementation group C; nuclear envelope component	Proliferation	Whitney et al. (1996)
	Fancl	Falconi anemia, complementation group L, proliferation of germ cells (*Pog*), germ cell-deficient mutation (*Gcd*); nuclear envelope component	Proliferation	Pellas et al. (1991), Agoulnik et al. (2002)
	Zfx	X-linked zinc finger protein; zinc finger protein	Proliferation and/or survival	Luoh et al. (1997)

Resident gonocytes	*Bmp7*	Bone morphogenic protein 7; cytokine	Proliferation	Ross et al. (2007)
	Hetwigs macrocytic anemia	Unknown	Proliferation and/or survival	Russell et al. (1985)
	Atrichosis	Unknown	Proliferation and/or survival	Handel and Eppig (1979)
	Nanog	Nanog homeobox; transcription factor	Proliferation and survival	Yamaguchi et al. (2009)
Oogonia	RA	Retinoic acid; cytokine	Meiosis entry, survival	Bowles et al. (2006), Koubova et al. (2006)
	Stra8	Stimulated by retinoic acid gene 8	Meiotic competence	Baltus et al. (2006)
	Dazl	Deleted in azoospermia-like; RNA binding protein	Meiotic competence	Ruggiu et al. (1997), Schrans-Stassen et al. (2001), Saunders et al. (2003)
	IGF-1	Insulin growth factor 1; cytokine	Survival	Lyrakou et al. (2002)
Spermatogonia	PDGF	Platelet derived growth factor; cytokine	Proliferation and survival	Li et al. (1997), Hasthorpe et al. (1999), Thuillier et al. (2003)
	E2	17β Estradiol; sex hormone	Proliferation and survival	Thuillier et al. (2003), Vigueras-Villasenor et al. (2006)
	PI3K signaling	Phosphatidylinositol-3'-kinase; intracellular signal transducer enzyme	Proliferation and survival	Morita et al. (1999), Trautmann et al. (2008)
	TGFβ	Transforming growth factor beta; cytokine	Proliferation	Godin and Wylie (1991), Richards et al. (1999b)
	Activin	Activin; cytokine	Proliferation	Matzuk et al. (1995), Richards et al. (1999b)
	Pten	Phosphatase and tensin homolog, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; protein phosphatase	Proliferation	Kimura et al. (2003)
	Pin1	Peptidyl-prolyl isomerase 1; phosphoprotein modifier	Proliferation	Atchison et al. (2003)

proliferation and/or survival include stem cell factor (SCF), leukaemia inhibitory factor (LIF), interleukin-4 (IL4) and basic fibroblast growth factor (bFGF/FGF-2). Spontaneous mutations that affect PGC proliferation include White spotting (*W*), steel (*Sl*), teratoma (*Ter*), Fanconi anaemia complement groups (FANC), Dead-end (*Dnd*) and X-linked zinc-finger protein (*Zfx*). (For a comprehensive review of these mutants, see Olaso and Habert 2000.)

- *Interleukin-4*

A cocktail of cytokines have been shown to have mitogenic effects on PGCs. Specifically, IL-4, which signals through two receptors, IL-4 receptor (IL4-R) and the IL-2R-ϒ subunit (Harada et al. 1990; Kondo et al. 1993), increases 8.5 dpc PGC numbers in culture (Cooke et al. 1996). Both IL-4 and IL-4R subunit transcripts are expressed in 8.5 dpc embryos and within genital ridge tissue at 10.5 and 12.5 dpc. Analysis of BrdU incorporation in the presence of IL-4 in culture showed no significant increase in PGC proliferation suggesting that IL-4 functions as a survival factor for PGCs, preventing apoptosis as opposed to stimulating proliferation (Cooke et al. 1996). Somewhat surprisingly, deletion of either *IL-4* or *IL-2R-γ* did not result in germ cell defects (Kuhn et al. 1991; Kopf et al. 1993; DiSanto et al. 1995), although redundancy within this system may be responsible.

- *Stem cell factor*

The potent cytokine SCF (also called steel factor, c-kit ligand or mast cell growth factor) has been shown to promote PGC survival and proliferation both in vivo and in vitro (reviewed by Pesce et al. 1994; Buehr 1997). Migrating PGCs express the tyrosine kinase receptor c-kit transcript, while the surrounding soma along the migratory path expresses its ligand, SCF (Matsui et al. 1990). Homozygous *W* and *Sl* mutations, which disrupt c-kit and SCF function, respectively, display more than 99% reduction in germ cell numbers prior to genital ridge colonisation (Matsui et al. 1990; Buehr et al. 1993; Loveland and Schlatt 1997). While SCF can be expressed in membrane-bound or soluble forms, the signalling cascades triggered by ligand–receptor binding remain largely uncharacterised.

- *Leukaemia inhibitory factor*

LIF has an established role in promoting mouse PGC survival in in vitro culture systems (Pesce et al. 1993). Binding of LIF causes dimerisation of LIF receptor with another cytokine receptor, gp130, to activate the STAT3 signalling pathway (Yoshida et al. 1994; Matsuda et al. 1999). Despite promoting migratory PGC survival in vitro, deletion of LIF in vivo does not affect fertility (Stewart et al. 1992). Conversely, mice lacking gp130 apparently have reduced numbers of germ cells (Hara et al. 1998). Therefore, a physiological role for LIF controlling PGC migration in the mouse embryo remains speculative.

- *Fibroblast growth factors*

Several members of the FGF family have been implicated in promoting PGC proliferation in vitro. In culture, FGF-2 promotes PGC proliferation (Matsui et al.

1992; Resnick et al. 1992), although in vivo deletion does not affect PGC numbers (Dono et al. 1998; Zhou et al. 1998). Other FGF members (FGF-4, FGF-8 and FGF-17) are expressed by neighbouring somatic cells during PGC migration (Kawase et al. 2004). Of these, FGF-4 and FGF-8 can also increase 8.5 dpc PGC numbers in vitro. Through assessment of BrdU incorporation, these factors were shown to increase proliferation of the PGCs, highlighting FGF-4 and FGF-8 as mitogenic factors for migrating PGCs. The combination of FGF-4 with LIF and another cytokine, tumour necrosis factor-α (TNF-α), could enhance the mitogenic effect above that of FGF-4 alone (Kawase et al. 2004). While PGCs are evidently capable of responding to these cytokines in vitro, evidence is lacking for physiological roles during embryo development.

- *Dead-end*

The genetic mutation named *Ter* was shown to result in defective PGC proliferation from 8.0 dpc onwards. The *Ter* mutation was mapped to the Dead-end (*Dnd*) locus and was found to result in a nonsense mutation in that gene (Youngren et al. 2005). The *Dnd* transcript is expressed in XY germ cells between 12.5 and 15.5 dpc (Cook et al. 2009) and encodes an RNA-binding factor that prevents miRNA degradation of specific mRNA transcripts. In the $Dnd^{Ter/Ter}$ mutants, PGC migration to the genital ridges occurs as normal, but by 12.5 dpc, total numbers are severely reduced such that males are sterile on most genetic backgrounds (Sakurai et al. 1995). When this mutation is present on a 129Sv/J genetic background, in which some germ cells survive, the teratoma incidence rises from 1% in wild types to 94% in homozygous $Dnd^{Ter/Ter}$ mutants (Noguchi and Noguchi 1985). Female 129Sv/J $Dnd^{Ter/Ter}$ mutants are sub-fertile. These results indicate defective cell cycle control of PGCs in the absence of *Dnd*. When the $Dnd^{Ter/Ter}$ mutation was crossed onto a *Bax*-null background (eliminating Bax-mediated apoptosis), teratomas were also formed in mixed genetic backgrounds, due to partial germ cell rescue (Cook et al. 2009). This study indicated that germ cell loss in $Dnd^{Ter/Ter}$ mutants is largely mediated by apoptosis and that *Dnd* is required for correct proliferation of PGCs. The mRNAs to which DND binds to control proliferation have not been identified.

- *Fanconi anaemia complement groups A, C and L*

The human disease Fanconi anaemia (FA) is characterised by hypogonadism and sub-fertility, haematological defects and growth retardation. Cells from FA patients display numerous defects including hypersensitivity to DNA cross-linking agents, ionising radiation and oxygen radicals, prolonged late S and G_2/M phase of the cell cycle, damage-resistant DNA synthesis and accelerated telomere shortening (Leteurtre et al. 1999; Grompe and D'Andrea 2001; D'Andrea and Grompe 2003). It is believed that FA complement groups (FANC) form a nuclear complex necessary for DNA damage responses during S phase (Garcia-Higuera et al. 2001).

Fancc deletion was replicated in a mouse knockout model by Whitney et al. (1996), in which neonates and adults harbouring the *Fancc* deletion displayed reduced gonocyte numbers and sub-fertility. Because this germ cell phenotype resembles the c-kit/SCF pathway disruption, the authors suggest that FA and SCF

pathways may interact (Whitney et al. 1996). In line with this, ex vivo *Fanca*$^{-/-}$ bone marrow cells displayed defective expansion capacity and increased apoptosis in response to SCF, and it is therefore possible that this abnormal response may also occur in the PGC population during their proliferation.

Targeted disruption of *Fanca* also revealed a 50% reduction of PGC numbers at genital ridge colonisation at 11.5 dpc, although PGC specification was normal at 8.5 dpc, as assessed by alkaline phosphatase staining (Wong et al. 2003). Normal numbers of ectopic PGCs at 11.5 dpc indicated normal PGC migration. Assessment of spermatogenesis showed further defects in meiosis in *Fanca* mutants, with mispaired chromosomes and increased apoptosis. Conclusive data showing *Fanca* expression in wild-type PGCs are lacking (Abu-Issa et al. 1999; Nadler and Braun 2000; Wong et al. 2003).

Consistent with an important role in PGC proliferation and survival, mutation of a third FANC member also results in PGC loss. Originally described as the germ cell-deficient (*gcd*) and proliferation of germ cell (*Pog*) mutations, these are now known to occur within the *Fancl* gene. Disruption of *Fancl* in both *gcd*/*gcd* and *Pog*$^{-/-}$ mutations gave rise to a 25–40% reduction of PGCs at 11.5 dpc (Pellas et al. 1991). Further characterisation using alkaline phosphatase staining revealed that PGC loss occurred between 9.5 and 10.25 dpc, during initial genital ridge colonisation. As there was no increase in alkaline phosphatase-positive cells in ectopic locations, the authors concluded that PGC migration occurred normally, but proliferation was disrupted (Agoulnik et al. 2002). It was noted that *Fancl* deletion does not appear to induce apoptosis in the PGCs, as assessed by TUNEL staining (Agoulnik et al. 2002), suggesting that another mechanism must be involved.

- *X-linked zinc-finger protein*

An X-linked zinc-finger protein encoded by *Zfx*, thought to function as a sequence-specific transcriptional activator, is also required for correct male and female PGC development. Indeed, *Zfx*$^{-/-}$ mutants display defects in PGC proliferation and/or survival, as PGC numbers are reduced by 50% at 11.5 dpc (Luoh et al. 1997). Importantly, the Y-linked genes *Zfy1* and *Zfy2*, expressed testis-specifically (Koopman et al. 1989), failed to compensate for the *Zfx* mutation. Similarly, the autosomal *Zfa* gene did not compensate in a functionally redundant manner.

13.4 Genital Ridge Colonisation and Germ Cell Sex Differentiation

Once the PGCs have successfully made their journey to the genital ridges, they are referred to as gonocytes. The change in name from PGC to gonocyte reflects an alteration in potentiality as these cells have a decreased ability to form germ cell-derived tumours (teratomas) when grafted into host testis capsules (Stevens 1966). XY gonocytes, in particular, appear as an intermediary between pluripotent cells

that can form embryonic germ (EG) cell colonies and spermatogonial stem cells (SSC) with potency restricted to differentiation into spermatozoa.

13.4.1 Morphology and Identity of Resident Gonocytes

Morphological and behavioural changes occur at the time of genital ridge colonisation. Gonocytes are easily distinguished from the surrounding somatic cell lineages by their large rounded shape, prominent nucleus containing one or two nucleoli and surrounding ring-like cytosol (Baillie 1964). Gonocytes also lose the ability to spread in culture, which corresponds to a loss of motility of these cells (Donovan et al. 1986). Following motility loss is a loss of cell polarisation and the ability to elongate.

The gene and protein expression profile also changes, with germ cell nuclear antigen (GCNA) expressed by resident gonocytes (Enders and May 1994), while alkaline phosphatase expression is lost after 14.5 dpc (Richards et al. 1999b). Markers expressed by the gonocytes include stage specific embryonic antigens (SSEAs), Thy-1 (CD90), c-kit (CD117) (Ling and Neben 1997), podocalyxin (sialomucin related to CD34) recognised by TRA-1-60 and TRA-1-81 (Andrews et al. 1984; Schopperle and DeWolf 2007), POU5F1 (OCT3/4) (Nichols et al. 1998), NANOG, STELLA, the TGF-related ligand growth and differentiation factor 3 (GDF3) and the RNA-binding protein DAZL (Yen 2004; Ezeh et al. 2005; Kerr et al. 2008).

13.4.2 Mitotic Proliferation of Resident Gonocytes

Gonocytes in the genital ridges continue to proliferate for several days before sex-specific differentiation takes place (discussed in Sect. 13.5). The cell cycle machinery has recently been investigated in these proliferating cells at 11.5 dpc and compared to an embryonic germ (EG), embryonic stem (ES) cells and an embryonic somatic cell (STO) control (Sorrentino et al. 2007). EG cells, derived from PGCs, are similar to ES cells in that they grow permanently in vitro and maintain pluripotentiality (Matsui et al. 1992; Resnick et al. 1992). PGCs, EG and ES cells all express various pluripotency-related genes and proteins and display high alkaline phosphatase activity. Using a focussed cell cycle cDNA array, Sorrentino et al. (2007) investigated differences in cell cycle markers between these populations. In contrast to the situation in ES cells, the authors identified strong expression of G_1 phase genes, specifically *Ccnd3*, *Cdkn1c* (*p57*), *Rb1* and *Tceb1l* in the PGCs (Fig. 13.2). The authors correlate the growth factor-dependent roles of these factors with PGC dependence on growth factors in the gonadal environment (discussed earlier). Ubiquitin control of the PGC mitotic cell cycle was also highlighted with Skil1-Cull-F-box protein complex members *Cullin1*, *2*, *3*, *Cul4a* and *Cul4b*, as well

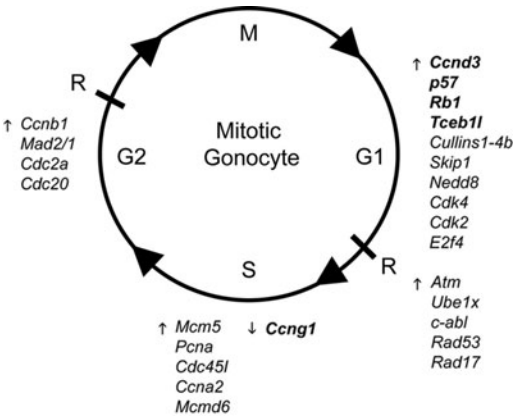

Fig. 13.2 Cell cycle genes expressed by mitotically proliferating XX and XY gonocytes at 11.5 dpc. Mitotic gonocytes are characterised by high expression of many (10) G_1 phase genes. Within S phase, downregulation of *Ccng1* characterises gonocytes. Fewer (4) G_2/M phase genes are highly expressed in the gonocyte population. *Atm* and *Ube1x* genes are the most highly expressed within of the checkpoint/DNA damage genes. Genes in ***bold*** are differentially expressed between gonocytes and EG/ES cells. Based on studies by Sorrentino et al. (2007)

as *Ned8* and *Ube1x*, all expressing high levels. Genes that control S phase and G_2/M transition included many cyclin-dependent kinase (CDK) inhibitors and appeared consistent across the PGC, EG and ES cell populations. There were relatively few DNA damage and checkpoint genes expressed within the PGC population, but of these, *Atm* and *Ube1x* were expressed at high levels. From the PGC cell cycle-gene profile, the authors conclude that mitotic PGCs utilise cytokine-dependent G_1–S phase regulation, utilise inhibitors of CDKs and possess RB1 and ubiquitination activity (Sorrentino et al. 2007). Somewhat predictably, the cell cycle profile of PGCs was most similar to those of the EG and ES cells and dissimilar to that of somatic cell populations. However, this study did suggest that EG- and ES-specific expression of genes such as *c-myc* and *Eras*, which can stimulate cytokine-independent proliferation, may underlie the proliferation and tumorigenic capabilities of EG and ES cells.

13.4.3 Factors that Affect Gonocyte Proliferation

Factors that affect XX and XY resident gonocyte proliferation are now emerging. These include members of the BMP signalling family, two uncharacterised genetic mutations and the pluripotency gene *Nanog*.

- *BMP Signalling*

As mentioned in Sect. 13.2, *Bmp4* and *Bmp8b* are involved in PGC specification during early development. As BMPs are also required for many other aspects of

gastrulation, knockout models are often embryonic lethal, preventing analysis of these factors on late foetal gonoctye development. *Bmp5*, *Bmp6*, *Bmp7*, *Bmp8a* and *Bmp8b* are all expressed in XX and XY genital ridges at 12.5 dpc (Ross et al. 2007). *Bmp7*, also expressed as early as 10.5 dpc in XX and XY gonads, was shown to affect gonocyte proliferation during the narrow window of 10.5–11.5 dpc. Loss of *Bmp7* caused decreased gonocyte numbers in both sexes, although with greater severity in XY gonads, suggesting a longer period of dependence for XY gonocytes on this signalling pathway (Ross et al. 2007). An established mechanism for BMP family redundancy exists (Oxburgh et al. 2005), and therefore in order to dissect the roles these factors play in gonocyte proliferation, combinations of gonocyte-specific *Bmp* deletions will be required.

- *Hertwig's macrocytic anaemia*

Hertwig's macrocytic anaemia (*An*) mutants display reduced numbers of gonocytes by 12.0 dpc and reduced proliferation and rapid degeneration of gonocytes over the 12.0–15.0 dpc period such that testes are virtually sterile at this time (Russell et al. 1985). Similarly in the ovary, gonocyte numbers were reduced, and a high level of degradation was detected in 12.0 and 13.0 dpc timepoints (Russell et al. 1985). Adult female ovaries were sub-fertile (Taney et al. 1991), suggesting some male-specific effects of this mutation. While the exact genetic mutation for this disease has not been identified, Pearson's syndrome, which also displays macrocytic anaemia, is a result of mutations to the gene encoding ALA synthase 2 (Fitzsimons and May 1996). A role for this synthase in gonocyte development has not been investigated to date.

- *Atrichosis*

The atrichosis (*at*) mutation results in complete male and female adult sterility, suggesting an embryonic cause (Handel and Eppig 1979). In these mutants, Sertoli cell development appears normal, suggesting a germ cell-specific defect. In 2004, Rockett et al. performed a DNA array comparing the *at*/*at* model of male infertility with wild-type adult testes. From this array, 47 genes involved in cell receptor activity, signal transduction, transcription regulation, cell cycle regulation and apoptosis were differentially expressed (Rockett et al. 2004). Interestingly, the transcript of angiotensin I-converting enzyme (Ace, CD143), a zinc-metalloprotease, was significantly downregulated in the *at*/*at* testes. This enzyme is known to be expressed in foetal gonocytes but absent from spermatogonia and spermatocytes and has also been identified in human intratubular germ cell neoplasm, seminomas and other testicular tumour phenotypes (Pauls et al. 1999). A role for this enzyme during normal foetal gonocyte development is indicated but remains uncharacterised.

- *Nanog*

As mentioned previously, Nanog is an important pluripotency marker for PGCs from the time of specification. PGC-specific shRNA knockdown of *Nanog* led to reduced PGC numbers from 10.5 dpc onwards, mediated by an increase in apoptosis

(Yamaguchi et al. 2009). Single-cell microarray analysis of *Nanog* knockdown PGCs at 10.5 dpc revealed a large transcriptional network (>700 genes) disrupted by reduced *Nanog* expression. These data indicate that Nanog plays a functional role in PGC proliferation and survival and is responsible for maintaining a large PGC-specific molecular network (Yamaguchi et al. 2009). The exact mechanism by which Nanog promotes survival/proliferation has not yet been investigated thoroughly.

13.4.4 Somatic Cell Signals for Germ Cell Sex

Having made the journey to the developing testes or ovaries, gonocytes await direction from the surrounding somatic cells before differentiating into either spermatogonia or oogonia, respectively. Two distinct cell cycle states characterise the differentiation pathways available to the gonocytes. In an ovary, XX gonocytes enter into the first phase of meiosis I from 13.5 dpc onwards and become committed to the oogonial fate (Johnston 1981; McMahon et al. 1981; Monk and McLaren 1981). In a testis, XY gonocytes cease proliferation and arrest in G_1/G_0 of the cell cycle around 12.5 dpc and subsequently develop as spermatogonia (also called prospermatogonia) (Hilscher et al. 1974).

Gonocytes are completely dependent on their surrounding somatic cell environment for both survival and sex differentiation cues. XX gonocytes that find themselves in a testis (by naturally occurring, or artificially manipulated sex reversal) enter G_1/G_0 arrest and commit to spermatogenesis. Conversely, XY gonocytes in an ovary are directed into meiosis and become oogonia (Ford et al. 1975; Palmer and Burgoyne 1991).

The search for the identity of sex-specific cues originating from the soma has continued for decades, but only recently has significant progress been made with regard to initiation of meiosis. Recently, retinoic acid (RA) was shown to stimulate meiosis entry of XX foetal gonocytes (Bowles et al. 2006; Koubova et al. 2006) (to be discussed in Sect. 13.5), but the factor(s) that initiate G_1/G_0 arrest in XY gonocytes in the testis remain unknown (to be discussed in Sect. 13.6). Factors known to modulate gonocyte cell cycle states as well as the cell cycle machinery utilised by XX and XY gonocytes are discussed in the following sections.

13.5 Oogonia: Induction and Control of Meiotic Entry

For decades, the question of whether meiosis entry of XX gonocytes is directed by somatic cell cues or is an intrinsic "default" pathway for these cells has perplexed researchers. Only recently have we discovered the meiosis-inducing effects of RA in foetal oogonia. The cell cycle machinery engaged at this time is also under current examination and is discussed below.

13.5.1 Induction of Meiosis

As mentioned previously, the first sign that female germ cell sex determination has occurred is entry of XX gonocytes into the first phase of meiosis. This occurs from 12.5 dpc onwards and by 2 days *postnatum* (dpn), all oogonia are arrested in diplotene of prophase I (discussed in detail in Sect. 13.5.2; Borum 1961; Speed 1982; Pepling and Spradling 2001).

- *Retinoic acid*

RA, a small molecule that signals through multiple nuclear RA receptors (RARs), alters target gene expression to modulate many different growth and differentiation processes during embryogenesis (Chambon 1996; Mark et al. 2006). RA signalling was first implicated in gonadal sex determination when the transcript for a specific RA-degrading enzyme, cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1), was found to be expressed in both male and female gonads at 10.5 dpc, but testis-specifically by 13.5 dpc (Menke and Page 2002). Furthermore, the gene that encodes an RA synthesising enzyme, retinaldehyde dehydrogenase 1A2 (RALDH2), was detected in the mesonephroi of both testes and ovaries at 10.5 dpc (Bowles et al. 2006). These data suggested that both testes and ovaries produce RA, but RA is degraded in the testis during the period of sex differentiation. Several in vitro culture systems and in vivo transgenic models have now confirmed this theory. Firstly, addition of exogenous RA induced XY gonocytes to erroneously enter meiosis in culture (Bowles et al. 2006; Koubova et al. 2006). Conversely, inhibition of RA function using an RAR panantagonist prevented meiosis induction in XX gonocytes in culture, determined by reduced expression of multiple meiosis-related genes (stimulated by retinoic acid gene 8, *Stra8*; synaptonemal complex protein 3, *Sycp3*; and dosage suppressor of mck1 homologue, *Dmc1*) (Bowles et al. 2006; Koubova et al. 2006).

In vivo evidence for RA's role in meiosis induction was observed in analysis of the *Cyp26b1*$^{-/-}$ animal model in which RA is not degraded in the foetal testis. In this model, gonocytes in XY gonads were observed entering meiosis (some through pachytene) in parallel with XX gonocytes, although by 16.5 dpc this RA exposure also led to increased apoptosis and complete gonocyte loss by neonatal life (Bowles et al. 2006; McLean et al. 2007). The intermediaries between the RA signal and the meiotic cell cycle machinery now await investigation.

- *Stra8*

While RA triggers meiosis in gonocytes of the foetal ovary, no other somatic cell types that are subject to RA signalling respond in the same manner. Therefore, it is assumed that gonocytes contain an inherent competence that allows RA signalling to be interpreted in this way. *Stra8* is a key factor that is an obvious target for RA signalling and entirely necessary for meiosis. Analysis of *Stra8*$^{-/-}$ ovaries and testes revealed only pre-meiotic oogonia and spermatogonia, respectively (Baltus et al. 2006). Intriguingly, the DNA content of the oogonia analysed revealed only

2N, not 4N DNA content. This indicated that in addition to defective meiosis, the final pre-meiotic S phase was also defective in the absence of *Stra8*. While *Stra8* is expressed at low levels during pre-meiotic proliferation, it is required for expression of both *Spo11* and *Dmc1,* which induce and repair meiotic DNA double strand breaks (DSBs), respectively (Bannister and Schimenti 2004), during the pachytene stage of prophase I.

- *Dazl*

The gene for the RNA-binding protein deleted in azoospermia-like (*Dazl*) is expressed gonocyte-specifically between 10.5 and 11.5 dpc (Seligman and Page 1998), and deletion results in sterility (Ruggiu et al. 1997; Schrans-Stassen et al. 2001; Saunders et al. 2003). Analysis of oogonia revealed reduced expression of *Stra8* and absent chromatin condensation (the first stage of meiotic prophase, leptotene), indicating that Dazl presumably functions upstream of Stra8. Furthermore, transcript and protein expression of SCYP3, a component of the synaptonemal complex, and REC8, a meiosis-specific cohesion factor (Scherthan 2003), was reduced or absent in $Dazl^{-/-}$ gonocytes (Lin et al. 2008).

- *Other growth factors*

While it is now established that RA induces XX germ cells to enter prophase I of meiosis from 12.5 dpc onwards in the foetal ovary, earlier in vitro studies also highlighted other growth factors that can enhance this unique cell cycle state. Using a culture system, 13.5 and 14.5 dpc ovaries were cultured until the 17.5 dpc-equivalent timepoint in the presence or absence of a growth factor cocktail containing SCF, IGF-1 and LIF. In this culture system, increased survival of oogonia was evident, although numbers remained lower than the in vivo control. A significant increase in the proportion of oogonia reaching pachytene at both timepoints was observed, indicating that these factors can promote meiotic progression in addition to survival (Lyrakou et al. 2002). Although pachytene was confirmed using anti-SCP3 antibody in the culture system, these cells were not able to complete recombination, indicated by the absence of MLH1-stained foci (Lyrakou et al. 2002). Importantly, it must be noted that this study did not establish whether the growth factors promoted survival of pre-meiotic oogonia or preleptotene oogonia or both.

13.5.2 Cell Cycle Control of Meiosis

Following RA's induction of *Stra8* to trigger initiation of meiosis, the XX gonocytes begin a slow progression through the first meiotic prophase I. Between 13.5 dpc and 2 dpn, XX gonocytes complete DNA synthesis of the final pre-meiotic S phase, continue past G_2 and enter prophase I to arrest in diplotene (Borum 1961; Speed 1982). Within prophase I, sister chromatid pairs condense (leptotene) and align at the synaptonemal complex with homologous chromatid pairs (zygotene).

Here, crossover occurs between chromatids, involving many DSBs and subsequent DNA repair (pachytene), giving rise to genetic diversity. By 17.5 dpc approximately 70% of oogonia have reached pachytene (Speed 1982; Lyrakou et al. 2002) and it is at this point that extensive apoptosis occurs within the gonocyte pool (Borum 1961). The synaptonemal complex then degrades to allow slight separation of sister chromatid pairs and some transcription of DNA (diplotene). At 2 dpn, prior to metaphase, XX gonocytes arrest for a prolonged period until hormonal stimulation and ovulation, post-puberty (Borum 1961; Speed 1982).

Recently, Miles et al. (2010) proposed that core regulatory machinery controlling G_2/M arrest of the mitotic cell cycle may be involved in XX gonocyte prophase I arrest also. (It is important to note that gonocyte arrest in diplotene is not analogous to mitotic G_2/M arrest, as XX gonocytes progress past this stage.) Expression of various G_2/M cyclins and DNA repair regulators were subsequently profiled within the XX gonocyte population from 12.5 to 15.5 dpc. Of the B-type cyclins, which regulate progression past G_2/M, *Ccnb1* was suppressed, and *Ccnb3* upregulated in meiotic gonocytes (Fig. 13.3) (Miles et al. 2010). This is an interesting finding as CyclinB1 associates with CDC25a to promote G_2/M progression, while CyclinB3/CDC2a association suppresses G_2/M progression. These data indicate that CyclinB3 may also play an important function in the early stages of prophase I, although evidence of protein expression has not been shown. The DNA damage response regulators ATM and ATR were also specifically detected in their phosphorylated (active) forms during meiosis. This finding is consistent with the many DSBs and repairs that occur during this time. Several ATM and ATR downstream targets were also analysed. Checkpoint kinases CHK1 and

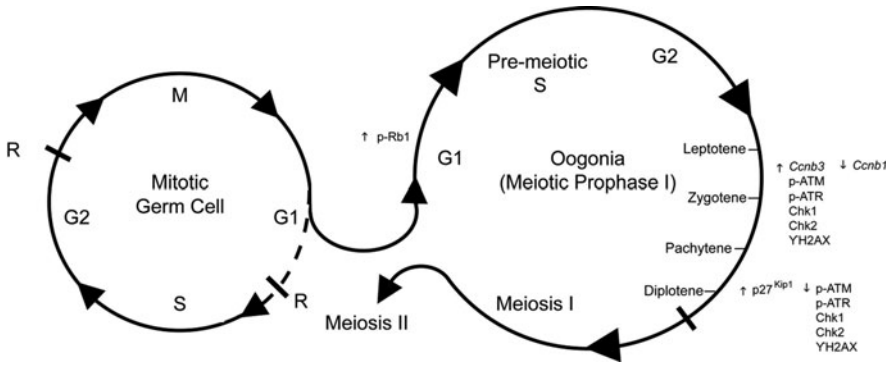

Fig. 13.3 Genes and proteins expressed by oogonia as they pass from leptotene (13.5 dpc) through diplotene (2 dpn) in prophase I of meiosis. As oogonia progress into pre-meiotic S phase, Rb1 remains phosphorylated (inactivated). Entry into leptotene of prophase I is characterised by expression of *Ccnb3* and suppression of *Ccnb1*. Additionally, phosphorylation (activation) of DNA damage/checkpoint proteins ATM, ATR, CHK1, CHK2 and γH2AX is detected. As oogonia arrest in diplotene of prophase I, phosphorylated ATM, ATR, CHK1, CHK2 and γH2AX are detected at low levels, but $p27^{Kip1}$ is strongly expressed. Based on studies by Miles et al. (2010), Spiller et al. (2009) and Western et al. (2008)

CHK2, as well as DNA breakpoint marker γ-histone H2AX (γH2AX), were all detected at the protein level, and in the case of CHK1 and CHK2, were also active (phosphorylated) in meiotic XX gonocytes at 15.5 dpc. The important role of ATM in this process is highlighted in the $Atm^{-/-}$ mutant, which displays infertility due to complete oogonia apoptosis at 17.5 dpc (Barlow et al. 1998).

Analysis of the DNA repair proteins at 2 dpn, when diplotene arrest is finally achieved for the majority of XX gonocytes, revealed only low levels of phosphorylated CHK1 and γH2AX (Miles et al. 2010). These findings were largely expected, as it follows that key DNA repair factors would be upregulated during the time of meiotic recombination (15.5 dpc, pachytene) and downregulated as the gonocytes complete the DNA repair and prepare for metaphase I (2 dpn, diplotene). Interestingly, analysis of CDK inhibitor $p27^{Kip1}$ revealed strong nuclear staining in diplotene-arrested XX gonocytes at 2 dpn (Miles et al. 2010). Because $p27^{Kip1}$ is a well-established regulator of G_1/S phase progression, these data suggest that it may play a novel role in diplotene arrest in XX gonocytes. Loss of $p27^{Kip1}$ leads to premature activation of follicles at puberty, indicating an important role in maintaining diplotene arrest during neonatal stages through adulthood (Rajareddy et al. 2007).

13.6 Spermatogonia: Initiation and Control of G_1/G_0 Arrest

In the mouse testis, gonocytes undergo two main phases of proliferation: one at around 12.5–14.5 dpc and another from 1 to 4 dpn, separated by several days of G_1/G_0 arrest. It should be noted that while G_1/G_0 arrest lasts for several days in the rodent model, in the human model this period extends for several months, starting at approximately 6 months gestation and continuing to 2–3 months postnatally (for further reading, see Culty 2009).

13.6.1 Factors that Modulate Spermatogonia Proliferation

As mentioned previously, the factor(s) that induce G_1/G_0 arrest in XY foetal gonocytes have not yet been identified. Factors that have been implicated in mouse and rat, foetal and/or neonatal gonocyte proliferation include platelet-derived growth factor (PDGF) signalling, oestrogen signalling, LIF, TGFβ signalling and RA; and many of these appear to utilise the PI3K pathway. Additionally, the genetic mutations of both PTEN and PIN1 have also implicated these factors in spermatogonia proliferation control and are discussed below. While data are discussed pertaining to both foetal and neonatal gonocyte proliferation control, it is important to note that in some cases responses differ between the two developmental stages (Boulogne et al. 1999a; Livera et al. 2000), suggesting phenotypic differences in these two gonocyte populations.

- *PDGFs*

PDGFs (consisting of A, B, C and D isoforms) are secreted ligands that facilitate paracrine signalling to control various cellular processes including proliferation, apoptosis and chemotaxis. (For a review, see Yu et al. 2003.) PDGFs function as homo- or heterodimers that bind to PDGF-α or PDGF-β (tyrosine kinase) receptors (PDGFRs). Importantly, PDGFs are considered "competency" factors for progression past G_1/G_0 arrest, as cell cycle progression requires other factors such as insulin and insulin-like growth factor 1 (Stiles 1983; Olashaw and Pledger 1987). Concomitant activation of PDGFR with epidermal growth factor receptor (EGFR) resulted in growth arrest and/or apoptosis in several in vitro studies (Eastman 1995; Kottke et al. 1999). PDGF signalling has, therefore, been suggested to both positively and negatively control proliferation, dependent on the cellular context.

In the rat testis, PDGFR-α, and PDGFR-β, as well as a cytosolic truncated variant of PDGFR-β (VI-PDGFR-β), are detected in gonocytes within the first 5 days of birth (Thuillier et al. 2003; Wang and Culty 2007; Basciani et al. 2008). Rat Sertoli cells at this time also produce PDGF-BB. In mice, PDGFR-β is detected in gonocytes from 18.0 dpc to 5 dpn and the Sertoli cells produce both PDGF-B and PDGF-D (Basciani et al. 2008). Using in vitro systems in the absence of supporting somatic cells, addition of PDGF-B to purified neonatal mouse and rat gonocytes stimulated proliferation (Li et al. 1997; Hasthorpe et al. 1999; Thuillier et al. 2003). In vivo analysis of the PDGF-$B^{-/-}$ and PDGFR-$\beta^{-/-}$ mutants that are embryonic lethal by 19.5 dpc were reported to show no defects in gonocyte morphology at either 13.5 dpc (Brennan et al. 2003) or 18.0 dpc (Basciani et al. 2008), although gonocyte proliferation and total number were not assessed in these mutants. Using the drug Imatinib that inhibits tyrosine kinase functions of the PDGFRs, Basciani et al. (2008) reported that neonatal gonocyte proliferation was reduced at 2 and 3 dpn in the absence of active (phosphorylated) PDGFR-β and V1-PDGFR-β. Together, these studies indicate that XY gonocytes are capable of responding to PDGF signalling that is present within the foetal and neonatal testes, but a role in modulating G_1/G_0 arrest in 13.5–18.5 dpc gonocytes (either positively or negatively) has not been investigated.

- *Oestrogen*

Oestrogen signalling has many functions throughout development, and foetal and neonatal exposure to oestrogenic compounds has been linked to cryptorchidism and testicular cancer (Skakkebaek et al. 2001). Neonatal XY gonocytes express oestrogen receptor β (ERβ), and the surrounding Sertoli cells produce 17β-estradiol (E2) (van Pelt et al. 1999; Saunders et al. 2000). Additionally, ER signalling-associated proteins Hsp90, p23 and Cyp40 are expressed by the neonatal gonocyte population (Wang et al. 2004). Stimulation of gonocyte proliferation by E2 was evident in an in vivo study by Vigueras-Villasenor et al. (2006). The authors used daily subcutaneous injection of E2 from 1 dpn and saw a twofold increase in gonocyte numbers at 3 dpn, although continuation of this treatment to 8 and 16 dpn increased gonocyte apoptosis (Vigueras-Villasenor et al. 2006). Studies by Thuillier et al. (2003), using

an in vitro 3 dpn gonocyte-only culture system, revealed increased proliferation of neonatal gonocytes and also increased PDGFR expression in these cells. The positive effect of E2 on proliferation was seen to require ER, as ER antagonists ablated the effect (Li et al. 1997; Thuillier et al. 2003). Simultaneous addition of both E2 and PDGF had no additive effect on proliferation, and the authors suggest that cross-talk exists between these two signalling pathways. In contrast to these findings, analysis of the mutant mouse model harbouring inactivated ERβ revealed a 50% increase in gonocyte numbers at 2 dpn, due to reduced neonatal gonocyte apoptosis (Delbes et al. 2004). In line with a protective role of ER signalling, E2 has been shown to act as a survival factor in human adult gonocytes (Pentikainen et al. 2000).

The most recent study to investigate ER signalling in the gonad identified the expression of ERα in 11.5 and 12.5 dpc gonocytes (La Sala et al. 2010). Using an in vitro culture system, the authors were able to show a positive effect of E2 on PGC proliferation that required the activation (phosphorylation) of AKT, ERK and SRC via the PI3K pathway. Together, these studies confirm the ability of gonocytes to express receptors and respond to ER signalling, but they highlight opposing effects of E2 treatment on gonocyte proliferation between developmental stages. In order to assess a role for ER signalling in gonocyte G_1/G_0 arrest, the foetal gonocyte phenotype in the inactive-ERβ mouse model needs to be investigated. Additionally, the interplay between PDGF and E2, whether synergistic or autonomous, should be explored.

- *LIF and SCF*

LIF, when added to 1 dpn rat testis cultures, increased the number of proliferative gonocytes by 3 dpn, although this effect was not observed if added from 3 dpn onwards. This finding may represent a role for LIF in making quiescent gonocytes (1 dpn) capable of responding to proliferative signals earlier than normal, while at 3 dpn, the gonocytes have already responded to these signal(s), making the exogenous LIF redundant (discussed in Sect. 13.3; De Miguel et al. 1996). SCF is a vital factor for the survival and maintenance of PGCs in culture (Dolci et al. 1991) and acts synergistically with LIF to stimulate proliferation of PGCs (Matsui et al. 1991). The entry of goncytes into mitotic arrest may be correlated with a decrease in the expression of c-kit (Manova and Bachvarova 1991) or an uncoupling of the receptor and intracellular signalling (Rottapel et al. 1991). Addition of soluble or membrane-bound SCF in rat gonocyte culture did not positively affect proliferation in 1 dpn gonocyte culture (Hasthorpe et al. 1999).

- *RA*

XY gonocytes in vitro have also been shown to respond to RA. In rat 14.5 dpc testes, RA induces a small increase in proliferation and a large increase in gonocyte apoptosis (Livera et al. 2000). At 16.5 dpc, RA decreased the number of proliferating gonocytes in the rat testes, co-cultured with Sertoli cells (Boulogne et al. 1999a). In mouse foetal testis organ culture, RA increased gonocyte proliferation at 11.5, 12.5 and 13.5 dpc and prevented gonocyte entry into G_1/G_0 arrest at 14.5 dpc. This effect was dependent

on PI3K signalling activity, and in each of these cases, a large increase in apoptosis was observed (Trautmann et al. 2008). Rather than offering an answer to the induction of G_1/G_0 arrest, we now view these studies in the light of RA being the trigger for meiosis entry, an ovary-specific event at this time.

- *PI3K signalling*

As discussed, the PI3K pathway appears necessary for PGC survival promoted by SCF, LIF (Morita et al. 1999) and RA (Trautmann et al. 2008) during PGC migration and colonisation of the genital ridges. ER-stimulated proliferation during this time also requires an active PI3K pathway, and therefore, re-entry of mitosis in neonatal XY spermatogonia may also utilise ER signalling. Conversely, it appears that PI3K signalling needs to be suppressed in XY gonocytes (by PTEN, discussed below) in order for them to enter G_1/G_0 arrest (La Sala et al. 2010). Therefore, we might hypothesise that control of the PI3K pathway determines the sex differentiation of XY gonocytes, although the in vivo activation of this pathway has not been directly studied.

- *TGFβ signalling*

The transforming growth factor β (TGFβ) family of morphogens (Tgfβ-1, Tgfβ-2 and Tgfβ-3) are widely expressed throughout embryogenesis (Pelton et al. 1991) and generally function to enhance extracellular matrix formation and inhibit progression past G_1 of the cell cycle. Their mechanism of action is believed to involve the RB1 protein and CDKs (reviewed by Lawrence 1996). All three TGFβ isoforms have been identified within various somatic populations of the mouse and rat foetal gonad (Teerds and Dorrington 1993; Gautier et al. 1994; Olaso et al. 1997; Cupp et al. 1999). The TGFβ transmembrane serine threonine kinase receptors TβR-I (ALK5) and TβR-II have also been identified within 11.5 dpc mouse (Richards et al. 1999a) and 13.5 dpc rat (Olaso et al. 1998) gonocytes, suggesting a direct effect of these morphogens on the gonocyte population. TGFβ-1 was the first growth factor shown in vitro to suppress migratory (8.5 dpc) (Godin and Wylie 1991) and post-migratory (11.5 dpc) (Richards et al. 1999a) mouse PGC proliferation. Using rat 13.5 dpc gonad explant cultures, addition of both TGFβ-1 and TGFβ-2 decreased gonocyte number through increased apoptosis, detected by TUNEL staining. This effect of the TGFβs was limited to mitotically proliferating gonocytes, as apoptosis of G_1/G_0-arrested cells (at 17.5 dpc) remained unaffected (Olaso et al. 1998). In support of TGFβ-1's role in gonocyte proliferation control, analysis of 15.5 dpc testes lacking *TGFβ-1* revealed an increase in gonocyte number (Memon et al. 2008). By birth, *TGFβ-1*-deleted testes displayed a reduced number of gonocytes, possibly highlighting different functions for TGFβs at different developmental stages. Unlike TGFβ-1, deletion of *TGFβ-2* and *TGFβ-3* resulted in no obvious defects in gonocyte development (Memon et al. 2008). A direct role for TGFβ-1 in inducing G_1/G_0 arrest via interaction with RB1 and CDKs in foetal gonocytes has not been thoroughly investigated to date.

Activin is another member of the TGFβ family that displays a more restricted expression pattern during development. The genital ridge, in particular, expresses

the mRNA for both activin subunits βA and βB at 12.5 dpc (Feijen et al. 1994). Activin receptors ActR-IIB and ActR-IB were also identified within 11.5 dpc PGCs (Richards et al. 1999a), and addition of activin directly suppressed PGC proliferation in vitro. Surprisingly, no alterations to foetal gonocyte numbers were noted with the deletion of both activin subunits (Matzuk et al. 1995), suggesting that other ligands (perhaps TGFβ or Nodal) may signal through the activin receptors in its absence.

- *Pten*

The lipid phosphatase encoded by PTEN is an established tumour suppressor that regulates proliferation, differentiation, apoptosis and migration in various cell types (reviewed by Di Cristofano and Pandolfi 2000). Gonocytes lacking *Pten* display increased proliferation and an inability to enter G_1/G_0 arrest (Kimura et al. 2003). Loss of *Pten* also confers gonocytes an increased ability to form tumours (in vivo) and EG cells (in vitro). In other wild-type systems, PTEN induces G_1/G_0 arrest via suppression of the PI3K pathway (Li and Sun 1998; Ramaswamy et al. 1999; Sun et al. 1999; Chung et al. 2006). Specific PTEN interactions within XY gonocytes need to be sought in order to shed light on the mechanisms of cell cycle control of these special cells.

- *Pin1*

The proline isomerase PIN1 has also been implicated in XY gonocyte G_1/G_0 arrest (Atchison et al. 2003). In other systems, PIN1 interaction with cell cycle factors, such as CDC25c, TAU and the transcription factor CF2, leads to their phosphorylation and subsequent degradation (Lu et al. 1996; Yaffe et al. 1997). These roles for PIN1 have implicated it in cell cycle progression, DNA replication and checkpoint control (Lu et al. 1996; Winkler et al. 2000). *Pin1*-null mutants displayed a reduced number of PGCs due to prolonged cell cycle rate and an inability to enter G_1/G_0 arrest (Atchison et al. 2003). The precise role of *Pin1* within gonocyte cell cycle control awaits clarification and should have implications for spermatogonia G_1/G_0 arrest and migratory PGC proliferation.

13.6.2 Control of G_1/G_0 Arrest

While the search continues for the growth factor or other signalling molecule(s) that trigger G_1/G_0 arrest of spermatogonia, we are now beginning to identify the specific cell cycle machinery utilised to achieve this state. Two recent studies have investigated the cell cycle machinery used by mouse spermatogonia during G_1/G_0 arrest. Using a cDNA array, Spiller et al. (2009) profiled various cell cycle genes in proliferating (12.5 dpc) versus arrested (14.5 dpc) spermatogonia. Western et al. (2008) investigated transcript and protein expression of selected cell cycle regulators within 12.5–15.5 dpc spermatogonia (Fig. 13.4).

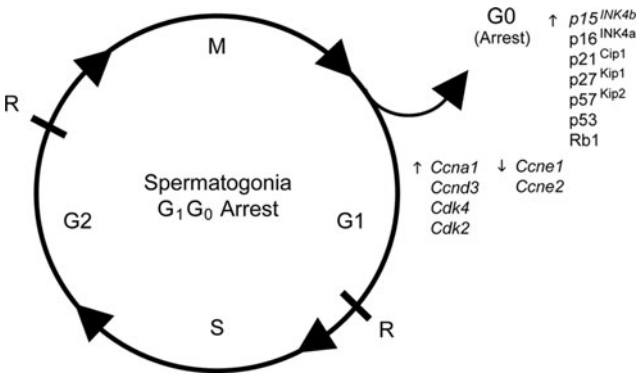

Fig. 13.4 Genes and proteins expressed by spermatogonia during G_1/G_0 arrest from 14.5 dpc onwards. G1 phase genes *Ccna1*, *Ccnd3*, *Cdk4* and *Cdk2* characterise G_1/G_0-arrested spermatogonia. Expression of cell cycle arrest genes and proteins $p15^{INK4b}$, $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ and p53 are detected within arrested spermatogonia. Hypophosphorylation (activation) of Rb1 is also detected at this time and necessary for correct arrest. Based on studies by Spiller et al. (2009, 2010) and Western et al. (2008)

Expression of the G_1 phase cyclins *Ccna1* (Spiller et al. 2009), *Ccnd3*, *Ccne1* and *Ccne2* (Western et al. 2008) was detected in arrested spermatogonia. *Ccna1* is considered the germ cell-specific cyclin, restricted to spermatogonia in the adult testis (Sweeney et al. 1996) and deletion results in infertility (Barbacid et al. 2005). *Ckd2* and *Cdk4* were also expressed in arrested spermatogonia (Spiller et al. 2009). Of these, *Cdk2* is required for entry into meiosis, and deletion results in male and female infertility (Barbacid et al. 2005).

CDK inhibitors were also investigated for a likely role in spermatogonia G_1/G_0 arrest. The INK and Cip/Kip family of inhibitors function to reduce the activity of the CyclinD–Cdk4/6 and CyclinE–Cdk2 complexes. Of these, the INK members $p15^{INK4b}$ and $p16^{INK4a}$ and the Cip/Kip members $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ were all expressed at the transcript level in arrested spermatogonia (Western et al. 2008; Spiller et al. 2009). At the protein level, expression of $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ increased in the gonocytes as they entered G_1/G_0 arrest, but displayed variable nuclear staining patterns that differed from that of surrounding somatic cells (Beumer et al. 1999; Western et al. 2008). These data indicate possible germ cell-specific roles for these common cell cycle regulators in modulating spermatogonia arrest. Importantly, evidence for in vivo function of these CDK inhibitors is also emerging. Following deletion of *Rb1*, $p27^{Kip1}$ and $p15^{INK4b}$ were upregulated in the gonocyte population to presumably initiate arrest in its absence (Spiller et al. 2010) (discussed below). Analysis of foetal testes in the absence of $p27^{Kip1}$ revealed normal gonocyte arrest and total cell number, indicating that it does not play a crucial role in the maintenance of this cell cycle state (Beumer et al. 1999). Analysis of $p27^{Kip1-/-}$ adult testes revealed increased testis size and elevated sperm production due to overproliferation of Sertoli and germ cells. (Holsberger et al. 2005). Although spermatogenic defects were noted in the absence of $p27^{Kip1}$, this

phenotype is believed to be a secondary effect of the primary Sertoli cell defect. A similar overproliferation defect was noted in the absence of $p21^{Cip1}$, although with less severity. Combined loss of $p27^{Kip1}$ and $p21^{Cip1}$ revealed an additive effect on increased sperm production and testis weight, although $p27^{Kip1}$ is believed to be the primary regulator (Holsberger et al. 2005). Combined deletion of $p27^{Kip1}$ with another INK4 member, $p18^{INK4c}$, also displayed additive effects for increased testis weight (50%), above that for deletion of $p18^{INK4c}$ alone (30%) (Franklin et al. 2000). To date expression of $p18^{INK4c}$ has not been investigated in foetal spermatogonia arrest. These data indicate that simultaneous germ cell-specific deletion of multiple CDK inhibitors will be required in order to determine an in vivo role for these factors in gonocyte G_1/G_0 arrest.

The well-known cell cycle regulator RB1 has also recently been implicated with spermatogonia G_1/G_0 arrest in vivo. RB1 plays an established role in inducing quiescence in somatic cells through its binding of E2F transcription factors, thereby preventing cell cycle progression (Dyson 1998; Stevens and La Thangue 2003). Expression of hypophosphorylated (active) Rb1 was detected specifically within XY gonocytes from 13.5 dpc, with expression declining by 15.5 dpc (Western et al. 2008; Spiller et al. 2010). Given the appropriate expression of RB1 within spermatogonia, it was hypothesised to play a role in initiating their arrest. The model of complete *Rb1* deletion ($Rb1^{-/-}$) was subsequently analysed by Spiller et al. (2010) for defects in spermatogonia arrest. At 14.5 dpc in the absence of *Rb1*, a significant increase in gonocyte proliferation was detected concomitant with an increase in gonocyte number. Surprisingly, by 16.5 dpc the proliferation defect was resolved, although total gonocyte numbers remained elevated. Increases in expression of both $p27^{Kip1}$ and $p15^{INK4b}$ were detected at this time, suggesting that these CDK inhibitors can affect spermatogonia arrest in the absence of functional RB1 (Spiller et al. 2010). The mild phenotypes of this study and those of the CDK inhibitors described above highlight the robust mechanism for ensuring correct G_1/G_0 arrest within the gonocyte population.

13.6.3 Spermatogonia and Radiosensitivity

It is interesting to note that the period of G_1/G_0 arrest corresponds precisely to a period of hyperradiosensitivity for gonocytes (Moreno et al. 2001). In rats, gonocyte arrest occurs from 18 dpc to 4 dpn (Hughes 1962) and irradiation during this time leads to complete loss of spermatogonia by 12 dpn due to delayed apoptosis beginning from 6 dpn. This apoptosis is Bax-independent, as Bax is not elevated following irradiation (Beumer et al. 2000). In an attempt to correlate this unusual radiosensitivity with metabolic behaviour, Moreno et al. (2001) investigated the expression of cell cycle regulators p53, $p21^{CIP1}$ and RB1 in gonocytes following irradiation.

Cytoplasmic expression of p53 was detected in wild-type rat gonocytes at 18 dpc during arrest and was lost as gonocytes began proliferating postnatally by 6 dpn (Moreno et al. 2001). As p53 requires nuclear localisation for controlling cell cycle

arrest in response to DNA damage (Shaulsky et al. 1991), its cytoplasmic expression in foetal gonocytes indicates a possible alternate role. In irradiated testes, p53 expression persisted at 6 dpn, suggesting involvement in irradiation-induced gonocyte apoptosis.

Expression of cytoplasmic $p21^{CIP1}$ was detected in proliferating rat gonocytes prior to arrest (16–17 dpc), in arrested gonocytes (21 dpc) and in gonocytes proliferating postnatally (6 and 12 dpn). Although $p21^{CIP1}$ staining was not altered in irradiated testes, it was seen to co-localise with the mitochondria at all stages. As for p53, the authors suggest that $p21^{CIP1}$ may be playing an alternate role in the cytoplasm, possibly performing an anti-apoptosis function.

RB1 was detected at low levels in arrested rat gonocytes at 21 dpn and high levels were detected in proliferating gonocytes at 6 dpn. In irradiated testes, RB1 was undetectable at 6 dpn, indicating that RB1 is normally involved in postnatal gonocyte proliferation. The phosphorylation status of RB1 at either timepoint or treatment was not determined.

Moreno and colleagues (2001) also suggest that rather than a block in G_0 phase spermatogonia progress extremely slowly through G_1 phase. Indeed, spermatogonia continue to increase in size during the period of arrest, and treatment with either cyclohexamide or actinomycin D (to prevent new protein synthesis) results in decreased $p21^{CIP1}$ expression over time (Moreno et al. 2001). These results indicate that, despite the DNA methylation that has occurred by this time, some metabolic processes are maintained in arrested gonocytes. As irradiation is known to be most effective in cycling cells, and in particular those undergoing G_1–S phase transition (Sinclair and Morton 1966), it seems possible that continued (albeit slow) G_1 phase progression of gonocytes might explain their increased sensitivity to radiation. It is interesting to note that XX oogonia, which also undergo a prolonged period of arrest in the dyctyate stage of meiosis, do not display increased sensitivity to radiation during this time.

13.7 Death in the Germline

During development, both XX and XY gonocytes undergo several rounds of regulated cell death. In the foetal ovary, oogonia undergo two rounds of cell death; the first is observed around 13.5 dpc (Coucouvanis et al. 1993) and the second from 15.0 dpc through perinatal life (Borum 1961; Coucouvanis et al. 1993; Ratts et al. 1995). In the human ovary, this second round of attrition accounts for the loss of over two thirds of the original pool of oogonia (reviewed by Tilly 1996). In the foetal testis, spermatogonia death occurs between 13.5 and 17.5 dpc and again around birth (Boulogne et al. 1999b). In the testis, there is an estimated ten times more gonocyte apoptosis at this time than in the ovary (Kasai et al. 2003). The second postnatal round of death in mice appears due to necrosis, rather than apoptosis (Wang et al. 1998), although apoptosis has been observed in the rat and hamster (Miething 1992; Boulogne et al. 1999b).

While germ cell death is an event that takes place in both the ovary and the testis, for male germ cells this attrition seems insignificant when considering the potentially limitless production of spermatozoa following puberty. For females, the remaining population of germ cells that survive this selection represent the total fertility potential for one lifetime of reproduction (Peters 1969), although this concept is being challenged and remains to be proved definitively. Given the consequences of oogonia death for fertility, research to date has predominantly focused on the factors involved in this process.

13.7.1 The Cause of Death

The reason for the extensive gonocyte apoptosis remains a mystery. The predominant hypothesis suggests insufficient growth factor presence during this time, perhaps as a mechanism to correlate total germ cell number to the appropriate levels of supporting somatic cells that produce these factors (Huckins and Oakberg 1978). A second hypothesis for germ cell death involves chromosomal abnormalities or defective mitochondrial genomes (Krakauer and Mira 1999), as a mechanism to ensure germline fidelity and avoid cancer and developmental defects in offspring. This may be acutely necessary for germ cells, due to their unusually high rate of proliferation (Clermont 1962). A third hypothesis proposes altruistic cell death in the ovary for the benefit of neighbouring oogonia. In this scenario, dying oocytes behave as nurse cells, ensuring that oocytes destined to become primordial follicles are fully functional by transferring mitochondria and endoplasmic reticulum via intracellular bridges (Pepling and Spradling 2001).

Early studies have attributed germ cell attrition almost exclusively to apoptosis rather than other forms of cell death (Coucouvanis et al. 1993; Pesce et al. 1993; Pesce and De Felici 1994). A more recent study by De Felici et al. (2008) revealed that cultured oogonia from 15.5 dpc possess the ability to activate other death mechanisms including caspase-independent and autophagic cell death. In this study, alternate cell death accounted for 10–20% of the total TUNEL-positive population (De Felici et al. 2008), indicating that apoptosis accounts for the majority of gonocyte attrition.

The work described below focuses on mechanisms of apoptosis in the germline. The induction and execution of apoptosis involves four stages: the stimulus, signal, regulator and effector (Morita et al. 2001b). There is no clear evidence for an extrinsic pathway or death receptor that triggers apoptosis in the foetal gonocyte pool, although the intrinsic pathway of apoptosis is triggered by both extracellular and intracellular stresses including DNA damage, growth factor insufficiencies and genotoxicants.

13.7.2 Stimuli and Signals for Death

Potent cytokines SCF and LIF have been shown to promote germ cell survival in vitro (reviewed in Pesce et al. 1994; Buehr 1997), and studies by Morita et al.

(1999) discovered that this anti-apoptosis response was dependent on the PI3K signalling pathway. The relevance of the in vitro studies surrounding these growth factors is underscored by the in vivo analysis of mice lacking either SCF or c-kit, exhibiting fertility defects (discussed in Sect. 13.3.2; Mintz and Russell 1957; Motro et al. 1991; Besmer et al. 1993).

Several other growth factors have been implicated in promoting PGC survival or proliferation in vitro and include interleukin-4 (Morita and Tilly 1999), basic fibroblast growth factor (FGF) (Morita and Tilly 1999) and TNF-α (Kawase et al. 1994). IGF-1 alone and in combination with SCF and LIF has also been identified as an anti-apoptotic signal, also requiring PI3K signalling to mediate its effects in vitro (Morita et al. 1999). Conversely, pro-apoptotic effects of TGFβ have been observed in both male and female germ cells in culture and can partially inhibit the positive effects of SCF, LIF and IGF-1 (Morita et al. 1999). TGFb1 and TGFb2 in organ cultures of rat 13.5 dpc testes were also shown to increase gonocyte apoptosis (Olaso et al. 1998).

Ceramide, a lipid second messenger belonging to the sphingosine molecules, is now known to function as an intracellular effector molecule in apoptosis triggered by the stress response (TNF-α, Fas or ionising radiation) (Spiegel et al. 1998). Although a specific apoptotic pathway for ceramide action has not been identified, null mutants for the sphingomyelinase gene displayed a greater number of ovarian follicles during neonatal life (Horinouchi et al. 1995; Morita et al. 2000).

In addition to growth factor signalling, chromosomal abnormalities and/or defects in DNA replication represent critical stimuli for induction of cell death pathways. Indeed, there are numerous mutations in meiotic regulatory genes that result in oocyte death or defects in prophase I progression. These include *Atm* (Barlow et al. 1998), *Msh4* (Kneitz et al. 2000), *Msh5* (de Vries et al. 1999; Edelmann et al. 1999) and *Dmc1* (Yoshida et al. 1998).

13.7.3 Regulators and Effectors of Death

While relatively little is known about the extracellular signals controlling gonocyte apoptosis, the *Bcl-2* (B-cell lymphoma/leukaemia-2 protein) gene family's role in transducing these intracellular signals has been extensively characterised (Reed 1994). This family of proteins consists of both pro- and anti-apoptotic members, and the balance of expression of these members is thought to regulate apoptosis in many cell types (Vaux et al. 1988; Cory and Adams 2002). Loss of *Bcl-2* function in mouse ovaries sees a reduction in the total primordial follicle pool, indicative of perinatal germ cell loss (Ratts et al. 1995). Transfection of PGCs with *Bcl-2* reduced degeneration in culture (Watanabe et al. 1997). Similarly, deletion of *Bcl-x* (BCL-2-like 1) leads to complete, and partial, male and female sterility, respectively, by 15.5 dpc (Rucker et al. 2000; Riedlinger et al. 2002). Conversely, deletion of *Bax* results in increased follicle and spermatocyte numbers (Knudson et al. 1995) and rescued the effects of *Bcl-2* deletion (Rucker et al. 2000). These in vivo models

convincingly highlight the anti- and pro-apoptotic effects of *Bcl-2* and *Bax*, respectively, in perinatal germ cell death.

Bax expression has been detected in oogonia from 15.5 dpc until birth (Felici et al. 1999). This study suggests that only cells that downregulated Bax expression before birth survived to arrest in the diplotene stage of prophase of meiosis I. Further investigation of these mouse models revealed that the pro- and anti-apoptotic effects of the Bcl-2 family differ depending on the developmental stage of oogenesis. Specifically, analysis of the *Bax*-null mouse revealed that PGC and pre-meiotic oogonia rely on Bax-mediated apoptosis, but apoptosis after prophase I arrest is Bax-independent (Alton and Taketo 2007). In culture studies by Felici et al. (1999), oocytes cultured past prophase I arrest upregulated the SCF receptor, suggesting that growth factor levels are important past this point, while Bax-mediated control functions within the intrinsic mitotic and meiotic checkpoints of DNA recombination prior to prophase I arrest (Felici et al. 1999).

Significantly, a p53-enhancer region was identified within the *Bax* promoter (Miyashita and Reed 1995) and a p53-repression element within the *Bcl-2* promoter (Miyashita et al. 1994a). Further in vitro studies established a role for p53 in mediating the expression of *Bax* and *Bcl-2* within the supporting ganulosa cells of the ovary (Miyashita et al. 1994b; Keren-Tal et al. 1995; Tilly et al. 1995). Upstream of p53, ATM protein, which functions within both mitotic and meiotic checkpoints, is important for suppressing *p53*, $p21^{CIP1}$ and *Bax* levels in the testis (Barlow et al. 1998). Interestingly, both *p53* and *Atm* were specifically upregulated in 14.5 dpc XY gonocytes (discussed in Sect. 13.6.2; Spiller et al. 2009). p53 expression has also been detected specifically within XY human gonocytes at 20 weeks of gestation (Tosun et al. 2007), highlighting a conserved role for this master apoptosis regulator. Furthermore, loss of p53 phosphorylation achieved by deletion of C2H2-type zinc-finger transcription factor *Zfp148* results in reduced PGC numbers and increased apoptosis (Takeuchi et al. 2003). A downstream modulator of p53-mediated apoptosis, *Mdm2* (Mostert et al. 2000), was also recently identified within 14.5 dpc XY gonocytes (Spiller et al. 2009), and mutations to *Mdm2* have been implicated in human germ cell tumours (Velasco et al. 2004).

Caspases are downstream targets of the Bcl-2 family (Chinnaiyan et al. 1996). The caspase family of (aspartic acid-specific cysteine) proteases function to cleave proteins required for homeostatic maintenance, and these actions mark the final stages of cell death execution (Martin and Green 1995; Fraser and Evan 1996). *Caspase-2*-deficient mice display an increase in follicle number during neonatal stages (Bergeron et al. 1998), although the dependence of this phenotype on granulosa cell defects has not been determined. In vitro culture of foetal (13.5 dpc) ovaries with caspase inhibitors (zVAD-fmk and zDEVD-fmk) showed reduced apoptosis specifically in the germ cell population (Morita et al. 1999).

PI3K signalling has been implicated in the anti-apoptosis functions of multiple cytokines, although relatively little is known about downstream signalling of this pathway in germ cells. The PI3K downstream effector p70S6 (Tsai et al. 1993) is not believed to be required for the SCF-PI3K-mediated survival in germ cells (Morita et al. 1999). A second PI3K effector, AKT, thought to couple the cytokine

signalling with the Bcl-2 family control of the cell death checkpoint (Franke and Cantley 1997), has not been examined in germ cell survival to date.

A model of cytokine insufficiency, the caspase-11-null mutant, which displays a severe reduction in oocyte number, could be rescued by caspase-2 gene inactivation, highlighting this caspase in oocyte death (Morita et al. 2001a). Caspase-2 deficiency could not overcome meiotic recombination defects in the *Atm* mutants (Barlow et al. 1996; Xu et al. 1996) that also undergo apoptosis, indicating multiple methods of cell death (Morita et al. 2001a).

The checkpoint kinase, CHK1, involved in DNA damage-induced G_2/M phase arrest and apoptosis has been identified within foetal gonocytes (discussed in Sect. 13.5.2). *Chk1* expression was decreased in testes of $p53^{-/-}$ mice (Cheung and Li 2001) and in 12.5 and 14.5 dpc foetal gonocytes. The CHK1 adaptor protein, Claspin, was detected in gonocytes at 14.5 dpc only. Given the coincident timing of *Chk1* expression with the regulated gonocyte apoptosis in the foetal testes and ovaries, the authors suggest that CHK1 and claspin may regulate this process (Hasthorpe et al. 2007). A second checkpoint kinase, *Chk2*, is also expressed abundantly in foetal gonocytes (Bartkova et al. 2001) and is also regulated by p53 (Chin and Li 2003).

13.8 Summary

Germ cell development from initial specification to sex differentiation can be characterised by multiple cell cycle states, finely controlled by ever-changing somatic cell environments. Rapid proliferation during early gastrulation sees the founding population of 6 PGCs increase to over 26,000 in just a few days. A multitude of growth factors and chemokines are required to mediate not only proliferation but also migration of PGCs from the primitive streak to the developing genital ridges. Here, sex differentiation occurs as gonocytes choose one of two differentiation pathways, oogenesis or spermatogenesis. Oogonia respond to RA in the developing ovary and proceed into prophase I of meiosis. Conversely, in response to as yet unidentified factor(s), spermatogonia arrest in G_1/G_0 of the mitotic cell cycle.

Many factors expressed or secreted by the soma and germ cells are now known to affect gonocyte proliferation at various stages of their development. Clues regarding the identity of these factors have been gleaned from decades of in vitro culture experiments as well as naturally occurring and artificial genetic mutations. In the later instance, the consequence of mis-regulated germ cell proliferation is often apparent as infertility or cancer. In an effort to better understand the cause and effect of these debilitating disorders, focus has now turned to identifying the specific cell cycle machinery utilised by germ cells throughout development. While this research is only now emerging, comprehensive knowledge of this process will allow for better diagnostic and therapeutic applications for the disorders of infertility and germ cell cancer.

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Chapter 14
Protein Kinases and Protein Phosphatases that Regulate Meiotic Maturation in Mouse Oocytes

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Abstract Oocytes arrest at prophase of meiosis I (MI) and in vivo do not resume meiosis until they receive ovulatory cues. Meiotic resumption entails two rounds of chromosome segregation without an intervening round of DNA replication and an arrest at metaphase of meiosis II (MII); fertilization triggers exit from MII and entry into interphase. During meiotic resumption, there is a burst of protein phosphorylation and dephosphorylation that dramatically changes during the course of oocyte meiotic maturation. Many of these phosphorylation and dephosphorylation events are key to regulating meiotic cell cycle arrest and/or progression, chromosome dynamics, and meiotic spindle assembly and disassembly. This review, which is subdivided into sections based upon meiotic cell cycle stages, focuses on the major protein kinases and phosphatases that have defined requirements during meiosis in mouse oocytes and, when possible, connects these regulatory pathways.

14.1 Introduction

Meiosis is the cellular process by which haploid gametes, which are essential for sexual reproduction, are generated from diploid cells. In marked contrast to male meiosis that takes place continuously after reaching puberty, meiosis in females is initiated during fetal development and has two points of arrest. It is generally accepted that females are born with a finite number of oocytes, many of which undergo apoptosis while in prophase of MI (Beaumont and Mandl 1961; Byskov 1974; Coucouvanis et al. 1993). After premeiotic DNA replication, meiotic cells undergo homologous recombination where genetic information is exchanged between maternal and paternal chromosomes (homologs). Following recombination, primary oocytes arrest in prophase of MI with homologs held together by points of chromosome crossover called chiasmata. Oocytes are surrounded by somatic

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granulosa cells that participate in bidirectional communication with the oocyte to support oocyte growth, granulosa cell proliferation, and development of one another (Eppig 2001). During the prolonged prophase arrest (commonly referred to as the GV for germinal vesicle or nucleus) oocytes grow in size and granulosa cells increase in number. During this growth period oocytes accumulate transcripts necessary to support meiotic maturation, fertilization, and preimplantation development (Moore 1975; Sorensen and Wassarman 1976; Moore and Lintern-Moore 1978). About mid-way through the growth phase transcription decreases such that fully grown oocytes are essentially transcriptionally quiescent. Fully-grown, meiotically competent oocytes contained within large antral follicles, resume meiosis upon receiving ovulatory signals, and complete MI and, as a result of an asymmetric cell division, extrude a significantly smaller and nonfunctional cell called a polar body (Fig. 14.1). Oocytes, now referred to as eggs, arrest at metaphase of MII and do not complete MII until fertilized by sperm. A second polar body is extruded upon meiotic exit at fertilization. Thus, a single haploid egg is produced from one starting diploid germ cell. The process of resuming meiosis with arrest at MII is called oocyte maturation or meiotic maturation. This review will focus on regulatory mechanisms that protein kinases and phosphatases use to control meiotic maturation in females,

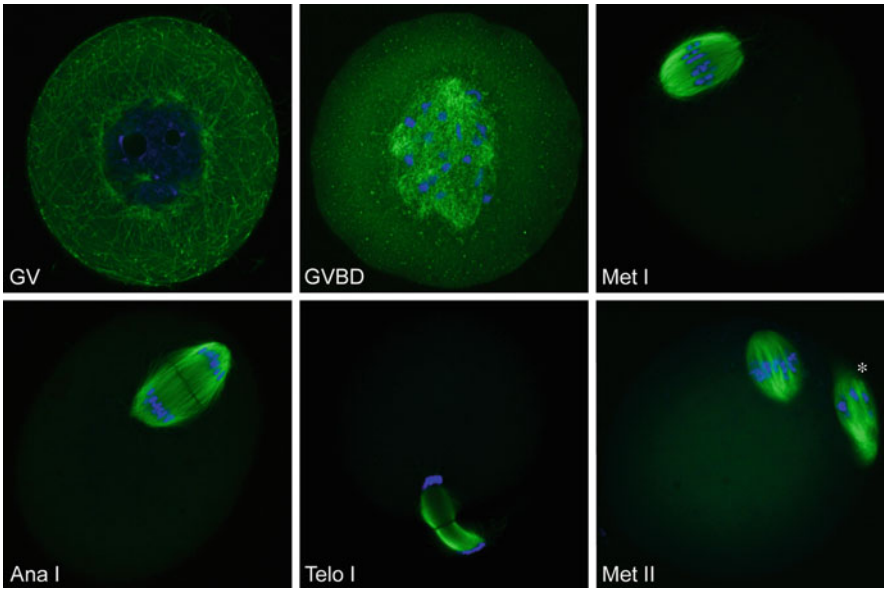

Fig. 14.1 Confocal images of oocytes in different stages of meiotic maturation. Fully-grown oocytes were collected from sexually mature female mice, matured in vitro, and fixed at various time points. Spindles (*green*) were detected with an anti-β-tubulin antibody (Sigma) and DNA (*blue*) was visualized with propidium iodide. The *asterisk* indicates the first polar body. *GV* germinal vesicle, *GVBD* germinal vesicle breakdown, *Met I* metaphase I, *Ana I* anaphase I, *Telo I* telophase I, *Met II* metaphase II

how their functions are similar and/or different from their roles in mitosis, and how alterations in their signaling capacity affect egg formation and fertility in females.

During meiotic maturation, there are dynamic waves of protein phosphorylation and dephosphorylation (Schultz et al. 1983; Bornslaeger et al. 1986, 1988). Historically, work in a myriad of biological systems, including meiotic maturation, has focused on protein kinases, resulting in a paucity of information about the roles of protein phosphatases. Generally, protein kinases display high sequence specificity for their cognate substrates whereas many protein phosphatases have relaxed specificity. This relaxed specificity permits a situation where cells encode many more protein kinases than protein phosphatases. For example, the mouse genome encodes 561 protein kinases compared to only 162 protein phosphatases (Caenepeel et al. 2004). Although protein phosphatases have a relaxed sequence specificity, recent data indicate the importance of sequence-specific docking sites on substrates that govern interactions between protein phosphatases and their substrates (Roy and Cyert 2009). Furthermore, control of transcription timing, cell-type expression, and subcellular localization of protein kinases, protein phosphatases, and their substrates add additional layers of specificity that are not mimicked by in vitro experiments (Virshup and Shenolikar 2009). Because reversible protein phosphorylation is a common mechanism used in biology, it is not surprising that oocytes employ these molecules to regulate meiotic maturation. These regulatory pathways are essential to ensure the development of high quality eggs required for fertilization and subsequent embryonic development.

14.2 Prophase I Meiotic Arrest

In female mammals, oocytes enter the first meiotic prophase during fetal development and remain arrested in prophase I until they resume meiosis in response to a preovulatory gonadotropin surge. High concentrations of cAMP are required to maintain meiotic arrest, and meiotic resumption, which resembles a mitotic G2-to-M transition (G2/M), is triggered by a maturation-associated decrease in oocyte cAMP (Eppig et al. 1983; Schultz et al. 1983; Eppig and Downs 1984). Oocytes will also resume meiosis when released from preovulatory antral follicles because they cannot maintain inhibitory concentrations of oocyte cAMP, which requires the companion somatic cells (Eppig and Telfer 1993; Conti et al. 2002). Addition of membrane-permeable cAMP analogs or inhibitors of the oocyte-specific phosphodiesterase PDE3A to the culture medium prevent spontaneous maturation in vitro (Dekel and Beers 1978; Vivarelli et al. 1983; Eppig et al. 1993; Conti et al. 2002).

14.2.1 Cyclin Dependent Kinase 1: Part I

The best-characterized signal transduction pathways in oocyte biology are likely the mechanism by which cyclin dependent kinase 1 (CDK1) (CDC2A in mouse

ontology) is regulated via phosphorylation and dephosphorylation to modulate meiotic arrest (Fig. 14.2). CDK1 is the catalytic subunit of Maturation Promoting Factor (MPF) initially isolated in frog and starfish egg extracts (Gerhart et al. 1984; Gautier et al. 1989; Labbe et al. 1989b). CDK1 must be bound to cyclin subunits to be active (Labbe et al. 1989a). Availability of cyclin is regulated at the translational level through a cytoplasmic polyadenylation element (Tay et al. 2000) and by ubiquitin-mediated proteolysis (Reis et al. 2007). Increased CDK1 activity triggers the meiotic G2/M transition (Masui and Clarke 1979); therefore, meiotic prophase I arrest requires low CDK1 activity. Several signaling pathways that are discussed below converge (Fig. 14.2) to ensure that CDK1 activity is low.

Although much is known about how CDK1 is regulated in mouse oocytes, additional levels of regulation exist in other organisms that have not been explored in mouse. For example, CDK1 is activated by phosphorylation on T160 in its activation loop by a CDK-activating kinase (CAK). In budding yeast, CAK (Cak1) is required for meiosis through activating CDK-dependent and -independent pathways (Schaber et al. 2002; Schindler et al. 2003). Mammals contain a monomeric CAK homolog called CCRK (Fig. 14.2), yet it is not known whether CDK1 is constitutively activated in oocytes or whether CCRK is required for meiotic maturation. Furthermore, CDKs can be bound to inhibitory subunits (e.g., p27) that inhibit their activity (Morgan 1995). This level of regulation has not yet been explored in oocytes.

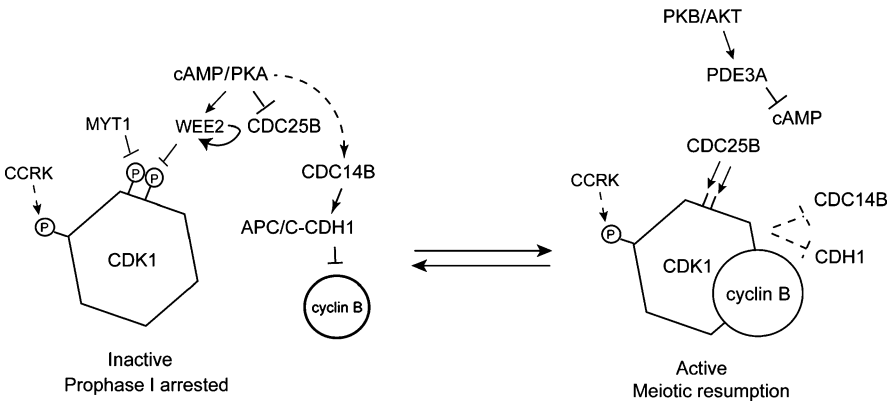

Fig. 14.2 Cartoon depicting the multiple layers of CDK1 regulation. The *arrows* indicate pathways of positive regulation and the *T-bars* indicate pathways of negative regulation. *Dashed lines* indicate pathways that are known to regulate CDK1 in other systems but have not been explored in oocytes. This cartoon does not take into account potential cyclin B-independent activation of CDK1 by RINGO or where PP1 feeds into to negatively regulate CDK1. *P* phosphorylated residue

14.2.2 WEE2 (Previously Called WEE1B) and MYT1 Kinases

CDK1 is a potent inducer of M-phase entry in both mitosis and meiosis (Masui and Clarke 1979) [reviewed in (Lindqvist et al. 2009)]. CDK1 activity is inhibited by phosphorylation at residues T14 and Y15 by MYT1 and WEE1 protein kinases, respectively (Fig. 14.2) (Nurse 1990; Morgan 1995). Crystallographic studies demonstrate that this phosphorylation event alters the environment in the ATP-binding pocket of CDK1 needed for generating stable interactions with the γ-phosphate of ATP (Welburn et al. 2007). Without these interactions ATP binding is not productive and substrate-binding affinity is reduced. Furthermore, the protein folds such that the phosphorylated region remains in contact with solvent, making it readily accessible for dephosphorylation and subsequent activation by CDC25 phosphatases (see Sect. 14.3.1.2). Inhibited CDKs maintain residual amounts of catalytic activity suggesting that basal CDK activity is primed and ready to amplify upon dephosphorylation.

Mammals contain two WEE1 tyrosine kinases, WEE1 (WEE1A) and WEE2 (WEE1B). WEE2 expression is limited to oocytes and embryos in both mice and nonhuman primates (Han et al. 2005; Hanna et al. 2010). Reducing the amount of WEE2 by RNAi or by a morpholino-oligonucleotide causes meiosis to resume in approximately 25–35% of oocytes in in vitro culture conditions that inhibit meiotic resumption (Han et al. 2005; Oh et al. 2010). In contrast, reduction of WEE1 does not induce meiotic resumption suggesting that WEE2 is the sole Wee-kinase required for negatively regulating CDK1 in mouse oocytes. The partial induction of meiotic M-phase entry suggests that another protein kinase overlaps with WEE2's inhibitory function. Indeed, reduction of MYT1 using a morpholino also causes 25% of oocytes to resume meiosis. Importantly, reducing the amount of both WEE2 and MYT1 causes ~50% of oocytes to resume meiosis in conditions normally inhibitory to germinal vesicle breakdown (GVBD), the first morphological marker of meiotic resumption (Fig. 14.1).

Recent data suggest that the action of these inhibitory protein kinases is regulated by their subcellular localization (Oh et al. 2010). WEE2 is restricted to the nucleus whereas MYT1 localizes throughout the cytoplasm suggesting that the oocyte requires both of the protein kinases presumably because they have different subcellular localizations (Fig. 14.3). Upon meiotic resumption, WEE2 is exported from the nucleus in a manner dependent upon nuclear localization and nuclear export sequences and CDK1 activity (discussed in more detail in Sect. 14.3.1.1). Interestingly, expression of a nuclear-targeted MYT1 mutant is less effective at preventing meiotic resumption than wild-type MYT1. As described in the legend of Fig. 14.3, there is active shuttling of many cell cycle regulators between the oocyte cytoplasm and nucleus. Having these two negative regulators in different cellular compartments is one way that oocytes ensure CDK1 activity is low regardless of its subcellular localization. Why oocytes require a meiosis-specific WEE kinase and why MYT1 is not effective when targeted to the GV are not currently clear.

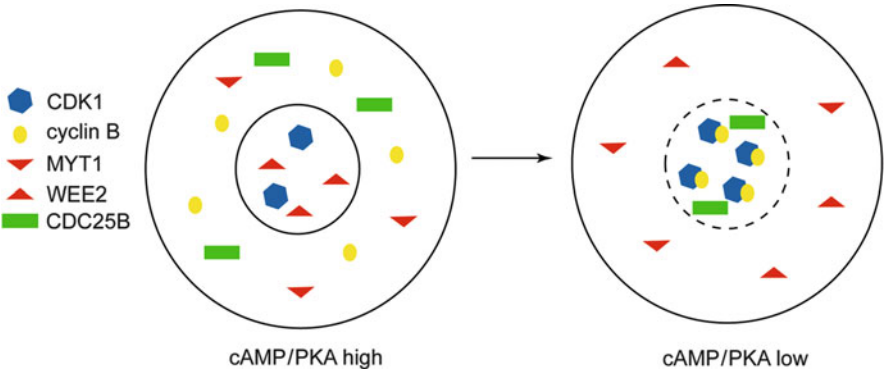

Fig. 14.3 Schematic demonstrating dynamic localization of CDK1 regulatory molecules. When cAMP levels are high, PKA is active and oocytes arrest at prophase of MI. Nuclear-localized CDK1 is subject to negative phosphorylation by WEE2 and positive regulators are localized in the cytoplasm. The cytoplasmic localization of CDC25B is regulated through the action of PKA. When cAMP levels are low, PKA is not active and meiosis resumes. Positive regulators of CDK1 (CDC25B and cyclin B) translocate into the nucleus to activate CDK1 triggering GVBD

14.2.3 Protein Kinase A

Protein kinase A (PKA) is a serine/threonine (S/T) cAMP-dependent protein kinase that exists as a holoenzyme tetramer containing two regulatory and two catalytic subunits [reviewed in (Kirschner et al. 2009)]. Upon cAMP binding to the regulatory subunits, active catalytic monomers are released and phosphorylate target substrates. PKA phosphorylates substrates within a K-K-L-S consensus sequence. In mouse oocytes, regulatory subunits RIa (PRKAR1A) and RIIa (PRKAR2A) are present and reducing RIa by RNAi strategies causes loss of the holoenzyme components (Duncan et al. 2006). Moreover, mice lacking the gene that encodes RIIa are fertile suggesting that RIa is the main PKA regulatory subunit in the oocyte. High PKA activity inside the oocyte is essential for meiotic prophase I arrest in fully-grown oocytes.

A screen for PKA substrates in oocytes identified WEE2, the CDK1 inhibitory kinase described above (Han et al. 2005). PKA phosphorylates WEE2 on S15 and promotes its activation by auto-tyrosine phosphorylation (Fig. 14.2). Thus, this interaction reinforces the inhibitory nature of WEE2 on CDK1 and ensures maintenance of the meiotic arrest.

Another substrate involved in this self-reinforcing inhibitory loop is CDC25B, a dual-specificity protein phosphatase. CDC25 phosphatases reverse the inhibitory WEE1/MYT1 phosphorylation of CDK1 (Fig. 14.2) (Sadhu et al. 1990; Nagata et al. 1991; Kakizuka et al. 1992; Sebastian et al. 1993; Zhang et al. 2008). PKA phosphorylates CDC25B on S321 (Zhang et al. 2008; Pirino et al. 2009; Oh et al. 2010). This action prevents nuclear accumulation of CDC25B by enhancing its interaction with a sequestering protein, 14-3-3, thus preventing CDC25B from

interacting with and activating CDK1/cyclin B. Therefore, PKA plays a direct role in maintaining prophase arrest by promoting CDK1 inhibition and preventing CDK1 activation. This regulatory mechanism appears specific to oocytes, because PKA does not phosphorylate these substrates in mitotic cells.

14.2.4 CDC14B Phosphatase

CDC14 is a dual-specificity protein phosphatase that is conserved from yeast to humans and has specificity for phosphorylated CDK1 consensus sites (T/S-P) (Gray et al. 2003). In mitotic cells of lower eukaryotes, CDC14 is required for exit from M phase and entry into G1 phase by negatively regulating CDK1 activity (Visintin et al. 1998). In mitotic and meiotic budding yeast cells, Cdc14 is regulated by periodic sequestration in the nucleolus (Stegmeier et al. 2002). During G1, S, and G2 phases, Cdc14 is held in the nucleolus by a complex of proteins called RENT (for *re*gulator of *n*ucleolar silencing and *t*elophase exit) (Shou et al. 1999). Release of Cdc14 from RENT is cell cycle-dependent and occurs in a highly regulated fashion through two signaling cascades called FEAR (for Cdc *f*ourteen *e*arly *a*naphase *r*elease) and MEN (*m*itotic *e*xit *n*etwork) (Shou et al. 1999; Visintin et al. 1999; Stegmeier et al. 2002). The FEAR pathway releases Cdc14 during early anaphase and one of its many substrates is Cdc15, the kinase that activates the MEN pathway to sustain Cdc14 release through mitotic exit.

Mammals contain two homologs, CDC14A and CDC14B. Although it is not clear if the release pathways that regulate Cdc14 in yeast exist in higher eukaryotes, CDC14A and B localization is cell cycle-regulated. CDC14A contains a nuclear export signal that prevents its accumulation in the nucleolus, and in human and mouse tissue culture cell lines undergoing mitosis, CDC14A localizes to centrosomes (Kaiser et al. 2002; Mailand et al. 2002). In contrast, CDC14B, which contains both a nuclear localization sequence and nuclear export signal, is sequestered in the nucleolus of somatic cells and is released into the nucleus during M phase (Kaiser et al. 2002; Mailand et al. 2002). Mitotic cells with reduced CDC14A have impaired centrosome separation and cytokinesis failure, suggesting that CDC14A is required for genomic stability, and reducing CDC14B by RNAi suggests it is required for centriole amplification, nuclear architecture maintenance, and microtubule stability (Cho et al. 2005; Wu et al. 2008). Moreover, CDC14B plays a key role in turning on the G2 DNA damage checkpoint to block the G2/M transition in damaged mitotic cells (Bassermann et al. 2008). Surprisingly, deletion of CDC14A and CDC14B either alone or in combination in tissue culture cell lines cells has no obvious phenotypic consequences (Berdougo et al. 2008; Mocciaro et al. 2010).

Mouse oocytes contain both CDC14A and CDC14B, and similar to somatic cells their localizations differ from one another (Schindler and Schultz 2009a, b). Surprisingly, the localizations of CDC14A and B appear to switch in the oocyte compared to mitotic cells because CDC14A localizes in the nucleus whereas

CDC14B is cytoplasmic, colocalizing with microtubules and microtubule-organizing centers (MTOCs) in prophase-arrested oocytes. Furthermore, neither CDC14 appears to localize in the nucleolus of the oocyte, suggesting that they are active during meiotic arrest. Oocytes depleted for CDC14B by RNAi undergo meiotic resumption in culture conditions normally inhibitory to GVBD (Schindler and Schultz 2009a) whereas when the function of CDC14A is blocked using an antibody approach oocytes maintained the prophase arrest (Schindler and Schultz 2009b). Similarly, oocytes overexpressing CDC14B, but not CDC14A, fail to undergo meiotic resumption. Collectively, these data suggest that CDC14B, and not CDC14A, is required to maintain the prophase I arrest. A role, if any, for CDC14A during the arrest is not currently clear.

The anaphase-promoting complex/cyclosome (APC/C) is a multisubunit E3 ubiquitin ligase that targets proteins for degradation by the 26S proteasome. APC/C activity is regulated by binding of activators and inhibitors. To trigger the G2 DNA damage checkpoint, CDC14B promotes the CDH1 (FZR1 in mouse nomenclature) activator to bind the APC/C (Bassermann et al. 2008) by reversing the deactivating phosphorylation of CDH1 by CDK1. In oocytes, the CDH1-bound APC/C targets proteins, including cyclin B, for destruction (Reis et al. 2006) (Fig. 14.2). Depletion of CDH1 using a morpholino stabilizes cyclin B1 protein to promote higher CDK1 activity, thus triggering meiotic resumption in culture conditions containing an inhibitor of GVBD (Reis et al. 2007). Consistent with its role in activating CDH1 in DNA-damaged cells, reduction of CDH1 in oocytes containing excess CDC14B rescues the delay these oocytes exhibit in undergoing the meiotic G2/M transition (Schindler and Schultz 2009a). Furthermore, oocytes overexpressing CDC14B contain less cyclin B1 protein suggesting that CDC14B activates CDH1 promoting APC/C ubiquitin-mediated proteolysis of cyclin B1. Because both the G2 DNA damage checkpoint and G2 meiotic arrest impinges on CDK1 activity, it is tempting to speculate that oocytes utilize additional signaling components from this checkpoint to prevent premature meiotic maturation (Solc et al. 2010). Perhaps similarities from these two systems will be useful in determining how CDC14B activity is positively regulated to maintain arrest and negatively regulated when cells are ready for transition into M phase.

14.2.5 Protein Phosphatase I and Phosphatase II (PP2)

Protein phosphatase I (PP1) belongs to a family of S/T protein phosphatases that participate in a variety of cellular functions such as cell cycle control and growth. In this family of enzymes, type-I phosphatases are inhibited by heat-stable inhibitors I-1 and I-2 and dephosphorylate the β-subunit of phosphorylase kinase. Type-II phosphatases (PP2A, B, C) are not inhibited by I-1 or I-2, and have specificity for the α-subunit of phosphorylase kinase. PP1 functions as a heterodimer containing a fixed catalytic subunit and different regulatory subunits that determine substrate

specificity and localization. The PP1 family of protein phosphatases is sensitive to toxins, such as okadaic acid (OA), that have been used extensively to study their function in oocytes (Rime and Ozon 1990).

Mouse oocytes contain both PP1 and PP2A (Smith et al. 1998). PPI localizes throughout the prophase-arrested oocyte, but is more concentrated in the nucleus, whereas PP2A is cytoplasmic (Wang et al. 2004). Furthermore, PP1 is phosphorylated following GVBD and during MI (Swain et al. 2003; Wang et al. 2004). Although the significance of this modification in oocytes is not known, in mitotic cells CDK1-mediated phosphorylation of PP1 on T320 inhibits its activity (Dohadwala et al. 1994). Treatment of oocytes from a variety of mammalian organisms with OA causes an increase in CDK1 activity and induces meiotic resumption in conditions that inhibit GVBD (Rime and Ozon 1990; Gavin et al. 1991; Schwartz and Schultz 1991; Hampl and Eppig 1995; Smith et al. 1998). These data indicate that a member of this protein phosphatase family negatively regulates CDK1 and meiotic resumption. Microinjection of an antibody against PP1 into prophase-arrested oocytes phenocopies the GVBD induction of oocytes injected with OA (Swain et al. 2003). In contrast, oocytes microinjected with an antibody against PP2A remain arrested in prophase suggesting that PP1 specifically regulates meiotic resumption. Conflicting evidence exists, however, because when I2, an inhibitor of PP1, was microinjected into oocytes meiosis did not resume. Moreover, when recombinant PP1 was microinjected into oocytes, oocytes resumed meiosis with normal kinetics, likely due to an inability to be phosphorylated and activated. Thus, the function and specificity of PP1 and PP2A during meiotic arrest remains elusive but it is highly likely that one or both negatively regulate CDK1 activity to maintain the G2 meiotic arrest.

14.3 Meiotic Resumption and Progression to Metaphase of MI (G2/M Transition)

Much progress has been made toward understanding the mechanism by which gonadotropins induce the maturation-associated decrease in oocyte cAMP (Tsafriri et al. 1996; Masciarelli et al. 2004; Mehlmann et al. 2004). Exit from meiotic prophase I and entry into metaphase of MI involves a major cell cycle transition that is similar to a mitotic G2/M transition. The first morphological hallmark of meiotic resumption is GVBD (Fig. 14.1). Because oocytes lack classical centrosomes, MTOCs that form during prophase from a network of cytoplasmic microtubules, migrate to the center of the oocyte, self-organize from a multipolar MTOC into a bipolar spindle that elongates and captures chromosomes that condensed prior to GVBD (Schuh and Ellenberg 2007). Finally, at metaphase of MI, chromosomes are aligned on the metaphase plate by stable microtubule–kinetochore attachments and are contained within a centrally localized, barrel-shaped spindle (Fig. 14.1).

14.3.1 Cell Cycle Regulators

14.3.1.1 CDK1: Part 2

Essential to both mitotic and meiotic G2/M transitions is an increase in CDK1 activity (Masui and Clarke 1979; Lohka 1998). Just as CDK1 is negatively regulated through several pathways to prevent premature meiotic resumption, CDK1 is positively regulated by several mechanisms to induce a rapid response to ovulatory cues. One way CDK1 is regulated is through the availability of cyclin B1 that is essential for CDK1 activity (Tay et al. 2000; Reis et al. 2007) (Fig. 14.2). During meiotic arrest, cyclin B1 is excluded from the nucleus where inactive CDK1 resides (Mitra and Schultz 1996; Marangos and Carroll 2004) (Fig. 14.3). Prior to GVBD, cyclin B1 translocates into the nucleus where it binds and activates CDK1. Timing of this translocation is consistent with predictions that CDK1 phosphorylates nuclear lamina proteins causing their depolymerization and membrane breakdown (Peter et al. 1990). In somatic cells, CDK1 phosphorylates the CDH1 activator of the APC/C (Zachariae et al. 1998; Kramer et al. 2000). This phosphorylation disrupts the interaction between CDH1 and APC/C, thus inactivating it. Because cyclin B1 levels are maintained at a low level through CDH1–APC/C (Reis et al. 2007), it is likely that CDK1 phosphorylates CDH1 generating a positive feedback loop whereby cyclin B1 levels stabilize and increase, thus increasing CDK1 activity further (Fig. 14.2). How CDK1 overcomes the activity of CDC14B for promoting CDH1–APC/C activity will provide insight as to how these on/off switches are regulated during critical cell cycle transitions.

In *Xenopus* oocytes, only a small fraction of CDK1 is associated with B-type cyclins (Ruiz et al. 2008). These oocytes contain a protein called RINGO (for *r*apid *in*ducer of *G*2/M progression in *o*ocytes) that also binds to and activates CDK1. This cyclin-independent activated CDK1 phosphorylates MYT1 thus relieving negative regulation of CDK1 generating yet another positive feedback loop to amplify CDK1 activity (Ruiz et al. 2008). Pig and mouse oocytes contain *RINGO* transcripts and overexpression of RINGO induces meiotic resumption in conditions mimicking high cAMP levels suggesting that mouse oocytes also contain alternative active CDK1 complexes (Terret et al. 2001; Kume et al. 2007). This cyclin-independent complex may explain how an initial burst of CDK1 activity could occur prior to cyclin B1 translocation and GVBD. Another way CDK1 is positively regulated is by dephosphorylation of T14 and Y15 by CDC25 phosphatases (Russell and Nurse 1986; Karaiskou et al. 1998) (Fig. 14.2) (see below).

14.3.1.2 CDC25A and CDC25B Phosphatases

The mammalian family of CDC25 dual-specificity phosphatases contains three members, CDC25A, B, and C that drive entry into M phase in mitosis and meiosis

through activation of CDK1 (Jinno et al. 1994; Lammer et al. 1998; Lindqvist et al. 2005; Zhang et al. 2008). Although these phosphatases have overlapping functions during mitosis, CDC25B is the family member required to trigger meiotic resumption in mouse oocytes. Female mice lacking the *Cdc25b* coding region are sterile because oocytes fail to resume meiosis indicating that CDC25A and CDC25C, which are present in the oocyte (Solc et al. 2008), cannot fully compensate for CDC25B's meiotic function (Lincoln et al. 2002). Mice lacking CDC25C are fertile and a role of CDC25C in oocyte maturation has not been explored further (Chen et al. 2001).

CDC25B localizes to the cytoplasm of arrested oocytes and translocates into the nucleus prior to GVBD (Zhang et al. 2008; Pirino et al. 2009; Oh et al. 2010) (Fig. 14.3). This translocation does not depend upon CDK1 activity and occurs prior to WEE2 nuclear export (Oh et al. 2010). As mentioned above, CDC25B action on CDK1 is inhibited by PKA phosphorylation of S321 that promotes CDC25B binding to and sequesteration by 14-3-3 (Fig. 14.2) (Zhang et al. 2008; Pirino et al. 2009; Oh et al. 2010). Cytoplasmic localization of CDC25B depends upon PKA activity because CDC25B is nuclear in oocytes from *Pde3A*$^{-/-}$ (an oocyte-specific phosphodiesterase) mice where PKA is inhibited (Oh et al. 2010). Furthermore, a mutant version of CDC25B (S321A) localizes in the nucleus and promotes accelerated GVBD kinetics (Pirino et al. 2009; Oh et al. 2010). It is not known, however, if PKA-mediated phosphorylation of CDC25B is reversed by a protein phosphatase to regulate translocation. A potential model is that PKA inhibits a protein phosphatase that, upon decline of cAMP levels and PKA activity, dephosphorylates PKA substrates to promote GVBD.

Although CDC25B is essential for GVBD, CDC25A also plays a role in positively regulating meiotic resumption because overexpression of CDC25A overcomes cAMP-mediated prophase I arrest (Solc et al. 2008). In contrast to CDC25B, CDC25A localizes in the nucleus (Fig. 14.3). A simple explanation why CDC25A cannot fully compensate for CDC25B in *Cdc25b*-knockout mice is that they have different localizations and potentially different substrate populations.

After GVBD and prior to metaphase of MI, CDC25A protein decreases. Overexpression and RNAi studies suggest the CDC25A and B have independent functions after GVBD (Solc et al. 2008). Only 10% of oocytes that undergo GVBD in the presence of excess CDC25A reach MII. Many of these oocytes have abnormal spindle morphology and chromosome alignment perturbations at metaphase of MI. In contrast, oocytes overexpressing CDC25B mature to MII similar to controls. One-third of oocytes with reduced CDC25A are unable to resume meiosis but of those that do, few reach MII. CDC25A is known to have non-CDK1 substrates in mitotic cells, which include components of the MAPK pathway (Xia et al. 1999; Kar et al. 2002; Nemoto et al. 2004; Wang et al. 2005). Thus, differences in substrate specificity and localization provide a simple explanation why oocytes require two, possibly three, CDC25 phosphatases.

14.3.1.3 Protein Kinase B/Akt

Protein kinase B (PKB) is a conserved S/T protein kinase from the AGC family (for homology to protein kinases A, G, and C) that is required for cell growth, cell cycle regulation, metabolism signaling, and apoptosis [reviewed in (Manning and Cantley 2007)]. Mammals contain three isoforms, AKT1, 2, and 3. Although they are ubiquitously expressed, isoform-specific knockout mice have distinct phenotypes. For example, *Akt1*$^{-/-}$ mice display growth defects and premature ovarian failure and *Akt2*$^{-/-}$ mice have glucose homeostasis defects; *Akt3*$^{-/-}$ mice do not have obvious phenotypic defects (Dummler et al. 2006).

PDE3A promotes the maturation-associated decrease in cAMP that leads to a decrease in PKA and therefore triggers resumption of meiosis. Ectopic expression of PKB in *Xenopus* oocytes increases PDE3A activity and induces meiotic resumption (Han et al. 2006). Furthermore, recombinant PKB phosphorylates and activates PDE3A in vitro. Minimally, PKB requires an R-X-R-XX-S/T motif within its target substrates (Alessi et al. 1996) and PDE3A contains five of these motifs. Furthermore, S290, 291, and 292 in PDE3A are phosphorylated by PKB in vitro and phosphorylation at these sites activates PDE3A in vivo (Han et al. 2006). Phosphoinositide 3 kinase (PI3K) activation of PKB is blocked by the PI3K inhibitor LY-294002 (King et al. 1997). CDK1 activation and meiotic resumption are transiently inhibited upon LY-294002 treatment, suggesting that PKB activity is required for initiating the meiotic G2/M transition (Kalous et al. 2006). Phosphorylated forms of PKB (T308 and S473) peak prior to GVBD and are quickly reduced after GVBD independent of CDK1 activity, a finding consistent with a role for PKB as an initiating stimulus for meiotic resumption (Fig. 14.2). In somatic cells, PDK1 phosphorylates PKB in its activation loop at T308 (Kim et al. 2001), but whether this occurs in oocytes is not known. Phosphatases sensitive to OA (e.g., PP1) likely deactivate PKB because treatment of oocytes with OA induces PKB hyper-phosphorylation (Kalous et al. 2006). Identification of a PKB activating kinase and a deactivating phosphatase will help connect PKB signaling in the PKA/CDK1 regulatory pathways.

14.3.2 Spindle Regulators

14.3.2.1 Aurora Kinases

Aurora kinases are a family of S/T protein kinases required for many cellular events including, but not limited to, mitotic entry, centrosome maturation, correcting improper kinetochore–microtubule connections, and cytokinesis. In mammals, there are three isoforms, Aurora kinase A, B, and C (named AURKA, B, C in mouse). Because overexpression of the different isoforms commonly occurs in cancers and as overexpression can induce aneuploidy and tumor formation in tissue culture cells, the Aurora kinases are a major focus for cancer pharmaceutical drug

therapy (Sen et al. 1997; Bischoff et al. 1998). Although AURKA and B are ubiquitously expressed in nearly all tissues, AURKC expression is largely limited to germ cells, although low levels are found in a number of cancer cell lines (Gopalan et al. 1997, 1998; Yanai et al. 1997; Tseng et al. 1998; Kimura et al. 1999). These kinases are highly similar at the sequence level, particularly in their catalytic domains. Their sequences diverge, however, at their N and C-terminal regions that are thought to provide substrate specificity and isoform-specific regulation. In mitotic and meiotic cells, AURKA is a centrosomally located protein, whereas AURKB and C localize to kinetochores and chromosomes (Gopalan et al. 1997; Hauf et al. 2003; Shuda et al. 2009). In mouse, AURKA clearly plays a role in progression to metaphase of MI, whereas AURKB and AURKC appear to function after metaphase of MI and will be discussed in the following section.

AURKA

Mouse oocytes express all three Aurora kinases with *AurkA* being the most abundantly expressed (Swain et al. 2008; Shuda et al. 2009). Similar to mitotic cells, AURKA localizes to MTOCs and to spindle poles during MI and MII (Saskova et al. 2008; Shuda et al. 2009). Furthermore, activated AURKA (phosphorylated on T288) associates with poles prior to and after GVBD, in a manner independent of CDK1 and PKB/AKT activity (Saskova et al. 2008). Overexpression of AURKA increases the number of MTOCs in prophase-arrested oocytes and these oocytes fail to mature to metaphase of MII arresting with long, distorted spindles (Saskova et al. 2008). In contrast, only 25% of oocytes where AURKA is reduced by RNAi undergo GVBD suggesting that AURKA is a positive regulator of meiotic resumption and progression to metaphase of MI. Moreover, TPX2, a known activator of AURKA in mitotic cells, is required to induce microtubule assembly and stabilize the metaphase I and II spindles in mouse oocytes, further supporting a role for AURKA in centrosome maturation and spindle morphogenesis as oocytes transition from prophase to metaphase of MI (Brunet et al. 2008).

14.3.2.2 Polo-Like Kinase

The conserved Polo-like kinase I (PLK1) is an S/T protein kinase that is required for mitotic entry and exit. Similar to AURKA, PLK1 is a centrosomal protein during metaphase that then relocalizes to the central spindle and midbody during mitotic exit and cytokinesis (Carmena et al. 1998; Simanis 2003; Brennan et al. 2007). PLK1 functions in an auto-amplification loop with CDK1 where CDK1 phosphorylated substrates serve as priming sites for PLK1 (Archambault and Glover 2009). Polo kinases contain a conserved Polo box domain in their C-termini that forms a binding pocket for CDK1-phosphorylated motifs of their target proteins (Cheng et al. 2003; Elia et al. 2003a, b). PLK1 also regulates the mitotic spindle and therefore many of its substrates are tubulins and associated proteins [reviewed in

(Archambault and Glover 2009)]. Additionally, PLK1 promotes mitotic exit by regulating cleavage and dissociation of cohesin complexes that hold sister chromatids together at metaphase (Alexandru et al. 2001; Hornig and Uhlmann 2004; Sumara et al. 2004; Yuan et al. 2007). Human PLK1 is phosphorylated and activated by AURKA in PLK1's activation loop (T210) and this activating phosphorylation is reversed by PP1 (Macurek et al. 2008; Seki et al. 2008). Mammals encode five Polo kinase homologs, but little is known about PLKs 2–5 and their functions have not been explored in mouse oocytes to date.

PLK1 is present in mouse oocytes and localizes in the nucleus of prophase-arrested oocytes, associates with spindle poles and chromosomes during metaphase of MI and with the spindle midzone during anaphase of MI (Wianny et al. 1998; Pahlavan et al. 2000; Tong et al. 2002; Yuan et al. 2007). Microinjection of an activity-blocking antibody causes slower GVBD kinetics suggesting PLK1 is a positive regulator of meiotic resumption (Tong et al. 2002). Consistent with this result is that PLK1 is posttranslationally modified by phosphorylation and has higher kinase activity during GVBD (Pahlavan et al. 2000). By metaphase of MI, PLK1 colocalizes to spindle poles. In budding yeast, the PLK1 homolog, Cdc5, which phosphorylates and removes meiotic cohesin from chromosomes, is required for proper kinetochore orientation, and regulates sister centromere segregation during MI (Clyne et al. 2003; Lee and Amon 2003). It is not currently known if these PLK1 or any of the other PLK homologs regulate these processes in mouse oocytes.

14.4 Metaphase of MI to Metaphase of MII Transition (MI Exit)

In mitosis, the cellular process transitioning from metaphase to G1 of the following cell cycle is often referred to as mitotic exit. This process includes segregation of homologous chromosomes, spindle elongation, establishment of a cytokinetic furrow and subsequent spindle disassembly, and cytokinesis. The first meiotic division (or exit) is distinct from mitosis in several ways. First, this division is reductional because sister chromatids remain attached while homologous chromosomes segregate. This chromosomal situation demands that cohesion between the sisters be maintained. Therefore, meiotic cells have adapted several unique methods to ensure fidelity of this process that includes use of meiosis-specific cohesin subunits (e.g., REC8) and stepwise removal of cohesin that begins with only removing the complex along the arms of chromosomes in MI and protecting centromeric cohesins (Brar and Amon 2008). Removal of arm cohesion allows chiasmata to resolve and permits chromosome segregation. Second, MI exit is distinct from mitotic exit because there is no round of intervening interphase or DNA replication. In oocytes, cytokinesis is an asymmetric division where cells extrude a small portion of cytoplasm containing one half of the genetic material in a structure called a polar body. Following cytokinesis, meiotic cells enter a second metaphase where they reassemble a spindle, align chromosomes on the metaphase plate, and prepare to

segregate sister chromatids. Although essential to generating healthy haploid gametes and key to understanding how meiosis is different from mitosis, it is not known how the exit from MI is regulated and how its regulation might differ from mitotic exit.

14.4.1 Cell Cycle Regulators

14.4.1.1 CDK1: Part 3

After meiotic resumption, CDK1 activity continually increases and peaks at metaphase of MI. Unlike mitosis, this increase is gradual and may explain why MI takes hours to complete (Choi et al. 1991; Gavin et al. 1994; Verlhac et al. 1994, 1996). This gradual increase in CDK1 activity is due to relatively slow accumulation of cyclin B (Hoffmann et al. 2006). The mitotic metaphase-to-anaphase transition requires a decrease in CDK1 activity. One mechanism employed by cells to negatively regulate CDK1 during mitotic exit is to destroy the activating cyclin B subunit (Murray et al. 1989; Yamano et al. 1996; Parry and O'Farrell 2001). This action is carried out by the APC/C bound to the CDC20 activator (Visintin et al. 1997). In addition, separase, the protease required to degrade REC8, also binds and inhibits CDK1 (Stemmann et al. 2001, 2006), and this binding is essential to complete MI (Gorr et al. 2006). Similar to mitiotic exit, CDK1 activity decreases upon anaphase I onset in oocytes (Masui and Clarke 1979), suggesting that oocytes employ a meiotic exit strategy that may be similar to mitotic cells. As described below, other nonubiquitin mediated processes exist to reverse the actions of CDK1 and some mechanisms appear to be conserved from mitosis.

14.4.1.2 CDC14A and CDC14B

As described above (Sect. 14.2.4) CDC14 is a dual-specificity protein phosphatase that has a high affinity for phosphorylated CDK1 substrates. In lower eukaryotes, such as the budding yeast *Saccharomyces cerevisiae*, Cdc14 is required for both mitotic and meiotic exit (Jaspersen et al. 1998; Buonomo et al. 2003; Marston et al. 2003). *CDC14* mutants arrest in late mitosis, with an elongated spindle and fail to divide (Hartwell et al. 1974; Visintin et al. 1998). Interestingly, during meiosis (sporulation in yeast), although *CDC14* mutants fail to disassemble the MI spindle, some chromosomes attempt to complete MII on a persistent MI spindle, rendering the dyad spores (gametes) inviable (Buonomo et al. 2003; Marston et al. 2003). These data suggest that although Cdc14 has the same function in reversing CDK1 action during MI exit, the chromosome segregation and spindle assembly/disassembly cycles are separate during meiosis unlike mitosis. During the past decade a myriad of CDC14 substrates have been described, most of which were discovered initially in budding yeast or human tissue culture cell lines as CDK1 substrates.

Many of these substrates include regulators of the central spindle, the region of the anaphase spindle with inter-digitating microtubules required for proper cytokinesis. Dephosphorylation of these substrates during anaphase (e.g., MKLP1 and PRC1) governs their localization to the central spindle where they are necessary for maintenance of proper ploidy (McCollum 2004; Zhu et al. 2006).

During meiotic spindle formation, CDC14B colocalizes with microtubules and remains associated with the entire spindle during metaphase of MI (Schindler and Schultz 2009a). In contrast, CDC14A is dispersed throughout the entire oocyte (Schindler and Schultz 2009b). Upon exit from MI, CDC14A and CDC14B colocalize on the central meiotic spindle where they persist until metaphase of MII, when CDC14A again is dispersed (Fig. 14.4) (Schindler and Schultz 2009b). These data suggest that both CDC14A and B regulate MII exit and are required to regulate the spindle and cytokinesis. Although oocytes depleted for CDC14B by RNAi extrude a polar body, many arrest in telophase of MI with unresolved cleavage furrows and disorganized chromosomes that are closer to the region of cytokinesis than the spindle poles (Schindler and Schultz 2009a). Moreover, oocytes microinjected with an antibody to block CDC14A activity have a delay in transitioning from metaphase of MI to metaphase of MII and an increased incidence of aneuploidy (Schindler and Schultz 2009b). Collectively, these data indicate that CDC14A and B have overlapping roles in promoting MI exit that may not be fully resolved until a double mutant mouse strain is generated.

14.4.1.3 Protein Phosphatase 2A

The S/T protein phosphatase 2A functions as a heterotrimeric complex that contains a core dimer of the catalytic subunit (PP2A–C), a scaffold A subunit (PP2A–A), and a variable regulatory B subunit (PP2A–B) that controls substrate specificity and localization of the enzyme complex. In mitotic cells, PP2A interacts with shugoshin (SGO1), a protein that protects cohesin at centromeres to prevent premature

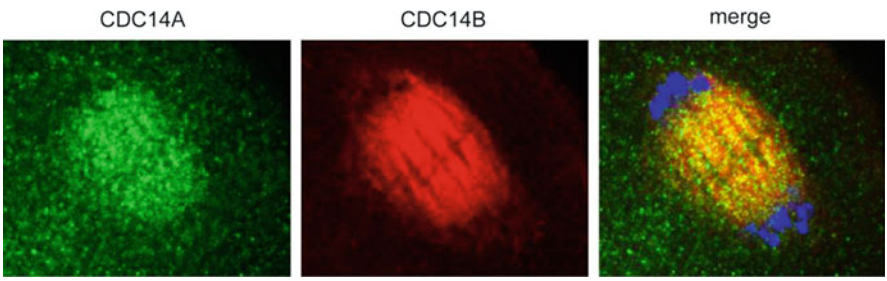

Fig. 14.4 CDC14A and CDC14B colocalize on the central meiotic spindle. Confocal image of CDC14A (*green*) and CDC14B (*red*) detected with polyclonal antibodies (Zymo Research and Abcam, respectively) during anaphase of MI. DNA (*blue*) was visualized with propidium iodide. This image was reprinted from (Schindler and Schultz 2009b) with permission from Landes Bioscience

chromosome separation (Kitajima et al. 2006). PLK1 phosphorylates a component of the mitotic cohesin complex (SA2), and this phosphorylation is critical for dissociation of cohesin from chromosomes and subsequent transition from metaphase to anaphase (Hauf et al. 2005). PP2A reverses this phosphorylation suggesting it is required to maintain sister chromatid cohesion prior to mitotic exit (Minshull et al. 1996). SGO1 acts as an adaptor for the phosphatase guiding specific PP2A complexes to centromeres through interaction of its coiled-coil domain with the catalytic and regulatory subunits (Xu et al. 2009). Moreover, this regulatory pathway is conserved in meiotic cells. For example, in *Schizosaccharomyces pombe*, the Sgo1 homolog that protects cohesin during meiosis physically interacts with Par1 (a PP2A homolog) and cells lacking Par1 precociously separate their sister chromatids after MI and randomly segregate their chromosomes during MII (Kitajima et al. 2006). Furthermore, ectopic localization of Par1 to a specific region of a chromosome arm forces maintenance of cohesion after MI.

Treatment of mouse oocytes with high concentrations of OA causes a metaphase I arrest (Smith et al. 1998). Furthermore, oocytes treated with lower concentrations of the toxin have increased numbers of prematurely separated sister chromatids that lead to hyperploidy and unpaired sisters (Mailhes et al. 2003). These data suggest that PP2A plays a similar role in protecting sister chromatid cohesion in meiosis as it does in mitosis. Moreover, PP2A colocalizes with SGO1 and SGO2 to centromeres at metaphase of MI and MII and overexpression of SGO1, to a level where it is no longer restricted to centromeres, recruits and thereby redistributes PP2A to chromosome arms (Xu et al. 2009). Oocytes with mis-localized SGO1 and PP2A fail to complete MI because they cannot remove REC8 from chromosome arms and therefore are unable to resolve chiasmata. Although it is clear that shugoshin [most likely SGO2 (Llano et al. 2008)] recruits PP2A to protect cohesion at centromeres in mouse oocytes, direct targets of the phosphatase have not been identified. One likely substrate is REC8 itself whose cleavage is regulated by phosphorylation on multiple residues in budding yeast (Brar et al. 2006). Interestingly, SGO1 and SGO2 have different specificity for different heterotrimeric complexes suggesting that the subset of substrates they direct PP2A to may vary. Because mice lacking SGO2 are infertile (Llano et al. 2008), this may be a powerful system for understanding which PP2A substrates are essential for centromeric protection.

14.4.2 Spindle Regulators

14.4.2.1 AURKB and AURKC

Although much is known about the mitotic functions of AURKB, little is known about its role in mammalian meiosis in oocytes. Furthermore, because AURKC is germ cell specific, few studies have been conducted to explore its function in these specialized cell types.

Using the ZM447439 inhibitor that has a reportedly higher affinity for AURKB than AURKA or AURKC (Ditchfield et al. 2003), several groups reported that oocytes arrest at MI with abnormal spindles, decondensed chromatin associated with a loss of histone H3 phosphorylation at S10 and misaligned chromosomes (Wang et al. 2006; Swain et al. 2008; Shuda et al. 2009; Vogt et al. 2009). Because AURKs B and C arose from a genome duplication, these two protein kinases are highly similar in sequence (Brown et al. 2004). The sequences of these protein kinases diverge at the N-terminus where AURKB has an extension containing destruction motifs. These motifs are recognized by the APC/C so that AURKB turns over upon mitotic exit (Nguyen et al. 2005). AURKB and C are thought to be functionally redundant because ectopically expressed AURKC localizes to centromeres during mitotic metaphase, where AURKB also localizes, and in somatic cells depleted for AURKB, expression of AURKC rescues their cytokinesis defects (Sasai et al. 2004).

In oocytes, AURKB concentrates at metaphase I centromeres whereas AURKC, although also at centromeres, is dispersed along the entire length of the chromosomes (Shuda et al. 2009; Vogt et al. 2009; Yang et al. 2010). Importantly, overexpression of AURKB, but not AURKC, partially rescues the chromosome alignment defect in oocytes treated with a low dose of ZM447439 suggesting that they have nonoverlapping functions during meiosis in oocytes (Shuda et al. 2009). Recent data where a dominant negative form of AURKC is overexpressed in mouse oocytes suggests that AURKC is required for microtubule–kinetochore attachment, chromosome separation, and cytokinesis (Yang et al. 2010). Analysis of AURKC female knockout mice that are reportedly subfertile (S. Kimmins, personal communication) will certainly help elucidate the requirements of these kinases during female meiosis.

In mouse sperm, AURKB and C have separate functions. Transgenic male mice expressing a catalytically inactive, dominant negative version of AURKB driven by a meiosis-specific promoter are subfertile, containing abnormal spermatocytes that arrest as binucleated cells failing to complete cytokinesis in MI (Kimmins et al. 2007). Because of this arrest, many of these defective spermatocytes undergo apoptosis. Male mice lacking AURKC are also subfertile but contain sperm with chromatin condensation defects, and have an abnormal morphology, e.g., loose acrosomes and blunt heads (Kimmins et al. 2007). Because AURKB and C cannot fully compensate for one another, these data suggest that they have unique functions during male meiosis and gametogenesis.

14.4.2.2 FYN: An Src Family Kinase

The Src family of nonreceptor tyrosine kinases (SFKs) is composed of nine members, three of which (FYN, YES, and SRC) are expressed in mouse oocytes. SFKs play critical roles in cell adhesion, invasion, proliferation, survival, and angiogenesis (Kim et al. 2009). It is therefore not surprising that overexpression or increased activation of SFKs is common to tumors. Because of their integral role

in tumor survival, SFKs are a popular target of cancer therapeutic design. Several groups have utilized SFK inhibitors to explore the function of SFKs during meiosis, but the results are conflicting, likely due to specificity issues. For example, treatment of oocytes with the inhibitors PP2 and SU6656 inhibit GVBD and MII exit (Levi et al. 2010; Zheng et al. 2007), whereas treatment of oocytes with the highly selective FYN inhibitor SKI606 does not block GVBD but does result in a significant reduction of oocytes maturing to MII (McGinnis et al. 2009). Furthermore, treatment with SKI606 results in chromosome alignment and spindle defects at MI and MII. The phenotypes associated with SKI606 are most consistent with the phenotype exhibited by knockdown of FYN by RNAi and with FYN knockout mice (McGinnis et al. 2009). Reduction of FYN results in a 50% reduction of oocytes reaching MII. FYN knockout mice are viable but females are subfertile, producing only 2–3 litters before reproductive failure. Moreover, *Fyn* knockout mice ovulate fewer eggs with approximately 20% at MI and not MII. Because FYN and activated SRKs localize to the meiotic spindle and the cortex, it is likely that FYN also regulates the timing of MI-to-MII transition (Levi et al. 2010) although the mechanism is currently unknown. To fully understand the unique functions of the three *Src* family kinases, development of additional specific chemical inhibitors and knockout mice will be required.

14.4.2.3 Glycogen Synthase Kinase 3

Glycogen Synthase Kinase 3 (GSK3) is an S/T protein kinase that is involved in a variety of pathways including, but not limited to, metabolism (via glycogen synthesis), cell cycle regulation, gene expression, and development (Rayasam et al. 2009). GSK3 differs from most kinases because: (1) it is constitutively active and is only negatively regulated by cellular signals such as phosphorylation; (2) it inactivates most of its target substrates; and (3) it requires priming phosphorylation of its substrates four amino acids C-terminal to the GSK3 phospho-acceptor site (Frame et al. 2001; Harwood 2001; Doble and Woodgett 2003). Two different genes encode two different, yet highly homologous isoforms of GSK3: GSK3α and GSK3β. Although they are similar in sequence and have similar substrate specificity, GSK3β knockout mice die at embryonic day 16 due to hepatocyte apoptosis suggesting that GSK3α cannot compensate for loss of GSK3β (Hoeflich et al. 2000). In lower eukaryotes, such as budding yeast, GSK3 homologs (Rim11, Mck1) control entry into meiosis through activating transcriptional activators that induce a temporally regulated transcription cascade essential for sporulation (Mitchell et al. 1990; Mitchell and Bowdish 1992).

Despite the vast array of knowledge about GSK3 in somatic cell processes, little is known about the requirement for GSK3α or β during meiosis in mouse oocytes. Both isoforms are present in oocytes, as determined by western blotting (Wang et al. 2003). GSK3 is inhibited by LiCl and oocytes treated with LiCl have altered spindle configurations and functions and perturbed chromatin organization (Pesty et al. 1994). Furthermore, LiCl-treated oocytes have reduced amounts of

microtubule polymerization and aberrant separation of homologs (Wang et al. 2003). Because GSK3 phosphorylates microtubule-associated proteins in somatic cells, it is likely that GSK3 controls MI through acting on spindle regulators.

14.5 Metaphase II Arrest (MII Arrest) and Exit (MII Exit)

During completion of MI, oocytes undergo an asymmetric cell division where they extrude a small proportion of cytoplasm containing half of the chromosome content into a polar body. After cytokinesis, oocytes directly enter and arrest at the second metaphase. MII exit is intimately connected with Ca^{+2}-dependent egg activation events that prepare the egg for embryonic development (Stricker 1999). MII exit entails resumption of meiosis with segregation of sister chromatids to generate haploid gametes. Similar to MI cytokinesis, MII cytokinesis is also asymmetric where oocytes extrude a second polar body.

14.5.1 Cell Cycle Regulators

14.5.1.1 CDK1: Part 4

The decrease in CDK1 activity during MI exit is transient as CDK1 activity increases and is high at metaphase of MII (Masui and Clarke 1979). A stable level of CDK1 activity during MII-arrest is, in part, maintained through an equilibrated cyclin B synthesis and degradation (Kubiak et al. 1993). MII exit is initiated by a second decrease in CDK1 activity. Overexpression of a nondegradable version of cyclin B1 blocks MII exit, suggesting that cyclin B destruction is a key regulator of both MI and MII exit in oocytes (Madgwick et al. 2006). As mentioned above, separase can bind and inhibit CDK1 during MI exit. High CDK1 activity inhibits separase in other cell types (Gorr et al. 2006; Holland and Taylor 2006), and therefore it is likely that maintenance of high CDK1 promotes sister chromatid cohesion by inhibiting separase and promotes its own activity by inhibiting its own inhibitor.

14.5.1.2 Mos/MEK/MAPK Pathway

The mitogen-activated protein kinase (MAPK) signaling pathway consists of at least three components that function in a specific order: an MAPK kinase kinase (MAPKKK, MAP3K, or MEKK), an MAPK kinase (MAPKK, MAP2K, or MEK) and an MAPK. In general, these pathways transduce extracellular and intracellular stimuli, which range from growth factors, hormones, cytokines, to stressors, to elicit an intracellular response such as proliferation, differentiation, cell survival, or

death. Once a stimulus activates a signaling axis the upstream S/T MAPKKK phosphorylates and activates the dual-specificity MAPKK that then phosphorylates a Y and a T residue in the activation loop of the MAPK. The activated MAPK can phosphorylate a wide variety of targets to trigger the downstream intracellular responses. These signaling modules are highly conserved from yeast to humans, and mutations that result in altered signaling are attributed to a variety of human diseases such as cancers, neurodegenerative and inflammatory diseases.

In oocytes, MOS is the MAPKKK that initiates the MAPK signaling cascade to activate the downstream MAPKs ERK1 and ERK2 (Su et al. 2003). MAPK activity increases after GVBD, is highest in metaphase II-arrested eggs and slowly declines after exit from MII. Female mice lacking MOS are sterile because their eggs fail to maintain the MII arrest and therefore parthogenetically activate (Colledge et al. 1994; Hashimoto et al. 1994; Verlhac et al. 1994). These data indicate that the MAPK pathway is required to maintain the MII arrest. Furthermore, because CDK1 activity must decrease for MII exit, these data suggest that MAPK plays a role in keeping CDK1 activity elevated at metaphase of MII. Historically, the activity that maintained the MII arrest was termed cytostatic factor (CSF). CSF is now thought to be an inhibitor of the APC/C called EMI2 (Madgwick et al. 2006) (Fig. 14.5). The APC/C triggers the metaphase-to-anaphase transition by regulating ubiquitin-mediated proteolysis of cyclin B1 and cohesin. EMI2 binds and inhibits the APC/C and is itself regulated by ubiquitin-mediated proteolysis upon fertilization when CaMKII and PLK1 phosphorylate it, thus making it a substrate of the SCF ubiquitin ligase. A current model exists whereby the MOS pathway prevents the destruction of EMI2, thereby maintaining high CDK1 activity and preventing MII exit (Fig. 14.5).

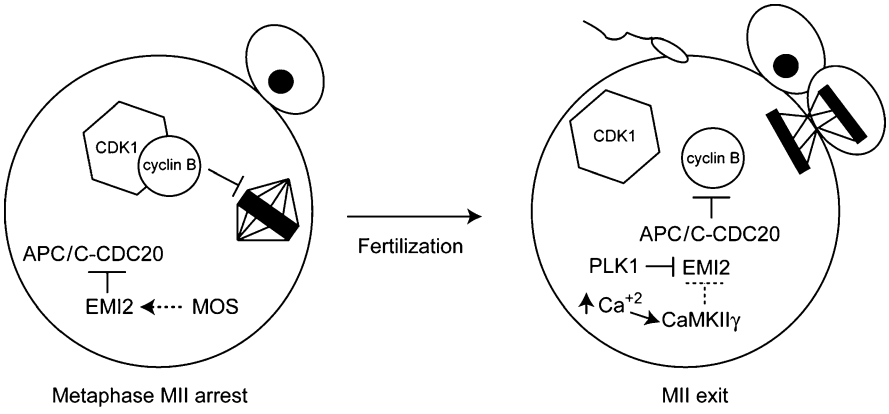

Fig. 14.5 Schematic depicting metaphase II arrest and exit. Arrest at metaphase II prior to fertilization is mediated by high CDK1 activity. CDK1 activity is sustained by EMI2 binding and inhibiting the APC/C–CDC20 ubiquitin. It is speculated (*dashed bars*) that upon activation via Ca^{2+} signaling, CaMKIIγ phosphorylates EMI2, which serves as a priming site for PLK1. Phosphorylated EMI2 dissociates from the APC/C and cyclin B is targeted for proteolysis thereby causing a decrease in CDK1 activity and promoting MII exit

14.5.1.3 Ca^{2+}/Calmodulin-Dependent Kinase II

CaMKs belong to a family of S/T protein kinases (consisting of CaMKI, CaMKII, and CaMKIV) that function in response to second messenger Ca^{2+} signals. Mammals contains multiple isoforms (over 30) of CaMKII that are derived from four genes: α, β, γ, and δ. In the absence of Ca^{2+}, Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is negatively regulated by an auto-regulatory domain that acts as a pseudo-substrate blocking the ATP-binding and substrate-binding pockets (Kemp et al. 1994). Upon Ca^{2+} binding, the auto-regulatory domain is displaced and CaMKII auto-phosphorylates and activates itself. CaMKII functions as a multi-subunit holoenzyme with mixed populations of 8–12 subunits.

Historically, CaMKII was suspected to be involved in egg activation during fertilization because CaMKII inhibitors block completion of MII, and expression of a constitutively active form of CamKIIα triggered MII exit and egg activation (Knott et al. 2006; Ducibella and Fissore 2008). It was not known, however, which isoform is responsible for responding to the Ca^{2+} signals and which events of egg activation are driven by CaMKII. Recently, two groups independently shed light on the isoform specificity and the requirement for CaMKII in different egg activation events (Fig. 14.5) (Chang et al. 2009; Backs et al. 2010). CaMKIIα and β knockout mice are fully fertile with defects in the hippocampus (Silva et al. 1992a, b; van Woerden et al. 2009) and CaMKIIδ knockout mice are also fully fertile but have cardiac defects (Backs et al. 2009). Importantly, female CaMKIIγ knockout mice are sterile (Backs et al. 2010), which is consistent with MII-arrested eggs containing transcripts only for CaMKIIγ (most likely splice variant 3) (Chang et al. 2009; Backs et al. 2010). Eggs from either CaMKIIγ knockout mice or eggs where CaMKIIγ has been reduced by antisense morpholino microinjection have no apparent defects in oocyte maturation. Although intracellular Ca^{2+} increases following fertilization or chemical activation, neither CDK1 nor MAPK activities decrease and therefore these eggs fail to exit from MII (Fig. 14.5) (Chang et al. 2009; Backs et al. 2010). Interestingly, the MII exit defect is rescued when oocytes are microinjected with cRNA encoding either CaMKIIγ or CaMKIIδ, suggesting that CaMKII's role in regulating MII exit is not isoform specific (Backs et al. 2010). In frog oocytes, association of EMI2 with the APC/C activator CDC20 inhibits APC/C activity. CaMKII-mediated phosphorylation of EMI2 creates a docking site for PLK1, which phosphorylates EMI2 triggering its ubiquitin-mediated proteolysis (Schmidt et al. 2006). Active CDC20–APC/C triggers cyclin B1 destruction, thus reducing CDK1 activity and thereby promoting MII exit. It is likely that mouse eggs also use this mechanism to trigger exit from MII.

14.5.1.4 FYN Kinase

Fertilization triggers an increase in protein tyrosine phosphorylation. As in other organisms, *Src* family kinases are thought to mediate this increase in phosphorylation, and in particular, FYN kinase is implicated in regulating MII exit upon

fertilization. For example, small molecule FYN inhibitors block MII exit and disrupt spindle morphology and chromosome alignment (McGinnis et al. 2007). Conversely, microinjection of active FYN kinase promotes MII exit in the absence of fertilization (Sette et al. 2002; Talmor-Cohen et al. 2004). Although these data suggest that FYN is required for MII exit, whether FYN knockout mice exhibit an MII exit defect has not been described.

14.5.2 Spindle Regulators

14.5.2.1 Protein Kinase C and Gycogen Synthase Kinase 3

The protein kinase C (PKCζ) family is comprised of ten S/T protein kinases that regulate cell proliferation, survival, and cell death. MII egg spindles contain several PKC isoforms (α, γ, δ, and ζ) and PKC regulates second polar body extrusion and downstream egg activation events after fertilization (Baluch and Capco 2008). Not only does Gycogen synthase kinase 3 (GSK3β) play a role in MI, but it is also required for MII exit. GSK3β is enriched at MII spindle poles and at kinetochores, and colocalizes with other signaling molecules such as PKCζ and PAR6 (Baluch and Capco 2008). Phosphorylation of GSKβ on S9 inactivates the kinase and this modified form of GSK3β localizes to MII spindle poles and kinetochores. S9 within GSK3β is a known PKCζ substrate and an antibody specific for active PKCζ coimmunoprecipitates S9 phosphorylated GSK3β from MII eggs (Baluch and Capco 2008). Moreover, microinjection of active GSK3 protein into eggs disrupts the MII spindle. These data suggest that GSK3β mediates MII spindle disassembly during MII exit and that PKCζ prevents premature exit from MII. In mitotic cells GSK3β phosphorylates and thereby inactivates several substrates known to stabilize the spindle including MAP1B, CRMP2, APC, Tau, and EB1 that would be likely candidates for stabilizing the MII spindle.

14.6 Concluding Remarks

Meiosis in oocytes is an altered version of the mitotic cell cycle, most remarkably because of cell cycle arrests and two successive rounds of chromosome segregation without an intervening round of DNA replication. Similar to mitosis, protein kinases and protein phosphatases regulate meiotic maturation in oocytes. Oocytes therefore employ several mechanisms to generate meiosis. There are several themes employed by oocytes, however, that contribute to generating this unique cell cycle. For example, mammals contain several isoforms of a signaling molecule and often there are meiosis-specific isoforms only expressed in oocytes. Furthermore, the subcellular localization of many of these regulators is controlled in a temporal fashion. This limits their access to substrates and regulators to specific

compartments during different phases of the cell cycle. Because mammals encode hundreds of these enzymes, the protein kinase/phosphatase signaling networks that govern meiotic maturation will likely grow in complexity as more players are described.

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Chapter 15
Anaphase-Promoting Complex Control in Female Mouse Meiosis

Keith T. Jones

Abstract Entry into, and passage through, the two meiotic divisions of the oocyte has to be highly coordinated to ensure proper segregation of chromosomes. This coordination ensures that the hallmark stops and starts of the meiotic process occur at the right time to prevent aneuploidy. The Anaphase-Promoting Complex is an activity mostly studied in the mitotic cell cycle division, where it has essential functions during mitosis. As detailed here the Anaphase-Promoting Complex also plays vital roles in controlling at least three meiotic events: maintenance of prophase I arrest, timely and faithful segregation of homologous chromosomes in meiosis I, and the meiotic arrest following ovulation.

15.1 Introduction

15.1.1 Female Meiosis Overview in the Mouse

As in all mammals, most follicles in the ovary contain oocytes that are arrested in prophase of the first meiotic division. They have been that way since mid-gestation, when female primordial germs cells have committed to meiosis under the action of retinoic acid. Hormone-independent recruitment of primordial follicles eventually leads to Luteinizing Hormone (LH) responsive follicles in which the prophase I arrested oocyte is induced to resume meiosis following the LH-surge, triggered by rising blood estrogen levels. The first meiotic division starts within an hour, and is completed at about 10–11 h later (see Fig. 15.1 for summary). LH induced changes to the follicle eventually lead to follicle rupture and expulsion of the oocyte from the ovary. By this time, the oocyte has already entered the second meiotic division where it rearrests and does not complete its meiotic division until fertilized. Fertilization is usually complete by 6 h after ovulation (14–18 h post-LH). The

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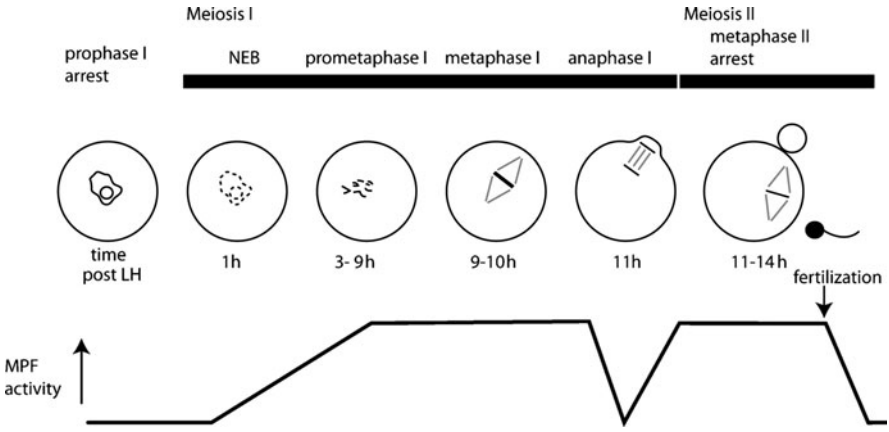

Fig. 15.1 Events of meiosis and associated changes in MPF. Schematic of the events leading from prophase I arrest, at the time of Luteinizing Hormone induced NEB, to eventual arrest at metII arrest. Timings are relative to the LH-surge. Also plotted are the associated changes in activity of Maturation-Promoting Factor, which is the primary kinase driving these meiotic transitions

second of the two meiotic divisions is thus only completed following fertilization. Here, I use the terminology "oocyte" to describe the immature gamete state, and "egg" as the mature gamete at the time it is normally fertilized.

15.1.2 MPF in Mitosis and Meiosis

Maturation-Promoting Factor (MPF) is the name given to the activity present in maturing oocytes capable of inducing nuclear envelope breakdown (NEB) when introduced into prophase I arrested oocytes (Doree and Hunt 2002; Jones 2004). Historically prophase I oocytes were described as having a prominent Germinal Vesicle, which is in fact the nucleolus within the nucleus: hence the terminology GV oocytes. More recently the term "G2" oocytes has started being used because the process of NEB mirrors the process of G2 to Mitosis (M-) phase transition of the mitotic cell cycle division.

MPF is a heterodimer of a kinase, Cyclin dependent kinase 1 (Cdk1; p34cdc2; cdc2) and a regulatory cyclin B subunit, which in mouse is cyclin B1. Speedy/Ringo is another known Cdk1 binding partner found in *Xenopus* and was originally described by its ability to induce NEB in oocytes (Ferby et al. 1999). There is currently no information regarding its role in mouse meiosis, therefore here it is taken that MFP activity in mouse oocytes translates to Cdk1/cyclin B1 activity.

The important feature of the meiotic divisions of mouse, as it is of all species, is that they are controlled by changes in MPF activity. Thus meiotic entry from G2/GV arrest is triggered by a rise in MPF. Completion of meiosis I requires an associated

loss in MPF, and rearrest of oocytes in meiosis II requires the reestablishment of MPF activity, and subsequent fertilization triggers its loss (Fig. 15.1). MPF activity is not limited to the meiotic divisions of oocytes, but instead is a universal factor involved in M-phase entry of somatic cells. As such it can be regarded as an M-phase Promoting Factor (MPF), and retain its name. This is not too surprising since NEB is common to the G2/M transitions of oocytes and adult cells.

Potentially hundreds of substrates for Cdk1 activity exist, at least in yeasts (Holt et al. 2009). In higher eukaryotes substrates include nuclear envelope associated nucleoporins, whose phosphorylation presumably promotes NEB, and condensins involved in chromosome condensation (Hudson et al. 2009), events associated with entry into meiosis and mitosis.

15.1.3 APC in Mitosis and Meiosis

MPF activation and inactivation occur by different mechanisms. Primarily, MPF activation is through changes in Cdk1 phosphorylation and MPF inactivation is through cyclin B1 degradation. This cyclin B1 degradation is directly induced by the action of the Anaphase-Promoting Complex-definition or Cyclosome (APC). The APC is a large multisubunit protein complex that earmarks substrates for proteolysis by polyubiquitination. The tagging of a substrate with ubiquitin causes its recognition by the 26S proteasome, which immediately proteolytically cleaves the protein.

The focus of this Chapter is how the APC is controlled in female meiosis. This control affects levels of cyclin B1, and consequently MPF and the meiotic divisions. As we shall see APC activity functions not only in MPF inactivation during the exit of the two meiotic divisions, but also surprisingly in the process of MPF activation at NEB. Furthermore APC activity during early meiosis I, at a time before MPF is fully active, appears important in controlling the length of meiosis I. This may represent a unique function of APC to mammalian oocytes.

A detailed account of the subunit structure of the APC should be sought elsewhere (Peters 2006). Important here is that the APC shows little activity towards substrates without binding one of two protein cofactors Cdc20 and Cdh1. APC^{Cdc20} activity is often limited to the metaphase-anaphase transition: primarily because Cdc20 needs Cdk1 phosphorylation to associate with the APC, and Cdk1 activity is normally confined to the period in M-phase leading to anaphase. There is less restriction imposed on APC^{Cdh1} activity, and in fact Cdk1-mediated Cdh1 phosphorylation is regarded as being inhibitory to APC binding. Thus there is some temporal separation in their period of APC association based on Cdk1 activity.

Cyclin B1 is not the only APC substrate, in fact APC^{Cdc20} and APC^{Cdh1} target a large range of substrates for degradation during mitosis (Pines 2006). These substrates contain discrete destruction motifs that target them for degradation. The most well characterized motif is the D-box, which is recognized by both APC^{Cdc20} and APC^{Cdh1}, and the KEN-box, recognized by APC^{Cdh1} only. We will here, however, focus on only one other APC substrate, securin. This is because the study of

the APC control in mammalian meiosis has really been limited to these two substrates. Securin (first described as Pituitary Tumor Transforming Gene, PTTG, in mammalian cells) is the molecular chaperone protein of the protease separase, whose target is the cohesin ring structures holding chromosomes together (Jallepalli et al. 2001; Oliveira et al. 2010). Cohesin cleavage, as we shall later (see Sect. 15.3.3), is necessary for separase-induced chromosome movement at the metaphase–anaphase transition (Oliveira et al. 2010).

The dogma established from mitosis is that at the metaphase–anaphase transition APC^{Cdc20} activity leads to degradation of both cyclin B1 and securin, reducing MPF activity and allowing chromosome separation respectively (Peters 2006). The loss in MPF causes a shift from APC^{Cdc20} to APC^{Cdh1} activity. This continues to target cyclin B1 and securin for degradation, but other substrates are also degraded at this time (including Cdc20 which contains a KEN-box). The functions of Cdh1 to the mitotic cell cycle division are not as clear as Cdc20 to the metaphase–anaphase transition. However, functions in maintaining an adequate gap phase between M- and S-phase have been found (Rape and Kirschner 2004).

15.2 The APC and Meiotic Resumption

15.2.1 Ability to Resume Meiosis of Small Oocytes

When mouse oocytes reach around 50 μm in diameter they become competent to undergo meiotic resumption. This suggests small oocytes lack a factor or factors critical for NEB. It is possible that this factor is in fact Cdk1 because levels are lower in small incompetent oocytes and higher in large competent oocytes, with the major rise associated with competency. In contrast, levels of cyclin B1 do not change (de Vant'ery et al. 1996). However, it is unlikely solely to be due to Cdk1, since over-expression of Cdk1 in incompetent oocytes fail to make them competent (de Vantery et al. 1997): changes in the balance of activating phosphatase and inhibitor kinases for Cdk1 may also play their part in competency (Kanatsu-Shinohara et al. 2000; Mitra and Schultz 1996; Schindler 2011). The competency to resume meiosis must be found in oocytes that are resident in follicles recruited into the growing cohort. Oocytes found in primordial quiescent follicles would be too small. It makes sense therefore for the growing oocyte to make itself ready for the meiotic divisions only at the time that its associated follicle is destined for ovulation.

15.2.2 Protein Kinase A and Prophase Arrest

Fully sized oocytes contained within growing follicles remain arrested at prophase I despite the ability to spontaneously undergo meiosis when dissected into culture

medium. The ovary is therefore believed to be a meiosis inhibitory environment. Ultimately it is protein kinase A (PKA) activity driven by cyclic AMP (cAMP) that is responsible for continued arrest. PKA phosphorylates both cdc25B, the activating phosphatase of Cdk1 in oocytes (Lincoln et al. 2002), while simultaneously phosphorylating wee1B, the inhibitory kinase of Cdk1 (Han et al. 2005; Han and Conti 2006). Phosphorylation of wee1B causes an increase in its activity and in turn phosphorylation of Cdk1. Although this Cdk1 phosphorylation event fails to affect cyclin B1 binding, it does inhibit Cdk1 kinase activity. The phosphorylation of cdc25 conversely inhibits activity. Thus the high intraoocyte levels of cAMP maintain prophase I arrest.

The trigger for continued cAMP within the oocyte appears to be constitutive activation of a G-protein coupled receptor (GPR3) (Freudzon et al. 2005; Mehlmann et al. 2004). However, it is unclear at present what constitutes the signal from LH leading to meiotic resumption. Oocytes lack LH receptors, so there is probably a signal from the surrounding somatic granulosa cells that targets the oocyte. One strong possibility is that LH induces a decrease in intraoocyte levels of cyclic GMP, which normally passes into the oocyte through gap junctions and acts to decrease phosphodiesterase activity responsible for hydrolysing cAMP (Norris et al. 2009). LH does this by reducing gap junction communication between the granulosa cells and the oocyte, effectively isolating the oocyte, lowering oocyte levels of cGMP, and raising phosphodiesterase activity (Norris et al. 2008).

Whatever is the mechanism of the initial LH trigger it has the effect of altering the balance of cdc25B and wee1B activities such that cdc25B is activated and wee1B inhibited. This balance ultimately activates Cdk1. This activation event is associated with a translocation of Cdk1 into the nucleus where it can act to trigger NEB. Before NEB, Cdk1/cyclin B1 is predominantly cytoplasmic. In fact, translocation of cdc25B and wee1B also appears to be associated with NEB. Nuclear wee1B translocates to the cytoplasm, while cytoplasmic cdc25B translocates to the cytoplasm (Oh et al. 2010). All these events transpire to cause activation of Cdk1/cyclin B1 within the nucleus of a prophase I oocyte, and this event is the trigger for NEB.

15.2.3 APC^{Cdh1} Control of Cyclin B1

Until recently the process of NEB in oocytes was studied exclusively in terms of Cdk1 phosphorylation status. Surprising then was the observation that loss of the APC activator Cdh1 induced meiotic resumption in oocytes culture in vitro in a media designed to maintain arrest (a high cAMP drive by addition of the phosphodiesterase inhibitor milrinone) (Reis et al. 2006). Why?

Presumably, the simple answer is that APC^{Cdh1} activity during prophase I arrest is causing the degradation of a substrate whose high levels are able to induce NEB. In the initial report of APC^{Cdh1} activity in mouse oocytes the important substrate was suggested to be cyclin B1 itself. Overexpression of cyclin B1 has the capacity to induce NEB, presumably by altering the sensitive equilibrium that must exist in

oocytes which prevents excessive accumulation of active MPF from a pool of inactive kinase. A later report, however, suggested that the important substrate for APC^{Cdh1} was securin rather than cyclin B1 (Marangos and Carroll 2008). Thus during prophase I arrest excess securin was degraded in preference to cyclin B1. Here the idea was that the APC has greater capacity to degrade securin over cyclin B1. Thus securin was effectively offering itself up for degradation so as to help maintain adequate levels of cyclin B1 to induce NEB.

15.2.4 APC^{Cdh1} Regulation During Prophase I Arrest

Independent of what substrate(s) are targeted for degradation during prophase I arrest, the ultimate aim is probably (1) to maintain sufficient cyclin B1 so as to be hormonally receptive, while (2) maintaining low enough levels of cyclin B1 so as not to undergo premature NEB. Therefore although we now have an understanding that cyclin B1 levels, in addition to Cdk1, need to be controlled during prophase arrest, and APC^{Cdh1} achieves this, it naturally leads on to the question of how is APC^{Cdh1} activity regulated. What regulates the regulator?

Although we do not currently know the answer to the above question, there have been identified a couple of factors in mouse oocytes that can modify APC^{Cdh1} activity, and as such help control the process of arrest. These are Emi1 and Cdc14B (Marangos et al. 2007; Schindler and Schultz 2009). Emi1 is an APC^{Cdh1} inhibitor, whose activity is also found in interphase of the somatic cell cycle. One idea for its function there is that it prevents DNA rereplication in S-phase directly by inhibiting APC^{Cdh1} (Di Fiore and Pines 2008). Other studies have suggested a G2 APC inhibitory function for Emi1 that allows accumulation of cyclin B1 ahead of M-phase (Reimann et al. 2001) although this idea is now contended (Di Fiore and Pines 2007). However, despite the uncertainties on the exact roles played by Emi1 in the mitotic cell cycle, in prophase I oocytes Emi1 is present and appears to contribute in making sure that APC^{Cdh1} activity is curtailed. Loss of Emi1 causes oocytes to become refractory in their ability to undergo NEB due to APC^{Cdh1} induced cyclin B1 degradation (Marangos et al. 2007). The converse is found for Cdc14B. Loss of this APC^{Cdh1} activator consequently reduces APC^{Cdh1} activity and oocytes undergo NEB at higher rates (Schindler and Schultz 2009).

Finally, there is also a spatial aspect in the regulation of APC^{Cdh1} activity. APC^{Cdh1} is predominantly found in the nucleus of an oocyte, where it appears to act as a nuclear guardian protecting against nuclear entry of Cdk1/cyclin B1 (Holt et al. 2010). Although Cdk1/cyclin B1 is mostly cytoplasmic, there is a constant shuttling of MPF between the cytoplasm and the nucleus. This shuttling is not unique to oocytes, but rather is observed universally for this heterodimer. The shuttling becomes exceptionally one-sided in the moments before NEB when much of the Cdk1/cyclin B1 translocates to the nucleus, and this is caused by phosphorylation of cyclin B1 (Clute and Pines 1999). However, normally before this phosphorylation event most Cdk1/cyclin B1 is quickly moved back out of the

nucleus to the cytoplasm. Oocytes appear to use nuclear APC^{Cdh1} as a protective mechanism to ensure that nuclear Cdk1/cyclin B1 does not accumulate. It is logical to assume that the capacity of the APC^{Cdh1} to degrade cyclin B1 is not sufficiently large so as to effect MPF activity when LH induces the translocation of Cdk1/cyclin B1 to the nucleus at NEB.

15.3 The APC and Meiosis I

15.3.1 Overview of Meiosis I

Here we take meiosis I to be the time from NEB to extrusion of the first polar body. Prophase I arrest has been examined in Sect. 15.2. This section will examine the process that sometimes is called "nuclear maturation" and examines how the oocyte controls the process of chromosome condensation, congression, biorientation and segregation. The timing of these events as we shall see is under control of the APC. We are not concerned with other events that may be occurring during oocyte maturation that are transforming the immature oocyte into a mature egg ready for the fertilizing sperm. Often these events are thought of as nonchromosomal and collectively called "cytoplasmic maturation." The archetypal example would be the maturation of cortical granules to be released from the oolemma and which are responsible for the block to polyspermy at fertilization (Abbott et al. 1999; Ducibella et al. 1988). While there may well be molecular timers in place in oocytes which have no synchrony with the process of "nuclear maturation," it is argued by this author that many of the events historically described as "cytoplasmic maturation" will ultimately be found to be controlled by switches or timers that have their origin in "nuclear maturation."

There are a few obvious, notable features of meiosis I to state before any in-depth analysis. First its length: meiosis I in mouse lasts about 10 h, compared to about 1 h for a mitotic division. Most of this time is spent in prometaphase. Second, it results in segregation of homologous chromosomes, often called a reductional division, rather than sister chromatids, an equational division (Fig. 15.2). Third, division is asymmetric as a result of spindle migration to the cortex. The first two events are thought to be connected: the unique type of reductional division in meiosis I consequently delays the whole process.

15.3.2 Assembly of the First Meiotic Spindle

Before NEB, chromosome show partial condensation around the nucleolus (Bellone et al. 2009), although not resolved to an extent to identify homolog pairs. The most obvious feature of the first few hours following NEB is the further condensation of

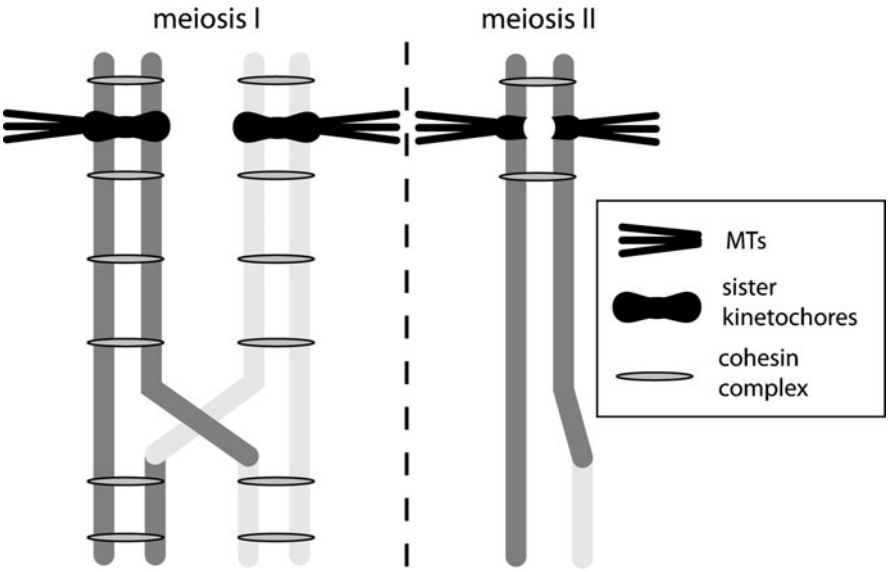

Fig. 15.2 Chromosome architecture during meiosis I and meiosis II. During meiosis I (*left*) homologous chromosomes are physically linked due to recombination events in fetal life. Cohesin complexes are positioned along the pair of sister chromatids. Sister kinetochores behave as a functional unit to ensure mono-orientation. Note the pulling of microtubules towards the two spindle poles at metaphase would only be counterbalanced by cohesin located distal to the site of recombination, here the bottom pair of complexes. During meiosis II (*right*) sister chromatids are only physically linked by cohesin complexes located in the peri-centromeric region. These have been protected from separase-mediated cleavage during meiosis I, but have become "deprotected" in meiosis II. Sister kinetochores in meiosis II do not behave as a functional unit, instead they have a back-to-back configuration. The architecture of sister chromatids in meiosis II is the same as in the mitotic division

chromosomes and microtubule dependent movement of chromosomes to form a bipolar spindle. In mouse by 4 h after NEB homologous chromosomes appear to have aligned on a spindle structure (Schuh and Ellenberg 2007). There are no centrosomes that help to nucleate microtubules in oocytes. Instead around 80 cytoplasmic microtubule organizing centers (MTOCs) coordinate microtubules (Schuh and Ellenberg 2007), the number of MTOCs diminishes during maturation as a result of their coalescence, such that by the time of metaphase I there are just two major MTOCs at each of the spindle poles. Interestingly the bipolar spindle structure may form due to the actions of MTOCs on MTOCs, rather than due to any influence of chromosomes (Brunet et al. 1998; Schuh and Ellenberg 2007).

It is important to remember that in meiosis I sister kinetochores, the structures on chromosomes that are the tethering sites for microtubules act as a functional unit and are orientated to the same pole (Brar and Amon 2008; Holt and Jones 2009). Sister kinetochores exhibit a side-by-side orientation and are mono-polar. In contrast, during meiosis II and a mitotic division, sister kinetochores exhibit a back-to-back

orientation and are bi-polar (Fig. 15.2). The molecular restructuring of the kinetochore required to do this is only understood in yeasts, and mammalian homologs appear currently lacking (Monje-Casas et al. 2007; Watanabe 2004). It remains a possibility that the very protracted length of meiosis I is because the special architecture of the mono-orientated sister kinetochore unit takes time to assemble. During meiosis I unusually for a cell division, kinetochore–microtubule interaction occurs very late on, just a short time before anaphase (Brunet et al. 1999; Schuh and Ellenberg 2007). Much of the prometaphase I period movement of homologs is orchestrated by microtubules associated with chromosome arms.

15.3.3 How Reductional Division Is Achieved in Meiosis I

During prometaphase I the resistive forces to the pulling of microtubules on chromosomes can only be achieved by the distal cohesive ties holding together recombined homologous chromosomes. During fetal life, recombination physically ties homologous pairs together (Revenkova and Jessberger 2005). Those ties remain until anaphase I when it is the action of separase on the kleisin component of the cohesin ring that breaks the ring structure (Kudo et al. 2006, 2009). Homolog cohesion is brought about primarily by these cohesin rings, structures that are thought to physically embrace the sister chromatid pair. In mitosis, the kleisin Scc1, a component of the ring-like cohesin structure is cleaved by separase, which at anaphase-onset has been freed from its molecular chaperone securin by the action of the APC. In meiosis Scc1 is largely replaced by Rec8, the molecular reason for this meiotic substitution are only just coming to light.

The unlocking of the cohesin rings must not happen along the entire length of the sister chromatids. Centromeric cohesin is protected from degradation. It must be spared in meiosis I because it will be the force holding sister chromatids together in meiosis II (Sakuno and Watanabe 2009). Centromeric cohesin in meiosis I is protected from degradation by a member of the Shugoshin family Sgo2 (Lee et al. 2008; Llano et al. 2008). Sgo2 targets the phosphatase PP2A to the centromere, where PP2A likely counteracts the phosphorylation of cohesin, which acts to make it more readily cleaved by separase. The kinase often thought to be counteracted by PP2A was polo kinase, because in mitosis it plays a similar role in removing arm cohesin during prophase. However, in meiosis, at least in yeasts, it is now thought to be Hhp2, an ortholog of casein kinase 1δ/ε (CK1) and Dbf4-dependent Cdc7 kinase (DDK) (Ishiguro et al. 2010; Katis et al. 2010). The distinction seems clear: in mitosis polo kinase is important to remove noncentromeric cohesin during prophase in a pathway that does not involve separase (Hauf et al. 2005); in meiosis I, CK1 and DDK are important to remove noncentromeric cohesin during anaphase-onset in a pathway that requires separase. Thus the sensitivity of meiotic Rec8 to separase is regulated by a balance of phosphorylation/dephosphorylation. Rec8 located away from centromeres is phosphorylated and separase sensitive; whilst centromeric Rec8 is dephosphorylated and so protected.

The mono-orientation of a sister kinetochore pair during meiosis I means that tension across that pair fails to develop as it would in mitosis. The tension instead transduces along the length of the chromosome to the point of recombination. This lack of tension on the sister kinetochore pair appears to be important in allowing Sgo2 to remain at the centromere. Thus one attractive idea is that as tension develops across a sister kinetochore pair, Sgo2, and hence PP2A and centromeric protection is lost (Lee et al. 2008). This would explain why the tensionless sister kinetochore pair causes protection of centromeric Rec8 in meiosis I, but the tension generated across the bipolar orientated sister kinetochore pair in meiosis II "deprotects" this centromeric Rec8.

15.3.4 Timing of the First Meiotic Division Through the SAC

If analogous to mitosis, one would predict that the APC is inhibited during the protracted period of prometaphase I arrest, and that such inhibition would be attributed to the activity of the Spindle Assembly Checkpoint (SAC). A detailed account of the functionality of the SAC in the mitotic cell division is outside the scope of this chapter but may be found in an associated chapter of this book (de Medina Redondo and Meraldi 2011). However, its ultimate target is Cdc20 and its binding to Cdc20 essentially prevents APC^{Cdc20} from being able to ubiquitinate either cyclin B1 or securin: events that appear prerequisites for successful mitotic completion (Musacchio and Salmon 2007; Peters 2006). Occupancy and/or tension of microtubules on kinetochores appear to be the main vehicle by which the SAC's activity is controlled. A lack of kinetochore occupancy, or a failure to generate tension across sister kinetochores would be the mechanism by which the SAC is activated. Thus the mitotic paradigm is that when all sister kinetochores are occupied and under tension this satisfies the SAC, allowing cyclin B1 and securin degradation by APC^{Cdc20}. Anaphase ensues.

In the late 1990s and early 2000s there were a few observations made which would seem to turn the entire model of SAC mediated APC control on its head for meiosis I. First was the observation that meiosis I appeared normal in oocytes containing a single (univalent) X chromosome (XO mouse) (Hodges et al. 2001; LeMaire-Adkins and Hunt 2000; LeMaire-Adkins et al. 1997). There was no delay in timing and segregation was often reductional in oocytes cultured from these mice. How would tension develop across the sister kinetochore pair on this single X chromosome? With no pairing homolog surely there would be no resistive forces to the microtubules so the SAC would never be satisfied? The conclusion seemed clear, the SAC was not present in mouse oocytes. A few years later the involvement of the APC in meiosis I was called into question from studies on maturing *Xenopus* oocytes where investigations appeared to demonstrate that this meiotic division did not need any APC activity or cyclin B1 degradation for its completion (Peter et al. 2001; Taieb et al. 2001).

The above experiments prompted many groups to examine the functionality of the SAC and the role of the APC in mouse oocytes. Reassuringly they all appear to point in the same direction in showing that the SAC is active and that the APC is needed for meiosis I in mouse. Antisense, oocyte specific knockout, or other inhibitory approaches all give a similar phenotype consistent with loss of the SAC when these members of the SAC family have been targeted: Mad2 (Homer et al. 2005a, b; Tsurumi et al. 2004); Mad1 (Zhang et al. 2005); BubR1 (Homer et al. 2009; Tsurumi et al. 2004; Wei et al. 2010); Bub1 (Leland et al. 2009; McGuinness et al. 2009; Tsurumi et al. 2004); and Bub 3 (Li et al. 2009). Thus acceleration of meiosis I and missegregation of homologs have both been observed when the SAC is inhibited suggesting that it is normally active during meiosis I and that it functions to delay anaphase I until homologous chromosomes are biorientated on the meiotic spindle. Similarly, nondegradable cyclin B1 or securin can both act to block the first meiotic division, demonstrating that their degradation, and hence APC activation is the target of the SAC in meiosis, and is needed for completion of meiosis I (Herbert et al. 2003; Hyslop et al. 2004; Ledan et al. 2001; Reis et al. 2007). All these studies are a reassurance that the meiotic division in mouse is not so very different to mitosis. However, they do leave open the question of whether differences do exist in the very large oocytes of *Xenopus* compared to mammalian oocytes and somatic cells.

The above experiments leave unanswered the question of why the meiotic division of the univalent X chromosome can occur reductionally. There are at least two possible reasons, (1) the sister kinetochores of the X chromosome could attach to microtubules of both poles so evading the SAC. This would potentially also generate an equational division the same as at meiosis II; (2) the SAC in meiosis can detect some types of kinetochore-microtubule error better than others, e.g., occupancy more than tension in the case of the univalent X.

15.3.5 SAC Independent Timer to Meiosis I

Earlier, in Sect. 15.3.4, it was stated that the long length of meiosis I compared to mitosis is an interesting feature of this division, made more intriguing by some observations suggesting absence of both SAC and APC function at this time. However, the consensus appears to have come full circle and the control of APC^{Cdc20} appears no different from mitosis. Therefore, is the length of meiosis I merely a reflection of how hard it is to establish mono-orientated sister kinetochores?

Puzzlingly a recent study has suggested that the APC remains active for the entire period of prometaphase I (Reis et al. 2007). Following NEB, APC^{Cdh1} mediated Cdc20 degradation was observed, and to some extent Cdh1 self-degradation- this is possible because like Cdc20, Cdh1 is also a substrate of APC^{Cdh1} activity. Eventually the rising MPF activity following NEB, and also the associated loss in Cdh1 protein, was found to turn off APC^{Cdh1}. This process would be analogous to the MPF mediated inactivation of Cdh1 during metaphase of a mitotic

cell division. The loss in Cdc20 would in itself present a problem for the oocytes to undergo the metaphase I–anaphase I transition, because APC^{Cdc20} is needed for cyclin B1 and securin loss at this time. However, the MPF-mediated loss in Cdh1 activity allows Cdc20 to rise.

Interesting the above presents us with a SAC independent mechanism of slowing meiosis I. Completion of meiosis will not occur until levels of Cdc20 have reaccumulated sufficient to degrade cyclin B1 and securin. In the absence of Cdc20, the meiotic process simply arrests at metaphase. However, in the absence of Cdh1, the meiotic division accelerates because of premature APC^{Cdc20} activity. This resulting acceleration leads to nondisjunction of homologous chromosomes due to a failure to align at the equator of a metaphase spindle. Under such a situation metaphase I is not halted by the SAC. It remains unclear if the SAC has been compromised by the absence of Cdh1, but in such Cdh1 knockdown oocytes it could still be activated by nocodazole or excess of Mad2. These observations lead to similar questions as those raised earlier for the XO mouse, what potential chromosomal missegregation errors can be detected by the SAC.

In the mitotic cell cycle certain SAC proteins have been suggested to fulfill a timing role in governing the onset of anaphase by a process that is not traditionally regarded as part of the SAC pathway, sensing kinetochore–mitrobule attachment and/or occupancy (see de Medina Redondo and Meraldi 2011). Thus based on the above, there may well be a similar timing mechanism in meiosis I which is based on Cdh1. It is possible that the reason why oocytes cannot adopt solely a SAC protein based mechanism of delay is because the APC actually needs to be active during the previous period of prophase I arrest (in order to maintain arrest- Sect. 15.2.4). Using a SAC-based mechanism would consequently inhibit APC activity at a time when its activity is vital for continued prophase I arrest.

Cdc20 may not be the only substrate of APC^{Cdh1} activity in prometaphase I. A very recent study confirmed APC^{Cdh1} activity at this time, but instead suggested that securin (similar to prophase I arrest, Sect. 15.2.3) may also be targeted for degradation (Homer et al. 2009). They also found that stability of Cdh1 and the SAC protein BubR1 were codependent, such that loss of one protein led to a dramatic loss in the other. The basis for this codependency is still unknown. It, however, establishes an intriguing corelationship between two activities that appear to control homolog segregation in oocytes.

15.4 The APC and Meiosis II

15.4.1 Overview of Meiosis II

In mammals, oocyte maturation is completed in the hours before ovulation. The egg is ovulated at a time when it is arrested at metaphase II (metII), and physiologically only completes its second meiotic division when it is fertilized. In mouse, this

happens within a few hours. If the egg is not fertilized, then it is destined to die. The physiological fertilization window is regarded as being 12–18 h post Luteinizing Hormone surge, with actual ovulation occurring at around 11 h. Although arrest at metaphase is usually quite stringent in the mouse, nonetheless the egg does undergo an aging process during arrest that is not related to cell cycle events. Therefore the quality of aged ovulated eggs is in general regarded as being inferior to those of freshly ovulated eggs.

The indeterminate length of metII arrest in mammalian eggs is probably an important feature of all eggs that are internally fertilized, and distinguishes them from their marine counterparts where spawning and fertilization are closely tied. The prolonged period of metII arrest is associated with high MPF activity. In contrast to the transitory nature of MPF activity in mitosis, here at metII, activity appears to stay at a constant, high-level until sperm fusion. Many years ago, work in *Xenopus* eggs demonstrated an activity present in the egg that was capable of maintaining MPF, and this was given the name Cytostatic Factor (CSF). The most important feature of CSF is that it is present in the mature egg but then is lost at fertilization. The loss of CSF activity leads to reduction in MPF, and exit from metII arrest. CSF activity is less easy to measure in mouse eggs but nonetheless fusion experiments have demonstrated an equivalent activity (Ciemerych and Kubiak 1998, 1999).

The fertilization event must trigger at least three important processes in the egg: (1) cell-cycle resumption and completion of meiosis II, (2) cortical granule exocytosis to block polyspermy, and (3) the switch-on of embryonic processes such as genome activation. Here we only discuss the first of these processes, although the inference is that the same sperm derived signal is involved in all three processes. The defining feature of fertilization with respect to the APC is therefore continued inhibition before gamete fusion, and then sperm induced activation once the sperm has fused with the oolemma.

15.4.2 Calcium Induced CamKII Activation at Fertilization

A Ca^{2+} signal in the egg is the necessary and sufficient physiological trigger for fertilization. In mammalian eggs the Ca^{2+} signal is oscillatory, composed of discrete spikes in Ca^{2+} that last a few minutes usually separated by a period of 10–20 min and which raise the resting Ca^{2+} level from around 100 nM up to levels greater than 1 M. This Ca^{2+} spiking activity can last several hours, and usually continues long past extrusion of the second polar body. MetII arrest continues if these Ca^{2+} changes are buffered, and conversely parthenogenetic activation is induced if Ca^{2+} changes in the egg are introduced that mimic those observed at fertilization (Jones 2005).

For many years the mechanism by which sperm caused these Ca^{2+} changes remained highly contentious. However, at present it seems likely they are induced by the release into the egg at gamete fusion of a sperm-specific member of the

phospholipase C (PLC) family, PLC zeta (Saunders et al. 2002; Swann et al. 2006; Yoon et al. 2008). Delivery of this PLC into the egg causes elevation in levels of inositol trisphosphate, which releases Ca^{2+} into the cytosol, following its binding to inositol trisphosphate receptors on the endoplasmic reticulum (Shirakawa et al. 2006; Swann and Yu 2008). The physiological relevance of sperm PLC remains to be demonstrated thoroughly by knockout studies; however, there is little disagreement that it is the primary trigger to Ca^{2+} changes at fertilization.

The rise in cytosolic Ca^{2+} induces activation a Calmodulin-Dependent Protein Kinase II (CamKII) (Knott et al. 2006; Madgwick et al. 2005; Markoulaki et al. 2003, 2004). Recent knock-down (Chang et al. 2009) and knockout studies (Backs et al. 2010) have established that CamKII (γ) is responsible for transducing the Ca^{2+} signal at fertilization. The CamKII family is large, with four distinct genes (α, β, γ, δ) that each contain a number of splice variants (Hudmon and Schulman 2002). Mouse eggs predominantly contain a single splice variant CamKIIγ3 (Chang et al. 2009) so this enzyme activity is likely to be activated in the egg by Ca^{2+}. It is unlikely that this splice variant plays a particular novel function in escape from meiotic arrest which cannot be mimicked by other variants, given a truncated constitutively active form of CamKII (α) is able to activate eggs (Madgwick et al. 2005).

The importance of CamKII to fertilization is observed by three main approaches: (1) knockdown or knockout of CamKII inhibits release from metII arrest (Backs et al. 2009; Chang et al. 2009); (2) constitutively active CamKII causes parthenogenetic activation (Madgwick et al. 2005); and (3) enzymatic measurements of CamKII that have demonstrated sperm-induced Ca^{2+} release has the ability to induce a burst of CamKII activity (Markoulaki et al. 2003, 2004). All these approaches point to CamKII being the physiological downstream target of Ca^{2+} action in terms of cell cycle resumption and there can be little doubt that it alone transduces the Ca^{2+} signal at fertilization.

15.4.3 Emi2 and c-mos: CamKII Targets

What is/are the downstream targets of CamKII? Recent evidence, especially in *Xenopus*, support the idea that CamKII phosphorylates an APC inhibitor Emi2, this phosphorylation event creates a docking site for polo kinase which also phosphorylates Emi2 (Hansen et al. 2006; Rauh et al. 2005; Schmidt et al. 2006; Tung et al. 2005). This double phosphorylation of Emi2 generates a recognition site for SCF, which like the APC is an E3 ubiquitin ligase. In metII arrested *Xenopus* eggs Emi2 expression during oocyte maturation probably leads to APC inhibition by the time the egg has fully matured. Although polo kinase appears to be active during metII arrest, Emi2 is not degraded probably because CamKII activity is not sufficient to cause the priming phosphorylation. It is not until the egg gets fertilized that this event happens.

In mouse the role of Emi2 appears the same as *Xenopus* (Madgwick et al. 2006; Shoji et al. 2006). It is synthesized during oocyte maturation to a level such that it is

able to inhibit APC activity at metII. If it is not synthesized, eggs fail to arrest at metII and go on to form a nuclear envelope, i.e., they cannot maintain MPF activity at high levels. Emi2 loss is needed in order to activate eggs and when measured its degradation precedes that of cyclin B1 (Madgwick et al. 2006).

Mechanistically it is easy to appreciate the role of an APC inhibitor in maintaining met II arrest, what then of c-Mos? Mos is a MAP kinase whose activity for many years has been closely associated with CSF. In *Xenopus*, the mechanistic link between c-Mos and Emi2 has recently been established. The MAP kinase pathway, driven ultimately by c-Mos activity, drives activation of p90-RSK (ribosomal S6 kinase), whose kinase activity directly phosphorylates Emi2 (Inoue et al. 2007; Nishiyama et al. 2007). This p90-RSK phosphorylation stabilizes Emi2, rather than driving its degradation as does CamKII. Hence in the absence of c-Mos and p90-RSK the instability of Emi2 leads to a failure to inhibit APC.

In mouse there may well exist a similar link between c-Mos and Emi2 stability, although here the connection is far less established. It is true that c-Mos activity is needed to maintain metII arrest, most readily demonstrated by the inability of eggs to arrest in knockout animals (Colledge et al. 1994; Hashimoto et al. 1994). However, in knockout animals of p90-RSK, eggs still arrest at metII (Dumont et al. 2005). p90-RSK exists as a family of four genes (RSKs 1–4). The three major isoforms that are believed to positively transduce actions of the MAP kinase pathway (RSKs 1–3) all have no effect on eggs either alone, or as a triple knockout. It remains possible, however, that the one remaining p90-RSK (RSK4) that has not been examined in mouse eggs can perform the task, although it is usually regarded as being an inhibitor of the MAP kinase pathway. Alternatively, other kinase(s) acting downstream from Mos/MAPK pathway could replace p90rsk activity in the mouse oocyte.

15.4.4 Activation of APC

The fertilization associated Ca^{2+} signal causes APC activation by inducing degradation of the APC inhibitor Emi2. This activation then leads to events that are analogous to mitosis. Cyclin B1 degradation is very quickly observed, with a sixfold increase in degradation following a Ca^{2+} signal (Nixon et al. 2002). Cyclin B1 degradation is complete by the time of second polar body formation. This leads to a rapid loss in MPF activity, a process that is needed in order to allow meiotic progression. Associated with cyclin B1 loss is also rapid degradation of securin, which would free separase to act on centromeric cohesin.

The need for a loss in MPF activity is readily observed when eggs are induced to express a nondegradable cyclin B1 (Hyslop et al. 2004). In such eggs arrest at metII is maintained despite the continued Ca^{2+} signal. In fact in such eggs the oscillatory Ca^{2+} signal persists for periods much longer than normal, possibly because PLC is sequestered into the pronucleus, and this may be the physiological signal for Ca^{2+} spiking termination (Kuroda et al. 2006; Larman et al. 2004).

High MPF activity has the ability to inhibit separase, an effect that is independent to securin binding (Nabti et al. 2008). However, in metII arrested eggs, it is securin, rather than MPF that is responsible for inhibiting separase. MPF-mediated inhibition of separase can only be observed in some nonphysiological situations, such as where nondegradable cyclin B1 is overexpressed (Hyslop et al. 2004; Madgwick et al. 2004).

Finally it is important to note that although the SAC is not the mechanism by which the APC is inhibited during metII arrest, it can nonetheless be activated by agents that disrupt the spindle structure such as nocodazole (Jones et al. 1995; Kubiak et al. 1993; Tsurumi et al. 2004). Thus the SAC is likely to operate in meiosis II in much the same way as it functions in mitosis. However, because sisters are equatorial and biorientated on the metaphase plate, the SAC is not responsible for APC inhibition. In situations where the SAC is activated, then eggs will remain metaphase arrested despite the continued presence of sperm Ca^{2+} signal (Jones et al. 1995). It is highly likely therefore under this scenario that although Ca^{2+} mediated Emi2 degradation has taken place (the only requirement being Ca^{2+}) APC inhibition continues because of the spindle poison activation of the SAC.

15.5 Conclusion

This chapter has highlighted the importance of the APC in the control of meiosis. Its activity governs prophase I arrest, the separation of homologues in meiosis I as well as sister chromatids in meiosis II. Currently we lack any detailed knowledge of the molecular make up of the APC in mammalian meiosis, but we do know its activity is influenced by Cdc20 and Cdh1, which are well described APC coactivators in mitotic studies. There are, however, subtleties to Cdh1 and Cdc20 control of the APC which may follow principles established from mitosis but hint at fundamentally different modes of control. Most surprising is the control of APC activity during meiosis I, which may be underlie the high susceptibility of oocytes to aneuploidy.

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Chapter 16
Established and Novel Cdk/Cyclin Complexes Regulating the Cell Cycle and Development

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Abstract The identification of new members in the Cdk and cyclin families, functions for many of which are still emerging, has added new facets to the cell cycle regulatory network. With roles extending beyond the classical regulation of cell cycle progression, these new players are involved in diverse processes such as transcription, neuronal function, and ion transport. Members closely related to Cdks and cyclins such as the Speedy/RINGO proteins offer fresh insights and hope for filling in the missing gaps in our understanding of cell division. This chapter will present a broad outlook on the cell cycle and its key regulators with special emphasis on the less-studied members and their emerging roles.

16.1 Introduction

The cell cycle machinery orchestrates the precise coordination of cell division. Numerous regulatory proteins direct cells through a specific sequence of events culminating in mitosis and the production of two identical daughter cells. Central to this process are the cyclin-dependent kinases (Cdks), which bind to cyclin proteins. Many advances in cell cycle regulation have been made since the discovery of cyclins 27 years ago by Tim Hunt and his colleagues. Observing the periodic increase and decrease of these novel proteins, Hunt stated that "it is difficult to believe that the behavior of cyclins is not connected with processes involved in cell

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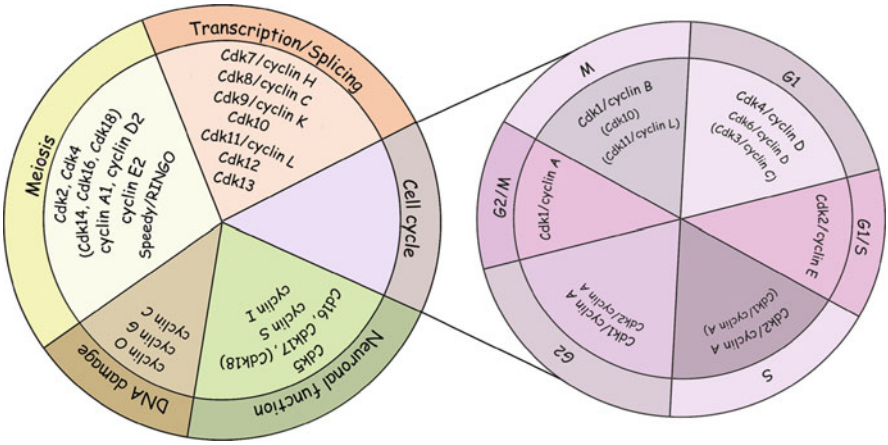

Fig. 16.1 Functional characterization of Cdks and cyclins. Cdks and cyclins complexes are depicted based on their cellular functions. Cdks/cyclins in parentheses indicate that evidence for involvement of these members in the cellular process is preliminary

division", suggesting that the synthesis of these proteins drives cells into mitosis and their destruction allows cells to finish one cycle and begin the next (Evans et al. 1983). Cyclins provided a crucial hint about the basic mechanism of the cell cycle and the first important role for regulated proteolysis in eukaryotes.

Cyclins are conserved throughout evolution. Cyclins and Cdks may have started life as integrating functions rather than components of a central oscillator. In bacteria, one round of DNA replication can take twice as long as a cell cycle, indicating that the coupling between DNA replication and cell division can be flexible (Murray 2004). As cell growth accelerates, the single replication origin can fire more than once in a cell cycle, and when it slows down, there are cycles in which it does not fire at all (Murray 2004). In most of today's eukaryotes (budding yeast being a notable exception), entering mitosis with partially replicated DNA leads to irreparable chromosome damage. Before such a feature could appear, a tighter coupling between replication and segregation was necessary. One form of coupling would be a clock that would tell previously independent processes when to begin, coupled with long intervals, so that one event could finish before the next begins.

Cyclins might have first appeared as molecules that accumulate continuously through the cell cycle and thus serve as a proxy for cell size, a role that one of the G1 cyclins in budding yeast still seems to play (Cross 1988; Nash et al. 1988). If the ancestral cyclins were stable throughout the cell cycle, the amount of cyclin would fall twofold when cells are divided, giving a narrow range for the thresholds associated with DNA replication and mitosis. This problem would be overcome if cyclins evolved to be destroyed by the proteolytic machinery that separated the sister chromatids. After this improvement, the cell cycle would start without cyclin, allowing cells to set well-separated thresholds of Cdk1 activity for replication and

segregation. Finally, as the segregation module sends the signal to separate the sister chromatids, it would also reset the clock initiating cyclin degradation.

The pattern of cyclin expression varies with progression through the cell cycle and the specific expression pattern defines the relative position of cells within the cell cycle. Cdk/cyclin complexes themselves become activated by phosphorylation at specific sites on the Cdks by Cdk7/cyclin H, also referred to as Cdk-activating kinase (CAK) (Kaldis 1999; Lolli and Johnson 2005). The cell interprets mitogenic signals in the G1 phase, leading to synthesis of the D-type cyclins. Cyclin D isoforms (cyclins D1, D2, D3) interact with Cdk4, Cdk6 (and Cdk2) and these active complexes initiate phosphorylation of the members of the retinoblastoma (Rb) family, which include Rb, p107, and p130. This leads to E2F-mediated transcription of genes including the A- and E-type cyclins. Cdk2/cyclin E complexes are active at the G1/S transition and promote entry into S phase. The Cdk2/cyclin A2 complexes drive S phase progression and Cdk1/cyclin A2 complexes at the G2/M transition lead to initiation of prophase. Cdk1/cyclin B complexes finally complete mitosis. Although this model was established on the basis of experimental evidence in eukaryotic cells, extensive work over the past decade using gene knockout technology has shown that it is not followed rigidly (Table 16.1). Mouse embryonic fibroblasts (MEFs) lacking Cdk4 and Cdk6, for example, respond appropriately to mitogenic stimuli after serum starvation (Malumbres et al. 2004). Cdk2 is dispensable for mitosis (Berthet et al. 2003; Ortega et al. 2003), and MEFs lacking E-type cyclins proliferate normally, except for a defect in cell cycle reentry from quiescence (Geng et al. 2003). The compensatory mechanisms in these knockout mice have been reviewed extensively elsewhere (Malumbres and Barbacid 2005; Satyanarayana and Kaldis 2009).

While significant attention has been paid to these Cdks and cyclins owing to their essential roles in the cell cycle, a number of other Cdks and cyclins, as well as closely related molecules, have been discovered in recent years. Functions for some of these are still emerging and in addition to cell cycle regulation, they appear to be involved in diverse roles such as transcription, RNA splicing, and neuronal development. This chapter will provide a broad overview of the mammalian cell cycle and then direct attention to the members that have not yet been studied extensively.

16.2 Cdk and Cyclin Family Members

So far, there are at least 15 different cyclins known from cyclin A to Y. Based on the current understanding, the human genome has 21 genes that encode Cdks. The first 11 members, Cdk1-11, represent the classical Cdks, with cyclin partners having been identified for all but Cdk10 (Table 16.2). To facilitate a comprehensive understanding of the Cdk family and encourage consideration of less studied members, those known by their cyclin-interacting motif (PFTAIRE, PCTAIRE), or others like CHED and CCRK, have now been named Cdks, even though their association with cyclins will have to be established in the future (Malumbres et al. 2009).

Table 16.1 In vivo outcomes of deletion of cyclins and Cdks

Disrupted gene	Phenotype/pathology	Viability	
Cyclin A1	Males mice exhibit sterility due to meiotic cell cycle arrest	Viable	Liu et al. (1998)
Cyclin A2	Embryonically lethal E5.5	Non-viable	Kalaszczynska et al. (2009), Murphy et al. (1997)
Cyclin B1	Embryonically lethal E10.5	Non-viable	Brandeis et al. (1998)
Cyclin B2	Normal and fertile	Viable	Brandeis et al. (1998)
Cyclin D1	Small body size, hypoplastic retinopathy, mammary gland defects, neurological abnormalities	Viable	Fantl et al. (1995), Sicinski et al. (1995)
Cyclin D2	Impaired proliferation of B-lymphocytes and pancreatic cells. Defects in cerebellar development, adult neurogenesis, hypoplastic thymus	Viable	Huard et al. (1999), Kowalczyk et al. (2004), Solvason et al. (2000)
Cyclin D3	Defects in T-lymphocyte development	Viable	Sicinska et al. (2003)
Cyclins D1 and D2	Retarded body size and impaired coordination, hypoplastic cerebella	Viable but die within first 3 postnatal weeks	Ciemerych et al. (2002)
Cyclins D2 and D3	Severe megaloblastic anemia. Lethality before E18.5	Non-viable	Ciemerych et al. (2002)
Cyclins D1 and D3	Neurological abnormalities. Death at P1, but a few survive to 2 months	Non-viable	Ciemerych et al. (2002)
Cyclins D1, D2 and D3	Proliferative defects in hematopoietic cells and cardiac myocytes. Lethality by E16.5	Non-viable	Kozar et al. (2004)
Cyclin E1	Overtly normal	Viable	Geng et al. (2003), Parisi et al. (2003)
Cyclin E2	Reduced male fertility	Viable	Geng et al. (2003), Parisi et al. (2003)
Cyclins E1 and E2	Severe defects in extraembryonic tissues. Lethality by E11.5	Non-viable	Geng et al. (2003), Parisi et al. (2003)
Cyclin F	Defects in extraembryonic tissues. Lethality by E10.5	Non-viable	Tetzlaff et al. (2004)
Cdk1	Lethality by E2.5 (insertional mutant; knockout has not yet been described)	Non-viable	Santamaria et al. (2007)
Cdk2	Male and female infertility, reduced body size, defective neural progenitor cell proliferation	Viable	Berthet et al. (2003), Ortega et al. (2003)
Cdk4	Male and female infertility, reduced body size, insulin-dependent diabetes due to abnormal development of pancreatic -cells	Viable	Rane et al. (1999), Tsutsui et al. (1999), Zou et al. (2002), Moons et al. (2002)
Cdk5	Severe neurological defects. Die immediately after birth	Non-viable	Ohshima et al. (1996)

(*continued*)

Table 16.1 (continued)

Disrupted gene	Phenotype/pathology	Viability	
Cdk6	Thymic and splenic hypoplasia	Viable	Malumbres et al. (2004)
Cdks 2 and 4	Heart defects. Lethality by E15.5	Non-viable	Berthet et al. (2006)
Cdks 2 and 6	Reduced body size, haematopoietic defects	Viable	Malumbres et al. (2004)
Cdks 4 and 6	Severe anemia. Lethality from E14.5 onward. The few live pups die soon after birth	Non-viable	Malumbres et al. (2004)
Cdks 2, 4 and 6	Cardiac and haematopoietic defects. Lethality by E13.5	Non-viable	Santamaria et al. (2007)
Cdk11	Mitotic defects, lethality by E3.5	Non-viable	Li et al. (2004)

Cyclins serve as regulatory subunits that activate protein kinases (Cdks). Initially discovered as a pair of cyclins, A and B (Evans et al. 1983), which associate with a single kinase subunit, Cdk1 (also known as Cdc2 and Cdc28), the family has expanded to contain multiple cyclins and Cdks involved in processes that include cell cycle, transcription, and differentiation. Each cyclin associates with one or two Cdks and most Cdks associate with one or two cyclins, although some, such as Cdk1 in budding yeast, associate with as many as nine distinct cyclins [reviewed in (Andrews and Measday 1998)].

All known cyclins are targeted for degradation by the addition of a chain of ubiquitins, but the details of this conjugation differ for each cyclin. G1 cyclins are ubiquitinated by the "Skp/cullin/F-box containing" (SCF) complex, whereas mitotic cyclins are ubiquitinated by the anaphase-promoting complex/cyclosome (APC/C). Both complexes [reviewed in (Jackson et al. 2000)] also degrade other proteins but they share a core complex (the association of a cullin-like protein [Cul1 in SCF and APC2 in APC/C] with a protein containing a particular zinc finger domain [Rbx1 in SCF and APC11 in APC/C]) and possibly a common origin. Despite these similarities, they are regulated in different ways. The SCF complex is active throughout the cell cycle and the destruction of its substrates depends on their phosphorylation, with different phosphate-binding proteins (F-box proteins) guiding various sets of substrates to destruction. The APC/C is activated at the onset of anaphase and degrades its substrates as cells exit mitosis, suggesting it might first have appeared as a specialized version of SCF to take over the role of cyclin destruction from some other proteolytic system.

The D-type cyclins are the first cyclins to be induced as G0 cells are serum stimulated to enter the cell cycle (Sherr 1994). D-type cyclins associate with and activate Cdk4 and Cdk6. Studies from knockout mice demonstrate that cyclin D1, D2, and D3 are, for the most part, functionally redundant but that each has unique tissue-specific functions (Ciemerych et al. 2002; Kozar et al. 2004; Sicinski et al. 1995). The primary substrate for D-type cyclin kinases is the retinoblastoma tumor suppressor protein (Rb). In cells lacking Rb, D-type cyclin kinase activity is not required for cell cycle progression (Lukas et al. 1995). In addition to functioning as regulatory subunits for Cdk4 and Cdk6, D-type cyclins also help to target Rb and

Table 16.2 The Cdk/cyclin family

Cdk	Alternative names	Cyclin-binding motif	Cyclin partners	Cellular function	
Cdk1	Cdc2	PSTAIRE	A1, A2, B1, B2, (B3), D, E, (F), (J)	G2–M phase transition (S phase entry and transition)	Althoff et al. (2009), Hershko (1999), Krude et al. (1997), Pagano et al. (1992), Sicinski et al. (1996), Won et al. (1992), Yang et al. (1999)
Cdk2	–	PSTAIRE	A1, A2, B1, B2, D, E1, E2, (J)	G1–S phase transition, S phase entry and transition	Gudas et al. (1999), Hershko (1999), Krude et al. (1997), Pagano et al. (1992), Sicinski et al. (1996), Won et al. (1992), Yang et al. (1999)
Cdk3	–	PSTAIRE	A1, A2, E1, E2, C	Transcriptional regulation, G0–S phase transition	Braun et al. (1998), Meikrantz and Schlegel (1996)
Cdk4	PSK-J3	PISTVRE	D1, D2, D3	G1–S phase transition	Zhang et al. (1997), Malumbres et al. (2004)
Cdk5	TPKII	PSSALRE	p35, p39, D-, E-, G-type cyclins, I	G1–S phase transition, DNA damage response	Brinkkoetter et al. (2009), Dhavan et al. (2002), Dhavan and Tsai (2001), Lalioti et al. (2010)
Cdk6	PLSTIRE	PLSTIRE	D1, D2, D3	G1–S phase transition	Russo et al. (1998)
Cdk7	CAK, MO15, STK1	NRTALRE	H	Cdk activation, transcriptional regulation, DNA repair	Fesquet et al. (1993), Poon et al. (1993), Solomon et al. (1993)
Cdk8	K35	SMSACRE	C, K	Transcriptional regulation, G0–S phase transition	Makela et al. (1994)
Cdk9	PITALRE, CDC2L4	PITALRE	K, T1, T2	Transcriptional regulation	Fu et al. (1999), Simone et al. (2002)
Cdk10	PISSLRE	PISSLRE	–	Transcriptional regulation, (G2–M transition)	Grana et al. (1994), Kasten and Giordano (2001)
Cdk11	CDC2L1, CDC2L2	PITSLRE	D3, L1, L2	G1–S phase transition	Loyer et al. (2008), Niu et al. (2005), Zong et al. (2007)
Cdk12	CRKRS, CRK7, CrkRS, CD2L7	PITAIRE	L1, L2	Transcription and RNA splicing	Chen et al. (2006)
Cdk13	CHED, CDC2L5	PITAIRE	L1, L2	Transcription and RNA splicing	Chen et al. (2007)
Cdk14	PFTAIRE1, PFTK1, ALS2CR7	PFTAIRE	D3, Y	G1–S phase transition, Wnt signaling in mitosis	Davidson et al. (2009) Malumbres et al. (2009)
Cdk15	PFTAIRE2, PFTK2	PFTAIRE	–	–	Malumbres and Barbacid (2005)
Cdk16	PCTAIRE1, PCTK1	PCTAIRE	–	Neuron biology, meiosis?	Graeser et al. (2002)

(*continued*)

Table 16.2 (continued)

Cdk	Alternative names	Cyclin-binding motif	Cyclin partners	Cellular function	
Cdk17	PCTAIRE2, PCTK2	PCTAIRE	–	Meiosis?	Hirose et al. (1997)
Cdk18	PCTAIRE3, PCTK3	PCTAIRE	K	Transcriptional regulation, meiosis?	Okuda et al. (1992)
Cdk19	CDC2L6, CDKL8	SMSACRE	C	Transcriptional regulation, G0–S phase transition	Malumbres and Barbacid (2005), Malumbres et al. (2009)
Cdk20	CCRK, CAKp42, p42, CDCH	PNQALRE	–	Cell cycle regulation	Malumbres et al. (2009), Wohlbold et al. (2006)
–	–	–	cyclin O (Ung2)	DNA repair	Akbari et al. (2004), Murray and Marks (2001)
–	–	–	cyclin S	(Memory processing)	Edelheit and Meiri (2004)
–	–	–	M-type cyclins	(Metal ion transport, biomineralization, retinal function)	Guo et al. (2005), Parry et al. (2009), Polok et al. (2009), Wang et al. (2003)

pocket proteins for phosphorylation through direct protein–protein interaction (Ewen et al. 1993; Kato et al. 1993). The Rb protein plays a critical role in regulating G1 progression and is likely a key component of the molecular network controlling the restriction point. Rb has been shown to bind and regulate a large number of cellular proteins, including members of the E2F family of transcription factors (Johnson and Schneider-Broussard 1998).

Through the activation of E2F, cyclin E is the next cyclin to be induced during the progression of cells through G1 (Geng et al. 1996; Ohtani et al. 1995). Cyclin E associates with Cdk2, and this kinase complex is important for cells to make the transition from G1 to S phase (Ohtsubo et al. 1995). Cdk2/cyclin E complexes participate in maintaining Rb in the hyperphosphorylated state (Hinds et al. 1992) and, thus, orchestrate a positive feedback loop for the accumulation of active E2F. Unlike the D-type cyclins, however, cyclin E kinase activity is still required in cells lacking Rb, suggesting that there are additional critical substrates for Cdk2/cyclin E complexes (Ohtsubo et al. 1995). Like many other Cdk/cyclin complexes, Cdk2/cyclin E phosphorylates histone H1 and this activity may be important for the chromatin rearrangement required during replication of the genome. Knockout mouse models revealed unexpectedly that E-type cyclins (E1 and E2) are largely dispensable for mouse development, but required for the endoreduplication of trophoblast giant cells and megakaryocytes (Geng et al. 2003). With the prevailing notion that E-type cyclins were essential for proliferation, it was again surprising when embryonic fibroblasts generated from these knockout mice proliferated normally, except for a defect in reentering the cell cycle from quiescence. This is attributed to a kinase-independent function of cyclin E in the loading of MCM proteins onto DNA replication origins (Geng et al. 2003, 2007; Pagano et al. 1992).

Cyclin A2, which is also regulated by E2F (Schulze et al. 1995), accumulates at the G1/S phase transition and persists through S phase. Cyclin A2 initially associates with Cdk2 and then, in late S phase, associates with Cdk1. Cyclin A2-associated kinase activity is important for entry into S phase, completion of S phase, and entry into M phase (Lehner and O'Farrell 1989; Walker and Maller 1991). Cyclin A2 colocalizes with sites of DNA replication, suggesting that cyclin A2 may actively participate in DNA synthesis or perhaps play a role in preventing excess DNA replication. At least some members of the E2F family are negatively regulated by cyclin A2. E2F1, E2F2, and E2F3 contain domains that directly bind cyclin A2. This allows cyclin A-associated kinases to phosphorylate the E2F heterodimerization partner DP1 resulting in an inhibition of E2F DNA-binding activity (Xu et al. 1994). As anticipated from its crucial regulatory functions in cell proliferation, cyclin A2 knockout embryos die shortly after implantation (Murphy et al. 1997). Unexpectedly, MEFs lacking cyclin A2 proliferate normally, owing to a compensatory role of cyclin E. However, cyclin A2 functions are essential in hematopoietic and embryonic stem cells (Kalaszczynska et al. 2009).

Cyclins A2, B1, and B2 in association with Cdk1 regulate mitosis (Arellano and Moreno 1997; Pines and Hunter 1991; Walker and Maller 1991). Cdk1/cyclin B complexes phosphorylate substrates that include cytoskeleton proteins such as lamins, histone H1, and possibly components of the mitotic spindle. For cells to exit mitosis, cyclins A2 and B must be degraded and experiments suggest that Cdk1/cyclin B kinases participate in the regulation of this destruction process. After

mitosis, cells again enter G1 and, at the restriction point, must decide whether to proceed into another round of cell cycle.

16.2.1 Cdks and Cyclins as Transcriptional Regulators

While Cdks were first identified as regulators of cell cycle progression, a number of members have been shown to be involved in other cellular processes, notably transcriptional regulation. Cdk7 is one such member with well-established dual roles in cell cycle control and transcriptional regulation. In association with the general transcription factor TFIIH, Cdk7/cyclin H complexes are involved in promoter clearance and progression of transcription by phosphorylation of the C-terminal of the large subunit of RNA polymerase II (Fisher 2005). Cdk8 and Cdk9 play similar roles in transcription and phosphorylate RNA polymerase components, in association with cyclins C and K, respectively [reviewed in (Akoulitchev et al. 2000; Garriga and Grana 2004; Wang and Fischer 2008)]. Cyclin K and the closely related cyclins T1, T2a, and T2b interact with Cdk9 to form the transcription elongation factor b [P-TEFb] (Peng et al. 1998). Cdk19 is very similar to Cdk8 and was suggested to interact with cyclin C. Cdk19 (also known as CDC2L6 or CdkL8) is one of the components of the mediator complex [required for induction of RNA polymerase II transcription by DNA-binding transcription factors] (Sato et al. 2004). Cdk10, a member whose role in cell cycle regulation is not yet clearly defined, inhibits transactivation of the Ets2 transcription factor, a regulator of Cdk1 expression (Kasten and Giordano 2001).

Roles in transcription and splicing have also been identified for Cdk11 (Hu et al. 2003; Loyer et al. 1998), Cdk12, and Cdk13. Cyclin L associates with Cdk11, the splicing factors S-35 (mice) or SC35 (human), and phosphorylates RNA polymerase II (Berke et al. 2001; Dickinson et al. 2002). A new member of the cyclin family, cyclin L, was identified in *D. melanogaster*, *C. elegans* (Berke et al. 2001; Boucher et al. 2001), mouse (Berke et al. 2001), and humans (Dickinson et al. 2002). In addition to the highly conserved "cyclin box" that interacts with Cdks (Kobayashi et al. 1992), cyclin L also possesses an RS domain characteristic of splicing factors (Berke et al. 2001; Boucher et al. 2001). Cdk11/cyclin L might participate in signaling pathways that link or regulate transcription and RNA processing. In mouse neurons, dopamine and glutamate induce expression of two isoforms of cyclin L (ania-6) by alternative splicing (Berke et al. 2001).

As predicted from the presence of arginine/serine (RS)-rich domains (present mainly in proteins regulating pre-mRNA splicing), both Cdk12 and Cdk13 are involved in regulation of splicing. Cdk12 colocalizes with components of the spliceosome and interacts in vitro with the C-terminal domain of RNA polymerase II as well as the splicing factors SF2 and ASF2 (Ko et al. 2001). Cdk13 interacts with the ASF/SF2-associated protein p32, a protein involved in regulation of splicing. Originally known as CrkRS (CDC2-related kinase, arginine/serine-rich gene), this kinase was named Cdk12 after an interaction with cyclins L1 and L2 was revealed (Chen et al. 2006). It was also recently demonstrated to be involved in MAPK regulation and

resistance to estrogen signaling inhibitors (Iorns et al. 2009). Cdk13 belongs to a high molecular weight subfamily of CDC2 family with PITAI/VRE motifs and bears 92% homology in its kinase domain to Cdk12. It was originally implicated in cholinergic signaling and cell division control in hematopoiesis (Lapidot-Lifson et al. 1992) and was named CHED (cholinesterase-related cell division controller). The rights to being called a Cdk were granted upon identification of cyclins L1 and L2 as interacting partners (Chen et al. 2007). Cdk13 also interacts with the HIV transactivator Tat and regulates viral mRNA splicing (Berro et al. 2008).

16.2.2 Cdks and Cyclins with Neuronal Functions

Cdk5 is the most prominent Cdk with neuronal functions. It is ubiquitously expressed in neurons and brain defects in *Cdk5*$^{-/-}$ mice have revealed critical roles for Cdk5 in neuronal differentiation and neuronal cytoskeleton structure (Ohshima et al. 1996). Complexes of Cdk5 with its activating partners, p35 and p39, phosphorylate several cytoskeletal substrates and substrates involved in transcription such as Stat3, mSds3, and p53 (Cruz and Tsai 2004).

Cdks16-18 are poorly studied kinases with reports indicating an involvement in neuronal functions. PCTK1 or Cdk16 interacts with p35, a major activator of Cdk5. Cdk16 is detected in many tissues but is highest in brain and testes. When Cdk16 is transfected into neuroblastoma cell lines, it can influence neurite outgrowth (Graeser et al. 2002). PCTK3 or Cdk18 mRNA is also expressed in many tissues with highest expression in the heart and brain. PCTK2 or Cdk17, on the other hand, is almost completely restricted to the brain and lung. These Cdks are also enriched in postmitotic neurons of adult brains (Cole 2009). Cdk16 and Cdk17 are part of a complex with Trap and Cables [Cables was identified as an adaptor molecule linking the non-receptor tyrosine kinase c-abl with Cdk5 in neurons] (Yamochi et al. 2001).

Among the cyclins, a correlation has been observed between the upregulation of cyclin S and memory processing in chicks (Edelheit and Meiri 2004). Since the expression of cyclin S is associated with both initial memory processing and subsequent memory consolidation, this cyclin is implicated in long-term memory formation through the regulation of neuronal gene expression. In mouse neurons, neuronal stimulators induce the expression of different isoforms of cyclin L (referred to in this study as ania-6) by alternative splicing (Berke et al. 2001). Cyclin L is associated with Cdk11 and components of the RNA processing complex (also see Sect. 16.2.1).

16.2.3 Cdks and Cyclins in DNA Damage Repair

One of the outcomes of DNA damage is inhibition of Cdk2 and Cdk1 activity to halt cell cycle progression and to allow for repair. Cdk2 and Cdk1 inhibition is mediated via the DNA damage checkpoint, a signal transduction cascade that directly or

indirectly affects the various regulatory pathways controlling Cdk2 and Cdk1 activity such as upstream kinases (Wee, Myt1, CAK), phosphatases (Cdc25), and Cdk inhibitors. Checkpoint kinases target p53 leading to the activation of the p53 transcriptional program and increased levels of the Cdk inhibitor, $p21^{Cip1/Waf1}$. Among the transcriptional targets of p53 are the G-type cyclins that were first identified serendipitously in screens for src family kinases in rat fibroblasts (Tamura et al. 1993) and later by differential screen for target genes of p53 (Okamoto et al. 1996). The precise function of cyclin G remains elusive; however, identification of multiple p53-binding sites (Zauberman et al. 1995), transactivation by wild-type p53, and induction following γ-irradiation (Hartwell 1992; Kastan et al. 1991) implicate a role in negative growth control or DNA damage repair. The absence of either prototypic protein destabilizing (PEST) sequences (Reed 1991; Rogers et al. 1986) or the "destruction box" sequence controlling the ubiquitin-dependent degradation of mitotic cyclins (Glotzer 1995) indicates alternate regulation of cyclin G protein expression. Cyclin G2 is implicated as a component of signal transduction pathways regulating cell growth (Horne et al. 1996).

Cyclin O (also known as Ung2) is implicated in DNA repair and regulation of apoptosis. Ung2 is a nuclear uracil-DNA glycosylase that removes misincorporated uracil and is also involved in immunoglobulin diversification (Krokan et al. 2002). In lymphoid cells, cyclin O induces apoptosis in response to glucocorticoid and radiation stimuli (Roig et al. 2009). How these diverse functions are regulated by cyclin O is unclear. Different phosphorylated forms of cyclin O have been identified in HeLa cells and their regulation appears to be related to the cell cycle (Hagen et al. 2008). A recent report implicates cyclin C in DNA repair in postmitotic neurons (Lalioti et al. 2010).

16.2.4 Mammalian CAK Activity

Together with cyclin H and MAT1 (ménage à trois), Cdk7 forms the Cdk activating kinase (CAK) complex (Makela et al. 1994; Poon et al. 1993; Solomon 1994), which phosphorylates Cdk1, Cdk2, Cdk4, and Cdk6 that are involved in cell cycle progression (Fisher 2005; Kaldis 1999). Also called p42 or CCRK (cell cycle-related kinase), Cdk20 shares 43% sequence identity with Cdk7. As predicted from its similarity to Cdk7, Cdk20 was reported to possess CAK activity (Liu et al. 2004). A later study that used purified monomeric Cdk20 found that it in fact had no detectable kinase activity toward Cdks (Wohlbold et al. 2006). Since no cyclin partners have yet been identified for this kinase, the possibility that Cdk20 acquires kinase activity upon binding with a cyclin remains elusive. Cyclin K, a component of transcriptional complexes (see Sect. 16.2.1), has been reported to display CAK activity in vitro (Edwards et al. 1998). This study did not identify the associated Cdk, making Cdk7, thus far, the only known kinase with proven CAK activity in mammalian cells, although there have been indications for additional mammalian CAKs (Bockstaele et al. 2006, 2009; Kaldis and Solomon 2000).

16.2.5 Cdks in Meiosis

Mammalian meiotic progression, like mitotic progression, is regulated by cyclins and Cdks expressed in mammalian germ cells (Rajesh and Pittman 2006). The generation of knockout mouse models has revealed specific functions for some cyclins and Cdks in meiosis. For instance, *Cdk4*$^{-/-}$ females are sterile due to a decrease in prolactin-producing pituitary lactotrophs (Rane et al. 1999; Tsutsui et al. 1999). *Cdk2*$^{-/-}$ males and females are sterile, and germ cells from these mice do not progress beyond Prophase I (Berthet et al. 2003; Ortega et al. 2003). Among the new members, Cdk14 and Cdk16 show a high level of expression in the testes. Cdk14 is expressed in late pachytene spermatocytes in the testis (Besset et al. 1998). Highest expression for Cdk16 and Cdk18 was observed in postmeiotic spermatids, suggesting a role in germ cell differentiation (Rhee and Wolgemuth 1995).

16.2.6 Emerging Players in Cell Cycle Regulation

The small group of Cdk/cyclin complexes identified so far as cell cycle regulators have provided a framework for how the mammalian cell cycle functions. Future work should determine how newer members fit into this established framework. Many regulators such as those described in previous sections function in other varied cellular processes; however, some such as cyclins F, Y, and J appear to be more likely candidates for cell cycle regulatory roles. Levels of cyclin F oscillate through the cell cycle, peaking in G2, and declining before mitosis; a pattern very similar to that of cyclin A (Bai et al. 1994). However, unlike other cyclins, degradation of cyclin F appears to be independent of proteasome and ubiquitin-dependent pathways (Fung et al. 2002). Functions in the cell cycle are largely unidentified for cyclin F and it remains an orphan cyclin with no known Cdk partners. MEFs lacking cyclin F exhibit reduced population-doubling time and a delay in cell cycle reentry from quiescence (Tetzlaff et al. 2004), indicating a role for this cyclin in the cell cycle. As part of the SCF (Skp/Cul/F-box containing) complex, recent work points toward a role for cyclin F in the degradation of CP110, a protein essential for centrosome duplication (D'Angiolella et al. 2010), during the G2 phase, thereby maintaining mitotic fidelity.

Cyclin Y is reported to interact with Cdk14 (PFTK1) and influences the activity and localization of Cdk14 (Jiang et al. 2009). Based on homology, it is likely that Cdk15 also interacts with cyclin Y, although this has not yet been reported. LRP5/6 (low-density lipoprotein receptor related protein) that bind Wnts are substrates for Cdk14/15 (Davidson et al. 2009). Cdk14/cyclin Y-mediated phosphorylation of LRP5/6 and other substrates may be involved in mitotic activation of Wnt signaling (Kaldis and Pagano 2009).

Cyclin J is a Drosophila cyclin with no known homologs in humans. Use of specific cyclin J-binding aptamers to inhibit cyclin J-associated activity revealed severe mitotic defects and a role for cyclin J in early embryogenesis (Kolonin and

Finley 2000), along with an association to Cdk2. However, Drosophila mutants lacking cyclin J display normal embryogenesis (Althoff et al. 2009). In additional contradiction to the earlier report, cyclin J binding is detected with Cdk1, but not Cdk2. Further, cyclin J is expressed only in females, but is not required for oogenesis. In contrast to other Cdk1 regulators such as cyclins A or B, cyclin J is not degraded during mitosis (Althoff et al. 2009) and its expression is limited to the germline. The lack of any obvious phenotypic defects in these Drosophila mutants lacking cyclin J may indicate functionally redundant pathways, and future efforts should be directed at unraveling the features of this interesting atypical cyclin.

In addition to its role in transcription and RNA processing (see Sect. 16.2.1), Cdk11 is involved in centrosome maturation and spindle formation (Petretti et al. 2006). Cdk11 was also implicated as a microtubule stabilization factor regulating spindle assembly (Yokoyama et al. 2008) and is required for sister chromatid cohesion (Hu et al. 2007).

Apart from its predicted CAK activity (see Sect. 16.2.3), Cdk20 may function in the cell cycle. The use of siRNA against Cdk20 in human cells resulted in impaired cell proliferation and a small increase in cells with sub-G1 content (Liu et al. 2004). Additionally, a specific chemical inhibitor of Cdk family members (RGB-286147) inhibits Cdk20 and promotes apoptosis (Caligiuri et al. 2005). These results together with identification of Cdk20 in an RNAi screen for kinases that inhibit apoptosis, suggest a role for Cdk20 in regulation of apoptosis (MacKeigan et al. 2005). Finally, Cdk20 has been reported to phosphorylate the male germ cell-associated kinase (MAK)-related kinase or MRK (Fu et al. 2006), and a link has been drawn between the two in the regulation of the cell cycle and apoptosis.

16.2.7 The Cell Cycle in Embryonic Stem Cells

Embryonic stem (ES) cells are the in vitro counterparts of the epiblast cells of the early postimplantation embryo. ES cells are pluripotent in that they can generate all cell types of the adult organism, which reflects the central role of the epiblast as the founder tissue of the whole embryo in rodents (Momčilović et al. 2011; Smith 2001; Wang and Blelloch 2011). Mouse ES cells display unusual proliferative properties and an unorthodox cell cycle [reviewed in (Singh and Dalton 2009)]. ES cell derivation does not rely on any immortalizing agent; they cannot enter a quiescence state, they do not undergo senescence, and they can proliferate over many generations. These cells display unusual mechanisms of Cdk activation and a cell cycle composed predominantly of the S phase (Stead et al. 2002). The G1 phase is very short (approximately 1.5 h), during which hypophosphorylated, G1-specific Rb is virtually undetectable (Savatier et al. 1994). The pocket proteins Rb and p107, though expressed in mouse ES cells, are hyperphosphorylated and inactive. E2F transcription factors are thus not subject to pocket protein repression and their target genes are transcribed in a cell cycle-independent manner (Stead et al. 2002; White

et al. 2005). Cdk4 and Cdk6/cyclin D-mediated activation of the Rb-E2F pathway does not appear to regulate the ES cell cycle (Faast et al. 2004; Savatier et al. 1996). Cyclin D1 and D3 are expressed at low levels in ES cells whereas cyclin D2 is not expressed. Low levels of D-type cyclins in ES cells compared to their differentiated derivatives reflects the situation in epiblast cells, which do not express appreciable D-type cyclins until gastrulation commences (Wianny et al. 1998). Cdk2, cyclin A, and cyclin E-associated kinase activities in these ES cells are precocious and cell cycle-independent, while Cdk1/cyclin B1-associated activity is the only one that appears to be cell cycle regulated (Stead et al. 2002; White et al. 2005). As ES cells differentiate, the pathways that maintain pluripotency are transformed to allow for lengthening of the G1 phase and the cell cycle and its regulation begins to resemble that of somatic cells. ES cells have proved to be a valuable system for modeling early embryonic events, and the distinct regulatory mechanisms of ES and differentiated cells reveal the adaptability of the cell cycle during development.

16.3 The Speedy/RINGO Family of Proteins

In addition to activation by cyclins, Cdks can be activated by a novel cell cycle regulator called Speedy or RINGO (Rapid INducer of G2/M progression in Oocytes), despite the lack of any primary sequence homology between Speedy/RINGO proteins and cyclins (Ferby et al. 1999; Lenormand et al. 1999). Speedy proteins were originally identified as regulators of the meiotic cell cycle in *Xenopus* oocytes and could promote G2 to M transition during oocyte maturation. This *Xenopus laevis* gene, called Speedy or Spy1, conferred radiation resistance to fission yeast deficient in the cell cycle checkpoint gene Rad1 (Lenormand et al. 1999). In a separate screen, a protein called RINGO was identified, that when expressed in G2 arrested *Xenopus* oocytes, stimulated the resumption of meiosis (Ferby et al. 1999). RINGO levels in *Xenopus* oocytes are regulated during meiosis by ubiquitin-mediated proteasomal degradation (Gutierrez et al. 2006). Ablation of endogenous RINGO expression in *Xenopus* oocytes resulted in a delay in progesterone-induced maturation, suggesting a role for this protein in the appropriate timing of meiotic maturation. RINGO also associated with and enhanced the kinase activity of Cdk1 (Ferby et al. 1999). These two genes are now known to be the same and are referred to as Speedy/RINGO.

Recently, five different mammalian Speedy family members have been reported (Table 16.3), with different expression profiles (Gastwirt et al. 2007). All members bind to and activate Cdk1 and Cdk2 (Cheng et al. 2005), although with different affinities. A human Speedy/RINGO homologue (Spy1 or Speedy/RINGO A1) appears to be important for S phase entry in cultured somatic cells in a Cdk2-dependent manner (Porter et al. 2002). Overexpression of human Speedy accelerates S phase entry and cell proliferation, and its inhibition by RNAi causes a cell cycle delay at G1/S. Speedy interacts with both Cdk2 (Porter et al. 2002) and

Table 16.3 The Speedy/RINGO family

Gene	Species	Tissue specificity	Associated Cdks	
X-RINGO	*Xenopus laevis*	Oocyte	Cdk1/Cdk2	Ferby et al. (1999)
X-Spy1	*Xenopus laevis*	Oocyte	Cdk1/Cdk2	Lenormand et al. (1999)
Speedy/RINGO A1	Homo sapiens/ Mus musculus	Ubiquitous (high in testis)	Cdk2	Porter et al. (2002)
Speedy/RINGO A2	Homo sapiens/ Mus musculus	Ubiquitous (high in testis)	Cdk1/Cdk2	Kume et al. (2007)
Speedy/RINGO B	Mus musculus	Testis only	Cdk1	Cheng et al. (2005)
Speedy/RINGO C	Homo sapiens	Testis, liver, placenta, bone marrow, kidney, small intestine	Cdk1/Cdk2	Dinarina et al. (2005)
Speedy/RINGO D	Mus musculus	–	–	Dinarina et al. (2005)
Speedy/RINGO E	Homo sapiens	–	Cdk1/Cdk2/ Cdk5	Dinarina et al. (2005)

the Cdk inhibitor, $p27^{Kip1}$ (Porter et al. 2003). The interaction with $p27^{Kip1}$ may suppress $p27^{Kip1}$-mediated inhibition of Cdk2, thereby promoting G1 to S phase transition (Porter et al. 2003). These biological functions of Speedy/RINGO proteins are dependent on Cdk activity since kinase-inactive forms of Cdk1 and Cdk2 abolish the effects of Speedy on cell cycle transitions (Cheng et al. 2005).

16.3.1 Activation of Cdks by Speedy/RINGO Proteins

The binding of Speedy/RINGO proteins to Cdks is mediated through a conserved central domain of about 100 residues called the Speedy-box (Cheng et al. 2005; Dinarina et al. 2005). Mutations in this domain abolish oocyte maturation and lead to impaired binding to and activation of Cdk1 and Cdk2 (Dinarina et al. 2005; Nebreda 2006). While both *Xenopus* and mammalian Speedy/RINGO proteins bind to and activate Cdk1 and Cdk2, it appears that binding to and activation of Cdk2 is more efficient than Cdk1 (Porter et al. 2002). These observations are, however, based on in vitro experiments, and future work with endogenous proteins should provide more accurate information. No interactions have yet been reported with Cdk4 and Cdk6; however, the human Speedy/RINGO E can activate Cdk5 (Dinarina et al. 2005; Nebreda 2006).

Interestingly, unlike Cdk activation by cyclins, activation of Cdks by Speedy/RINGO does not appear to require phosphorylation in the activation loop of the kinase domain (Karaiskou et al. 2001). Speedy proteins can activate Cdk1 and Cdk2 in vitro in the absence of activating phosphorylation of the Cdk (Cheng et al. 2005). Additionally, unlike cyclin-bound Cdk complexes, *Xenopus* Speedy/RINGO-activated

Cdk1 and Cdk2 are less susceptible to inhibition by negative regulators of Cdk activity, such as $p21^{Cip1}$ and Myt1, and may therefore be active under conditions where Cdk/cyclin complexes are inhibited (Karaiskou et al. 2001).

While much investigation has gone into understanding how cyclins affect the substrate choices of Cdks, a beginning has been made with characterizing the substrate specificity of Speedy/RINGO/Cdk2. In vitro assays have revealed that while Speedy/RINGO A2/Cdk2 displays lower activity toward traditional Cdk/cyclin substrates, it exhibits broader substrate specificity. Speedy/RINGO A2/Cdk2 was shown to have low enzymatic activity toward conventional Cdk2/cyclin substrates with the consensus site [S/T]PX[K/R] (Cheng et al. 2005). Speedy/RINGO A2/Cdk2 complexes show nearly 1,000-fold less activity toward histone H1, compared to that of Cdk2/cyclin A complexes, yet display broad substrate specificity with respect to the +3 position of the target sequence. Using GST-tagged pentapeptide substrates of the form KSPRX (where X is any amino acid), Speedy/RINGO A2/Cdk2 tolerated all but three amino acid substitutions at the +3 position. The best substrates contained tyrosine, arginine, and tryptophan, but not lysine as for Cdk2/cyclin A complexes, in this position. Furthermore, the CDC25 phosphatases were found to be phosphorylated tenfold less efficiently by Speedy/RINGO A2/Cdk2 compared to Cdk2/cyclin A (Cheng et al. 2005). In spite of the low in vitro activity of Speedy-activated Cdk2 toward conventional Cdk substrates, phosphopeptide mapping revealed that numerous noncanonical sites were phosphorylated by Speedy/RINGO A2/Cdk2 and not by Cdk2/cyclin A (Gastwirt et al. 2007).

Such differences in substrate preferences and Cdk activation between Speedy/RINGO and cyclin-associated Cdks suggest unconventional roles for Speedy/RINGO members in the regulation of the mitotic cell cycle and the meiotic program. The ability of Speedy/RINGO/Cdk complexes to phosphorylate noncanoniocal Cdk substrates raises the possibility of roles beyond cell cycle control. Efforts at identification of in vivo substrates and regulatory mechanisms of endogenous Speedy/RINGO members will reveal more about the physiological roles of this family.

16.3.2 Speedy/RINGO Proteins and Cancer

The originally characterized Speedy A1 isoform, (Spy1A), is expressed in multiple human tissues and immortalized cell lines (Cheng et al. 2005; Porter et al. 2002). Several immortalized cell systems has shown that overexpression of Speedy promotes a shortening of G1–S phase of the cell cycle, activation of Cdk2, degradation of the CKI $p27^{Kip1}$, and ultimately enhanced cell proliferation (Porter et al. 2003). Importantly, small interfering RNA (siRNA) knockdown of Speedy A1 prevents these events, thereby demonstrating the essentiality of Speedy A1 in cell growth mechanisms (Porter et al. 2002). Furthermore, Speedy A1 has been shown to

override the DNA damage response, functioning to inhibit DNA damage-induced apoptosis (Barnes et al. 2003). Hence, Speedy A1 could play a key role in regulating both cell growth and death processes. Nevertheless, these results will have to be verified in vivo.

16.4 Concluding Remarks

Among the members of the Cdk family, it appears that only a few (Cdk1, Cdk2, Cdk4, Cdk6) are exclusively associated with cell cycle control. In spite of significant homology to these Cdks, the "nonstandard" Cdks such as Cdk7-11 regulate transcription, while others are involved in neuronal function, or roles not directly connected to cell cycle progression. It has been suggested that these "transcription Cdks" coevolved with the C-terminal domain of RNA-polymerase II (Krylov et al. 2003) and originated after the cell cycle Cdks (Krylov et al. 2003). Among the cyclins, Cdk-association and cell cycle involvement are well-established for the A, B, D, and E-type cyclins. Others such as cyclin O and cyclin S do not have known Cdk partners and appear to be involved in DNA repair or memory duration. These varied roles may link transcription, growth signaling pathways, and developmental aspects to the cell cycle, establishing a secure homeostasis network. Regulation for many of these newer Cdks and cyclins is not understood and the functions assigned to some are at best predictive. Identification of binding partners, closer examination of expression and localization patterns, and genetic analyses will bring us closer to understanding their functional relevance.

Speedy/RINGO proteins represent a novel class of Cdk activators that play important roles in cell cycle progression. These proteins are not present in yeast, plants or insects and it is conceivable that Speedy proteins regulate cell cycle progression in all vertebrates. The mechanisms of action conferred by the Speedy/RINGO family represent novel modes by which Cdks are regulated and provide the possibility of multiple Cdk pools with different activation states, substrates, and functions. Cdk activation without requirement of phosphorylation events and in the face of inhibition allows for small pools to be active while still globally restricting Cdk activity.

It has long been known that misregulation of Cdks and cyclins is associated with oncogenesis. Misregulation of Cdk inhibitors such as p21 and p27, as well as inhibition of the tumor suppressor p53 and its pathways, has a strong correlation to cancer (Porter et al. 2003). The inability to properly respond to DNA damage and cellular stress through checkpoint activation and apoptosis has a role in oncogenic potential as well as therapeutic considerations. It is therefore not surprising to find that Speedy overexpression has been found in cancer tissues and cancer cell lines. The loss of control over a molecule like Speedy, which has effects on Cdk activation, growth control, checkpoints, and apoptosis, poses a threat to genomic stability and may be oncogenic in nature (Golipour et al. 2008). Thus, it may prove invaluable to know the implications of the Speedy/RINGO family members in

the diagnosis and treatment of cancer. The outcome of common and experimental chemotherapeutic and anticancer drugs may be greatly influenced by the status of the Speedy/RINGO family members. Future research should define an important role for this novel family of cell cycle regulators in cell biology and cancer biology.

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Chapter 17
Function of the A-Type Cyclins During Gametogenesis and Early Embryogenesis

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Abstract The cyclins and their cyclin-dependent kinase partners, the Cdks, are the basic components of the machinery that regulates the passage of cells through the cell cycle. Among the cyclins, those known as the A-type cyclins are unique in that in somatic cells, they appear to function at two stages of the cell cycle, at the G1-S transition and again as the cells prepare to enter M-phase. Higher vertebrate organisms have two A-type cyclins, cyclin A1 and cyclin A2, both of which are expressed in the germ line and/or early embryo, following highly specialized patterns that suggest functions in both mitosis and meiosis. Insight into their in vivo functions has been obtained from gene targeting experiments in the mouse model. Loss of cyclin A1 results in disruption of spermatogenesis and male sterility due to cell arrest in the late diplotene stage of the meiotic cell cycle. In contrast, cyclin A2-deficiency is marked by early embryonic lethality; thus, understanding the function of cyclin A2 in the adult germ line awaits conditional mutagenesis or other approaches to knock down its expression.

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17.1 Introduction to the A-Type Cyclins

Although cyclin A was the first cyclin identified and subsequently cloned in any organism (Swenson et al. 1986), our understanding of the mechanisms of regulation of expression and function of the A-type cyclins remains somewhat limited. Cyclin A was originally described as a protein that exhibited an unusual expression pattern in clam embryos (Evans et al. 1983). Subsequently, genes for cyclin A have now been found in all multicellular organisms, including humans (Pines and Hunter 1990). While only a single gene encoding cyclin A is present in the genomes of the nematode *C. elegans* and the fruitfly *Drosophila melanogaster*, we discovered that there are two distinct cyclin A genes in the mouse, one of which, *Ccna1*, is testis-specific and restricted to the germ line (Ravnik and Wolgemuth 1996; Sweeney et al. 1996). Two A-type cyclin genes have now been documented in many other organisms, including humans (Yang et al. 1997). The gene encoding the second mammalian A-type cyclin, cyclin A2 (*Ccna2*), is ubiquitously expressed in all proliferating cells and is upregulated in a variety of cancers (Pines and Hunter 1990; Wang et al. 1990).

Cyclin A2 is generally considered to be the critical mammalian S-phase cyclin (Hochegger et al. 2008; Pines and Hunter 1990; Yam et al. 2002), but is unique among the mammalian cyclins in that it is expressed in more than one stage of the cell cycle, specifically in both the S- and G2-phases. Cyclin A2 is induced at the beginning of S-phase (Erlandsson et al. 2000; Girard et al. 1991) and once synthesized, it binds and activates its catalytic partners, the cyclin-dependent kinases (Cdk) Cdk2 and Cdk1. The cyclin A2-Cdk complexes are the machinery that drive S-phase progression, at least in part by phosphorylating proteins that play important roles in DNA synthesis (den Elzen and Pines 2001; Girard et al. 1991; Pagano et al. 1992; Yam et al. 2002; Zindy et al. 1992; Fisher 2011). Its expression at the G2-phase further suggests a second function, involving the entry of cells into mitosis (Swenson et al. 1986). Indeed, injection of anticyclin A2 antibodies into cultured fibroblasts, or inhibition of cyclin A2 function by p21Cip1 during the G2-phase, blocked the progression of cells into mitosis (Furuno et al. 1999; Pagano et al. 1992). These and numerous other studies have led to the current model that the "core" components of the cell-cycle machinery consist of cyclins A and B and their associated Cdks and constitute the critical elements of the cell-cycle engine (Hochegger et al. 2008; Murphy et al. 1997). Perhaps not surprisingly then, targeted mutagenesis of the murine *Ccna2* gene resulted in early embryonic lethality, apparently around the peri-implantation stage (Murphy et al. 1997), as did loss of function of the cyclin B1 gene (Brandeis et al. 1998). However, as discussed in greater detail below, cyclin A2 appears to be dispensable for very early embryonic cell divisions.

17.2 Expression and Function of the A-Type Cyclins During Gametogenesis

17.2.1 Unique Features of Mammalian Gametogenesis from a Cell Cycle Perspective

Gametogenesis in higher organisms affords a unique opportunity for understanding the regulation of both the mitotic and meiotic cell cycles. Given the high level of conservation of key regulatory components across evolutionarily diverse organisms, it is likely that specific proteins will have highly conserved functions during both mitosis and meiosis in mammals, as they do in *Drosophila* and the yeasts in which they have been best characterized. However, it is clear that there are control points and checkpoints in the mitotic and meiotic cell cycles of higher eukaryotes that do not exist in simpler organisms and which further differ between the male and female germ lines (rev. in Handel et al. 1999; Wolgemuth 2002, 2003; Wolgemuth and Roberts 2010). The mitotic divisions of the gonocytes and the signals to enter and progress through meiosis are precisely controlled and exhibit sexual dimorphism with regard to their timing during development. For example, in female mammals, the oogonia commit to meiosis during embryonic development while in the male, this occurs at puberty and throughout adult life. As another example, the oocyte arrests in the diplotene stage of meiosis for a period that can last for months or years depending on the species. Progression through meiosis in the female germ cell pauses again in metaphase II awaiting fertilization to complete the second meiotic division.

In contrast, spermatocytes progress through meiosis with very different temporal hallmarks – once spermatogonia enter preleptotene S-phase, the first and second meiotic divisions proceed without interruption, yielding haploid spermatids which then undergo the dramatic chromatin remodeling events of spermiogenesis. The mitotic divisions that precede meiosis are also rigorously regulated and vary from species to species. The spermatogonial stem cells undergo mitotic divisions wherein one product of this cell division maintains self-renewing, stem cell qualities while the other one goes on to further mitotic divisions and form spermatogonia. Spermatogonia in mammals are usually classified as type A spermatogonia which do not exhibit heterochromatin histologically or B-type spermatogonia which do. In the spermatogonial compartment of mice, the stem cell is designated as A_{single} (A_s) and the subsequent products of mitotic divisions as A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}). These cells can be identified according to their characteristic location on the basal membrane of the seminiferous tubule. The A_{al} cells, 16 in number in the mouse, then undergo six mitotic divisions, yielding the A_1, A_2, A_3, and A_4 followed by Intermediate and type B spermatogonia, which then enter the preleptotene stage of meiosis.

17.2.2 The A-Type Cyclins in the Male Germ Line

The two A-type cyclins exhibit dramatically different patterns of expression: in the mouse, *Ccna2* is ubiquitously expressed in mitotically dividing cells while expression of *Ccna1* is highly restricted, being most abundant in the testis (Sweeney et al. 1996). Within the testis, *Ccna1* is further restricted to the germ line, specifically in stage IX to XII spermatocytes (Sweeney et al. 1996). Human *CCNA1* is also expressed at highest levels in male germ cells, again in pachytene to diplotene spermatocytes and possibly in round spermatids (Liao et al. 2004). We and others have also detected very low levels of cyclin A1 expression in hematopoietic progenitor cells (Ekberg et al. 2004; Kramer et al. 1998; Yang et al. 1997), although the significance of this expression remains to be determined. In addition, although mouse *Ccna1* has also been reported to be expressed in the brain (van der Meer et al. 2004) and ovaries (Sweeney et al. 1996); (and see discussion below), these studies remain to be confirmed.

Ccna1 and *Ccna2* also exhibit a distinct, nonoverlapping pattern of expression during male germ cell development: *Ccna2* expression is downregulated early in the meiotic cell cycle before *Ccna1* is expressed (Ravnik and Wolgemuth 1999; Sweeney et al. 1996). In the adult mouse testis, *Ccna2* is expressed in spermatogonia and preleptotene spermatocytes, suggesting that it could have an S-phase function in both the mitotic cell cycle of spermatogonial germ cells, analogous to its function in somatic cells, but also in germ cells entering meiosis (Ravnik and Wolgemuth 1996, 1999). As discussed in detail below, genetic ablation of cyclin A2 function in the mouse results in early embryonic lethality, thereby precluding our use of conventional gene targeting to elucidate its function in the germ line. In contrast, the strikingly restricted expression of *Ccna1* led us to hypothesize that its primary site of function is in the male germ line, specifically at the first meiotic division.

To test this hypothesis and to begin to address possible redundancy of the two A-type cyclin genes, we generated cyclin A1-deficient mice by targeted mutagenesis of the *Ccna1* gene (Liu et al. 1998a). *Ccna1*$^{-/-}$ mice were overtly healthy and the females were fully fertile; however, the males were sterile. Initial histological and cytogenetic analysis revealed an absence of cells from the late diplotene stage on and no spermatids or sperm were seen. Assessment for apoptosis by TUNEL assay showed a massive wave of cell death in diplotene cells. Markers for gene expression in earlier stages of meiotic prophase did not reveal any differences when compared to wild-type animals and examination of synaptonemal complex preparations appeared indistinguishable between normal and cyclin A1-deficient spermatocytes (Liu et al. 1998a). There was a striking reduction in the activation of the maturation promoting factor (MPF) kinase at the end of meiotic prophase, although both Cdk1 and cyclin B proteins were present. The apparent relatively normal appearance of the meiotic chromosomes was supported by subsequent studies in which pachytene cyclin A1-deficient spermatocytes were artificially driven into a meiotic configuration by treatment with okadaic acid (Liu et al. 2000a). Metaphase

I preparations from mutant and normal spermatocytes appeared similar, with no obvious defects in chiasmata. Cyclin A1 is therefore essential for passage into the first meiotic division in spermatocytes, a function that cannot be complemented by the concurrently expressed B-type cyclins (Chapman and Wolgemuth 1993).

Nickerson et al. were able to pinpoint the time of arrest in the cyclin A1-deficient spermatocytes to the late diplotene stage of meiosis after the resolution of chiasmata (Nickerson et al. 2007). Use of antibodies against the synaptonemal complex protein 3 (SCP3), which forms part of the lateral element of the synaptonemal complex (Lammers et al. 1994) and antibodies that recognize centrosomes, the human CREST autoimmune antisera (Earnshaw and Rothfield 1985), which are believed to recognize at least in part the CENP-A, B and C proteins (Brenner et al. 1981), enabled careful staging of chromosome spreads prepared from day 17, 21, and 28 wild-type and *Ccna1*$^{-/-}$ mice. This analysis revealed that there was normal meiotic progression until middiplotene and normal formation and resolution of chiasmata. However, instead of proceeding through diakinesis and forming metaphase I bivalents, the cyclin A1-deficient spermatocytes arrested and underwent apoptosis. Interestingly, this arrest is distinct from the meiotic arrest observed in mice deficient in the putative cyclin A1 kinase partner, Cdk2, at several levels. First, Cdk2-deficient mice exhibited meiotic defects in both the male and female germ lines (Ortega et al. 2003). Second, the arrest was observed in mid-pachytene spermatocytes and third, the pachytene chromosomes exhibited thin threads of SCP3 staining, perhaps indicating aberrant pairing (Ortega et al. 2003). In contrast, pachytene chromosomes in cyclin A1-deficient mice were normal with respect to staining of SCP3 (Nickerson et al. 2007) and were able to form apparently normal-appearing meiotic metaphase 1 bivalents upon treatment with okladaic acid to drive exit from prophase (Liu et al. 2000b).

Other interesting features of the arrested spermatocytes included a characteristic clustering of centromeric heterochromatin and the subsequent appearance of γ-H2AX foci, first at the centromere and then along the chromosomal axes (Nickerson et al. 2007). This unusual clustering preceded hallmarks of apoptosis, such as phosphorylation of Ser139 in γ-H2AX, which curiously appeared to begin at the centromeres and then spread along the length of the chromosomes. We speculated that this clustering of centromeric heterochromatin may represent mislocalization or aberrant association of centromeres and noted that it would be of great interest to know whether such clustering occurs in other mouse models exhibiting meiotic prophase arrest and apoptosis, such as the *Cdk2* (Ortega et al. 2003), *Spo11* (Baudat et al. 2000), *Mlh1* (Edelmann et al. 1996), and *Atm* (Xu et al. 1996) knockout mice.

Our earlier studies at the immunohistological level had revealed a clear nuclear localization of cyclin A1 in spermatocytes from midpachytene through diplotene (Ravnik and Wolgemuth 1996; Liu et al. 1998a). Subsequent analysis of meiotic chromosome preparations revealed a diffuse staining of chromatin but also specific cyclin A1 localization at foci in the pericentromeric region at late diplotene, coincident with the point of arrest in cyclin A1-deficient mice (Nickerson et al. 2007). Concomitant examination of the chromosomal distribution of Cdk2, a putative binding partner for cyclin A1 (Joshi et al. 2009; Liu et al. 1998b, 2000a),

revealed a distribution of Cdk2 protein in the centromeric region at telomeres and at foci along chromosomes during pachytene to diplotene (as reported by Ashley et al. (2001)). Interestingly, despite some overlap, cyclin A1 did not completely colocalize with its putative Cdk2 partner at the centromeres. Furthermore, the distribution of Cdk2 was not altered in cyclin A1-deficient mice.

Also of interest was the observation of an apparent lack of histone H3 serine 10 phosphorylation in the cyclin A1-deficient spermatocytes (Nickerson et al. 2007). Histone H3 becomes phosphorylated at serine 10 at the pericentromeric region in late diplotene of male meiosis and this phosphorylation persists through metaphase I (Cobb et al. 1999). Examination of histological sections of testes and of chromosome spread preparations revealed that phosphorylation of H3 serine 10 was dramatically reduced in heterozygous *Ccna1*$^{+/-}$ spermatocytes and undetectable in homozygous *Ccna1*$^{-/-}$ spermatocytes that are completely devoid of cyclin A1 (Nickerson et al. 2007). The kinase that performs this phosphorylation in both mitosis and meiosis is thought to be the aurora B kinase, a component of the passenger protein complex that also includes INCENP, survivin, and borealin (Vagnarelli and Earnshaw 2004). Aurora B kinase in particular is believed to be critical for correct chromosome alignment at metaphase (Ditchfield et al. 2003). The reduction of this phosphorylation in *Ccna1*$^{+/-}$ spermatocytes and its absence in *Ccna1*$^{-/-}$ spermatocytes prompted us to examine this complex in more detail (Nickerson et al. 2007). We found the levels and distribution of survivin to be indistinguishable between *Ccna1*$^{+/-}$ and *Ccna1*$^{-/-}$ spermatocytes when compared to *Ccna1*$^{+/+}$. In contrast, while immunoblot analysis of whole testicular lysates did not indicate a significant difference in levels of aurora B protein between control and cyclin A1-deficient testicular lysates, the amount of aurora B protein associated with meiotic chromosomes was strikingly different. In particular, we observed a pronounced reduction in staining intensity of aurora B protein localizing to the pericentromeric heterochromatin in late diplotene mutant spermatocytes. This suggests that in the absence of cyclin A1 protein, aurora B does not localize properly, likely contributing to the failure to complete the first meiotic division.

A second targeted mutant allele of *Ccna1* has been generated by the insertion of a *Lac Z* reporter gene (van der Meer et al. 2004), in order to disrupt cyclin A1 production. In this case, the entire cyclin A1 coding region is still in the genome rather than having deleted required coding regions. An unusual splicing event that deleted lacZ sequences could theoretically restore functional cyclin A1 protein, although this did not occur in this case. The males carrying this mutation were also sterile and exhibited genetic strain-dependent differences in the fertility of the heterozygous mice as well. That is, when these *Ccna1*$^{+/-}$ mice were on a mixed background of 129S6/SvEv and MF1, they were reported to have "reduced sperm production and fertility" as compared to mice carrying the wild-type *Ccna1* allele (van der Meer et al. 2004). Furthermore, mice that were heterozygous for this knockout on a pure 129S6/SvEv background were reported to be sterile due to a greatly reduced production of sperm. To the best of our knowledge, there has been no further characterization of this phenotype, and we have never observed this reduced fertility in our *Ccna1*$^{+/-}$ mice on a mixed C57Bl/6 and 129SvEv background (Liu et al. 1998a).

Finally, as mentioned above, the lack of cyclin A1 in spermatocytes resulted in a fully penetrant induction of apoptosis. However, it was not clear whether this induction represented a primary response to a lack of cyclin A function or a secondary response to a general degeneration of the highly structured seminiferous tubules. We therefore undertook studies examining the induction of apoptosis during the first wave of spermatogenesis in cyclin A1-deficient testes (Salazar et al. 2005). The temporal appearance of cell death was observed to coincide with the G2/M cell cycle arrest that occurred in late diplotene spermatocytes, suggesting that apoptosis was in fact a primary response to the cell cycle arrest. That is, at the time when the first wave of differentiating cells should be completing the first meiotic division, significantly higher numbers (by almost twofold; $p < 0.01$) of TUNEL-positive pachytene spermatocytes were observed in the cyclin A1-deficient as compared to the control testes. It was also shown that caspase 3 was clearly involved in apoptosis occurring in the cyclin A1-deficient spermatocytes, as increases in the amount of the procaspase protein and changes in the subcellular distribution of the activated form were observed.

Apoptosis in the testis can involve both cell-intrinsic (Bcl-2 family-mediated) and/or cell-extrinsic (Fas-mediated) pathways (Beumer et al. 2000; Lee et al. 1997). Some proteins of the cell-intrinsic pathway, the Bcl-2 family of proteins, including the proapoptotic protein Bax, are essential for normal spermatogenesis. Bax-deficient testes are characterized by an accumulation of spermatogonia, consistent with a proposed failure of germ cell death during the first wave of spermatogenesis (Knudson et al. 1995). The preleptotene spermatocytes failed to undergo meiosis, presumably because of the resulting aberrant Sertoli cell to spermatocyte ratio. Immunohistochemical analysis in cyclin A1-deficient testis showed an increase of Bax-positive spermatocytes and a redistribution of localization of Bax protein from a cytoplasmic to perinuclear and nuclear localization. A positive correlation of the detection of Bax expression and TUNEL-positive cells was also observed. Thus, apoptosis that occurs in the absence of cyclin A1 at least in part involves Bax signaling.

The role (if any) of p53 in regulating cell death in the absence of cyclin A1 in vivo was addressed by producing mice that were both cyclin A1- and p53-deficient (Salazar et al. 2005). Loss of p53 gene function could not rescue the cell cycle arrest in *Ccna1*$^{-/-}$ mutant testis; however, there was a significant reduction in the apoptotic index in the doubly mutant tubules. This observation led us to suggest that cyclin A1 may have roles in regulating two signaling cascades – one leading to progression through meiosis and a second function in regulating apoptosis in spermatocytes. The fact that there were apoptotic cells in the double mutant indicated that cell death in response to cyclin A1-deficiency also involved a p53-independent pathway(s). The mutant mice generated by van der Meer and colleagues (van der Meer et al. 2004) were also used in a study in which double cyclin A1 and p53-deficient mice were produced (Baumer et al. 2007). It was reported that these mice had increased numbers of giant cells in the testicular tubules, although the significance of this phenomenon was not pursued. The induction of an apparent apoptotic response (TUNEL-positive) and the appearance of giant cells has been

seen in various other gene knockout studies that resulted in impaired meiosis [rev. in (Salazar et al. 2003)]. It would thus appear that the induction of cell death is critical for insuring not only that the proper number of germ cells is produced but also that germ cells that have not gone through the proper reduction divisions of meiosis cannot form viable gametes.

17.2.3 The A-Type Cyclins in the Female Germ Line

Cyclin A2 has been shown to be present in protein extracts from total adult mouse ovary, (Sweeney et al. 1996) and cyclin A2 mRNA and protein were detected in fully grown oocytes (Winston et al. 2000; Fuchimoto et al. 2001). To elucidate the pattern of cyclin A2 expression throughout ovarian development, with a particular focus on the germ line, we undertook a detailed analysis of embryonic to adult ovaries using in situ hybridization, immunohistochemistry, and immunoblotting analysis (Persson et al. 2005). The progression of folliculogenesis in the adult mouse ovary can be staged according to oocyte size, morphological characteristics. and number of layers of the surrounding follicle cells (Pedersen and Peters 1968). In situ hybridization results showed that while *Ccna2* transcripts were detected in granulosa cells at all stages of follicular development, the expression was low in early stage follicles (stages 1–5), according to Pedersen and Peters (1968) and was much higher in growing follicles, particularly in the cumulus layer (cells immediately surrounding the oocyte) in stage 6–8 follicles. It should be recalled that oocytes are arrested at this time, in the diplotene stage of meiosis. However, *Ccna2* transcripts were present in oocytes at all stages of folliculogenesis, from the very early resting follicles to stage 6 and 7 follicles as well as in ovulated eggs within the oviductal ampulla. Immunohistochemistry revealed that the cellular distribution of cyclin A2 protein was similar to that observed for *Ccna2* transcripts, suggesting that cyclin A2 expression is not regulated at the level of translation. The levels of cyclin A2 expression in granulosa cells increased with follicular growth and differentiation whereas more mature oocytes contained less cyclin A2.

Examination of fetal ovaries between embryonic day (E) 13.5–18.5 by immunohistochemistry revealed that cyclin A2 protein was indeed expressed and, further, that the pattern of its distribution changed during development. At E13.5–E14.5, cyclin A2 was detected in mitotically active somatic cells, as well as oogonia and early meiotic oocytes, and was predominantly nuclear. This nuclear localization of cyclin A2 continued through E15.5, when germ cells enter meiotic prophase and are presumably leptotene or zygotene oocytes. Interstingly, at E16.5, about half of the oocytes still displayed nuclear cyclin A2 while in the remaining oocytes, it became predominantly cytoplasmic. At E18.5, when the majority of oocytes are in the pachytene to diplotene/dictyate stage, the localization was predominantly cytoplasmic with only weak nuclear staining.

The high levels of expression and nuclear localization of cyclin A2 protein in the embryonic ovary suggested that it is active in germ cells as they undergo

proliferation and enter into meiotic prophase. It is interesting to compare the striking change from a robust nuclear localization in mitotic oogonia to a predominantly cytoplasmic localization in oocytes meiotic prophase to the cyclin A2 expression in the corresponding stages in spermatocytes. The localization of cyclin A2 is always predominantly nuclear in mitotically proliferating spermatogonia and in preleptotene spermatocytes (Ravnik and Wolgemuth 1999). Cyclin A2 is then undetectable in leptotene, zygotene, pachytene, and diplotene stages of meiotic prophase (or later stages), but in late meiotic prophase, the novel cyclin A1 is expressed (Sweeney et al. 1996). We have speculated that germ cells need to exclude or prevent A-type cyclins from being active during the stages in which the cells are undergoing the "business" of meiosis, so as to prevent premature entry into a meiotic division while in the process of pairing, recombination, repair, etc. (Liao et al. 2005). We further suggest that spermatocytes could achieve this by degrading cyclin A2 and then activating cyclin A1, whereas the oocyte could sequester cyclin A2 to the cytoplasm. A differential sub-cellular distribution of cyclin A1 has also been observed in specific circumstances. While cyclin A1 is normally nuclear in both mouse (Liu et al. 1998a) and human (Liao et al. 2004) late prophase spermatocytes, it was found to be predominantly cytoplasmic in leukemic cells in a transgenic mouse model (Liao et al. 2001) and leukemic cells from patients (Ekberg et al. 2004).

One final note with regard to the two potential cyclin-dependent kinase partners of cyclin A2: readily detectable expression of both Cdk1 and Cdk2, two common partners for the A-type cyclins, was observed in granulosa cells and oocytes at all stages of folliculogenesis (Persson et al. 2005). Cdk1 was predominantly cytoplasmic, whereas Cdk2 was both cytoplasmic and nuclear in oocytes.

And what about a function for cyclin A1 in mouse oocytes? The presence of cyclin A1 protein had been reported in ovulated mouse oocytes in one study (Sweeney et al. 1996) and in another, both mRNA and protein were purported to be present in germinal vesicle-intact oocytes and to decline during meiotic maturation (Fuchimoto et al. 2001). The significance of these observations is unclear, given that the cyclin A1-deficient female mice are fully fertile (Liu et al. 1998a; van der Meer et al. 2004), and we have not detected *Ccna1* mRNA by in situ hybridization or immunohistochemistry in sectioned ovarian tissue (Ravnik S, Persson CL, Wolgemuth DJ, unpublished observations). To resolve the question of cyclin A1 expression in a definitive manner, immunoblot analysis was performed on extracts from total ovaries and oocytes from cyclin A1-deficient mice (and controls) using anticyclin A1 antibodies (Persson et al. 2005). The results revealed the presence of a weakly cross-reacting band, close in size to *bona fide* cyclin A1, in extracts from ovary, isolated oocytes, 1-cell, and 2-cell embryos. This same band was also detected in the testis of cyclin A1-deficient mice, ruling out the possibility its being *bona fide* cyclin A1. Further, in the aforementioned targeted mutagenesis experiment of van der Meer and colleagues (van der Meer et al. 2004), there was no beta-galactosidase expression in the adult ovary. This further suggests that the *Ccna1* gene was not being transcribed, an observation consistent with our conclusions that neither *Ccna1* mRNA nor cyclin A1 protein

are expressed in oocytes. Expression of beta-galactosidase was reported to be expressed in a few regions of the adult mouse brain in this same study; however, no studies elucidating the physiological relevance of this putative expression have been reported.

Finally, since cyclin A1 is expressed during meiotic prophase in the male germ line, we examined histological sections of embryonic ovaries using anti-cyclin A1 antibodies (Persson et al. 2005). Adult testis sections were included on the same slides as positive controls. No specific staining for cyclin A1 protein was found in embryonic ovaries from E13.5 through 18.5, the latter stages of which clearly contain pachytene and diplotene oocytes. We therefore conclude that, consistent with the results of our targeted mutagenesis experiments (Liu et al. 1998a), cyclin A1 is neither expressed during oogenesis nor required for oocyte function, at least in the mouse model.

17.3 The Early Mouse Embryo and the A-Type Cyclins

Several studies have examined expression of the A-type cyclins in the early embryo, to determine both the contribution, if any, of maternal stocks of mRNA and protein to early embryonic mitotic divisions, as well as to determine when the embryonic cyclin A2 gene is activated. Levels of cyclin A2 protein were reported to decrease with the progression of the oocyte through germinal vesicle breakdown and meiotic maturation (Fuchimoto et al. 2001). However, both maternally produced cyclin A2 mRNA and protein persist through the first mitotic division following fertilization and the activation of transcription of the embryonic genome at the two-cell stage (Winston et al. 2000). They are then reduced to undetectable levels in embryos between the two- to four-cell divisions. Using the model in which *LacZ* was inserted into the endogenous *Ccna2* gene (Murphy et al. 1997), clear evidence for *Ccna2* activation was observed in blastocysts (Winston et al. 2000). In this same study, cyclin A2 protein was also detected by immunofluorescence in individual blastomeres of 8-cell embryos.

The significance of this expression is not clear, however, in light of the very dramatic phenotype seen in cyclin A2-deficient embryos: early embryos divide perfectly well up to the blastocyst stage, but undergo a demise at the time of implantation (Murphy et al. 1997; Winston et al. 2000). The same early embryonic lethality was observed in recent conditional knockout strategies of the cyclin A2 gene (Kalaszczynska et al. 2009). So clearly, cells of the early embryo can undergo mitotic cell division in the complete absence of cyclin A2. These embryos can implant as assessed in vivo and in vitro by blastocyst outgrowth assays but die shortly thereafter (Winston et al. 2000). In addition, there are no obvious differences in their ability to undergo DNA replication, as assayed by BrdU incorporation at the blastocyst stage.

17.4 Regulation of Expression of the A-Type Cyclins in the Germ Line

As noted above, the two A-type cyclins exhibit strikingly different patterns of expression from one another that also differ between the male and female germ line cells. We therefore hypothesized that there will be specific regulatory elements unique to each A-type cyclin that are critical for their distinct regulation of expression. The essential role of *Ccna1* in male germ cell development and the concurrent expression of cyclin A1 mRNA and protein suggested the importance of understanding mechanisms controlling transcription of the *Ccna1* gene. The lack of cell lines derived from male germ cells made it necessary to analyze the *Ccna1* promoter in these cells in vivo in transgenic mice (Lele and Wolgemuth 2004). Serial deletions in the *Ccna1* upstream sequence allowed us to define two functional segments of the promoter.

Analysis of transgenes carrying the genomic fragment of mouse *Ccna1* spanning −1.3 kb to +0.8 kb (designated 1.3cyA1lacZ) of the putative transcriptional start site, using a combination of X-gal staining and in situ hybridization, revealed expression specifically in spermatocytes at stages IX to XII of the cycle of the seminiferous epithelium (Lele and Wolgemuth 2004). No expression was observed in spermatogonia or earlier meiotic stages, a pattern that is similar to the narrow window of expression during spermatogenesis seen for the endogenous mouse gene. In contrast, 1.3 kb of human *CCNA1* promoter directs expression of EGFP in a much less restricted pattern in male germ cells (Muller-Tidow et al. 2003), reflecting differences in the mouse and human promoters despite their sharing highly conserved regions.

Within this fragment of the mouse gene are consensus sequences for two sets of paired CDE/CHR elements (Lele and Wolgemuth 2004). These elements were first discovered in the proximal promoter of the *CCNA2*, *CDK1*, and *CDC25C* genes, which are expressed in S- and G_2-phases of the mitotic cell cycle, and are believed to control the timing of expression of these genes during the cell cycle (Zwicker et al. 1995). In vivo footprinting of the *Ccna2* proximal promoter revealed that the bipartite element was occupied at stages when the gene was not transcribed. Mutation of the CDE/CHR element in the context of *CCNA2* or *CDK1* promoter/reporter genes caused derepression of the reporters in G_1-phase. These elements have now been shown to be involved in controlling the timing of expression of other cell cycle-regulated genes, including the genes for cyclin B2 (Lange-zu Dohna et al. 2000), rabkinesin6 (Fontijn et al. 2001), polo-like kinase (Uchiumi et al. 1997), p130 (Fajas et al. 2000), m-survivin (Otaki et al. 2000), and aurora A (Tanaka et al. 2002). Also, the CDE/CHR element appears to downregulate expression of the human *CDK1* gene in response to TPA-induced differentiation of U937 cells (Sugarman et al. 1995) and in response to p53-dependent DNA damage (Badie et al. 2000).

We further observed that the promoters of the genes for mouse and rat cyclin A1 are unique from the human in that they contain two sets of CDE/CHR elements, but

like the human gene, have unpaired CDE and CHR consensus sequences (Lele and Wolgemuth 2004). There have been no previous reports of unpaired CDE elements, but the promoters of human *CCNB2* (Wasner et al. 2003) and mouse C*dc25C* (Haugwitz et al. 2002) are regulated by CHR elements that are not paired with functional CDE elements. Factors that bind CDE/CHR (Liu et al. 1997) or CHR (Kishore et al. 2002; Philips et al. 1999) have been detected in various cultured cell lines, but have not been identified. Although there are many studies examining the regulation of expression of the human and mouse cyclin A2 genes in various cell lines (rev. in Blanchard 2000), to the best of our knowledge, nothing is known with regard to the regulation of their expression in the male and female germ lines.

17.5 Insight from Other Model Organisms

17.5.1 An Evolutionary Perspective

The processes of gametogenesis, especially in the female germ line, result in oocytes with strikingly different morphologies among various species (e.g., a small, transparent mouse egg vs. a large, opaque frog egg!). Yet the overall goal to produce haploid cells involves common strategies. It has been suggested previously that several aspects of premeiotic germ cell development are in fact widely conserved (Pepling et al. 1999). The genes responsible for many of these processes may also be conserved across species, and thus functions identified for a gene in one species at the very least provide a framework within which to address function in other more complex models. The same is true for early embryogenesis – the syncitial early *Drosophila* embryo may seem a world apart from the totally cellularized early mouse embryo – yet as far as cell cycle control is concerned, many of the same regulators and machinery are not only present but are critical for normal development.

17.5.2 Drosophila

The *Drosophila* genome contains a single A-type cyclin first identified by Lehner and O'Farrell (1989) who further showed that its expression from the zygotic genome was essential for cell division after the maternal stores of cyclin A were exhausted. Both cyclin A and B proteins are distributed evenly throughout the embryo, but cyclin A becomes more concentrated in the cortex region at the blastoderm stage (Maldonado-Codina and Glover 1992). In the early stages of zygotic divisions (cycles 4–6), cyclin A localization is strongly nuclear during interphase with only low levels detected in the cytoplasm (Stiffler et al. 1999). Several cycles later, its subcellular distribution is predominantly cytoplasmic

during interphase, although there is some weak punctuate localization in the nucleus as well (Maldonado-Codina and Glover 1992). As the cell prepares to divide, cyclin A associates with the condensing chromosomes and subsequently segregates into the daughter nuclei during anaphase. After the separation of the two daughter nuclei, cyclin A is not degraded but rather returns to the cytoplasm. It should be recalled that the *Drosophila* embryo at this stage is syncitial, and interestingly there is little degradation of the cyclins until the embryo is cellularized (Maldonado-Codina and Glover 1992).

As far as cyclin A's function in the *Drosophila* germ line, it is clearly expressed in a subset of cells in both the stem cells and growing cystocytes of the germaria of adult females (as are cyclin B and cyclin E) (Lilly et al. 2000). Cyclin A was surprisingly found at the site of the fusome, a structure that is rich in vesicles, passes through ring canals, and physically connects all the cystocytes within a single cyst (de Cuevas et al. 1997; de Cuevas and Spradling 1998; Kishore et al. 2002). This association exhibits a periodicity and occurs during the late S to G2 stage of the cell cycle. Overexpression of cyclin A in the cystocytes results in an extra round of mitotic divisions and cysts with 32 rather than 16 cells (Lilly et al. 2000). Association of cyclin A with this structure is also present in male cystocyte mitotic divisions.

In early stages of meiotic prophase in developing fly oocytes, cyclin A protein levels have been reported to be at the posttranslational level by deadenylation of its mRNA (Morris et al. 2005) and by repression of translation by the Bruno protein during the arrest at the end of meiotic prophase (Sugimura and Lilly 2006). As meiosis resumes during meiotic maturation, cyclin A mRNA is repolyadenylated and the Bruno repressor is lost (Vardy et al. 2009). The cyclin A protein that is now made is also phosphorylated at multiple sites, likely involving autophosphorylation. Translation of cyclin A mRNA is also promoted by the PAN GU (PNG) kinase at this stage and at earlier stages of oogenesis. After the completion of meiosis, PNG kinase appears to promote further polyadenylation of the cyclin A mRNA. Cyclin A is degraded by the anaphase promoting complex/cyclosome (APC/C) at the end of the first meiotic division (Pesin and Orr-Weaver 2007). Such tight regulation of cyclin A levels is certainly consistent with our hypothesis outlined above regarding the importance of strict regulation of the A-type cyclins in mammalian gametogenesis, particularly during meiosis, and the likely occurrence of this regulation at various levels.

17.5.3 Xenopus

The *Xenopus* oocyte has been a major source of obtaining extracts for many of the pioneering studies identifying factors involved in cell cycle function and regulation (for example, Strausfeld et al. 1996). It has further been suggested that the early *Xenopus* embryo represents "a unique developmental context" in which to investigate the role of the A-type cyclins, particularly their function in both cellular

proliferation and cell death (Carter et al. 2006). After fertilization, a rapid series of cell divisions characterizes proliferation in the early frog embryo, until the developmental stage known as the midblastula transition (MBT) is achieved. These early cell divisions consist of rapidly alternating S- and M-phases, with essentially no G-phases, and are driven by cyclin E/Cdk2 complexes which control S-phase, and by a combination of cyclin A1/Cdk1 and cyclin B/Cdk1 which regulate the entry into M-phase (Hartley et al. 1996, 1997; Kimelman et al. 1987; Newport and Kirschner 1982; reviewed by Gotoh et al. 2011). Interestingly, in *Xenopus* egg extracts, both cyclin A1 and cyclin E have been shown to have S-phase promoting activity (Strausfeld et al. 1996). The regulation of their synthesis and turnover is quite distinct at these stages. Cyclin A1 and cyclin B are translated from maternally stored mRNAs and are degraded at each division and synthesized anew (Hartley et al. 1996). In contrast, cyclin E protein is maternally derived and is not turned over, but rather remains constant until the MBT (Hartley et al. 1997). The presence of large stores of cyclin A1 in the frog oocyte is in striking contrasts to its complete absence in mouse oocytes (discussed above). In fact, this was initially confusing because at the time of discovery of two A-type cyclins in *Xenopus*, only a single cyclin A had been identified in the mouse and human genomes (discussed in Sweeney et al. 1996).

At the MBT, the cell cycle and its machinery undergo distinctive remodeling. While cyclin A1 is the major A-type cyclin during the first 12 mitotic cell cycles, at the MBT, the zygotic genome encoded cyclin A2 gene is activated (Howe et al. 1995). This cyclin A2 is clearly related to murine and human cyclin A2 (Howe et al. 1995; Strausfeld et al. 1996; Sweeney et al. 1996), and as noted above, it is the only A-type cyclin present in mammalian oocytes. In *Xenopus*, cyclin A1 appears to form a complex with Cdk1, but not Cdk2, while cyclin A2 interacts with both Cdks (Minshull et al. 1990; Strausfeld et al. 1996). In mammals, cyclin A1 can interact with both Cdk1 and Cdk2 in vitro (Joshi et al. 2009) and in vivo (Liu et al. 2000a). When *Xenopus* embryos are exposed to ionizing radiation prior to the MBT, however, cyclin A1 protein was shown to persist beyond the MBT and further to complex with both Cdk1 and Cdk2 (Anderson et al. 1997). This may suggest that, depending upon the physiological context, cyclin A1 in *X. laevis* can complex with both of these Cdks, as it does in the mammalian system.

17.6 Unanswered Questions and Future Directions

17.6.1 What Are the Critical Interacting Proteins and Substrates of Cyclin A1 and A2 in the Germ Line and Early Embryo?

There have been a number of studies identifying proteins that interact with cyclin A2/Cdk2 complexes, particularly in cultured cell lines (Suryadinata et al. 2011). Much less is known about the interacting proteins for cyclin A2 in germ cells and almost nothing is known about interacting proteins for cyclin A1, due to the very

limited tissue distribution of its expression. Although several approaches have been used to identify proteins that interact with cyclin A1 and cyclin A1-Cdk complexes, almost all studies have been limited to identifying partners and substrates in the myelocytic leukemia cell lines where human cyclin A1 is known to be upregulated (Yang and Kornbluth 1999; Yang et al. 1997). For example, using a yeast triple hybrid strategy, several known proteins including Ku70 as well as several less-well characterized proteins including INCA1, KARCA1, and PROCA1, were pulled out in the screen and confirmed by GST pulldown experiments (Diederichs et al. 2004). The Ku70 DNA repair protein was of particular interest, as it was subsequently reported that cells lacking cyclin A1 were deficient in the repair of double-strand breaks (Muller-Tidow et al. 2004). A possible physiological relevance to the function of cyclin A1 interacting proteins in spermatogenesis was provided by the observation that INCA (inhibitor of Cdk interacting with cyclin A1) interacts with a novel testis protein, RSB-66 (Chen et al. 2008). However, RSB-66 was reported to be most abundant in round spermatids, where cyclin A1 is expressed at low levels, if at all. Clearly an area in much need of investigation is the identification of physiologically relevant interacting proteins and potential substrates for cyclin A1-Cdk (and cyclin A2-Cdk) complexes in vivo.

17.6.2 Could the A-Type Cyclins Have Cdk Independent Functions in the Germ Line?

The observations that (a) both cyclin A1 and Cdk2-deficient mice exhibited defects in spermatogenesis but with distinctive patterns, (b) cyclin A1 and Cdk2 proteins do not completely colocalize in wild-type spermatocytes, and (c) Cdk2 localization did not change in prophase spermatocytes that lack cyclin A1, raises the possibility that cyclin A1 may interact with proteins other than its predicted Cdk partner. There is increasing evidence for Cdk-independent functions for cyclins: in addition to activating Cdks, cyclins can interact with other proteins, and thus, have very different functions. It has actually been recognized for some time now that the D-type cyclins can serve as coactivators or corepressors of tissue-specific transcription factors (Zwijsen et al. 1997). Studies from the lab of Piotr Sicinski further showed that E-type cyclins also have kinase-independent functions (Geng et al. 2007), which is of particular interest since both the E-type cyclins, like the A-type cyclins, partner with Cdk1 and Cdk2.

17.6.3 Does Cyclin A2 Play an Important Role in the Germ Line?

The early embryonic lethality exhibited by cyclin A2-deficient mice has precluded our understanding of its potential functions in the male and female germ line (and in

any other adult tissue and lineages for that matter). It was therefore of great interest that mice carrying floxed alleles of the *Ccna2* gene were recently generated and, in combination with the *Ccna1*$^{-/-}$ strain, used to explore the function of the A-type cyclins in fibroblasts, embryonic stem cells, and in the hematopoietic lineage (Kalaszczynska et al. 2009). Mouse embryonic fibroblasts (MEFs) lacking both cyclin A1 and cyclin A2 were surprisingly capable of apparently normal cellular proliferation, due at least in part to the upregulation of cyclin E1. In this same study, a tour de force set of experiments involving complex breeding schemes, tetraploid blastocyst complementation rescue, and deletion of floxed *Ccna2* alleles generated MEFs that were devoid of cyclin A1 and A2 as well as cyclin E1 and E2. These MEFs were incapable of proliferation and DNA synthesis was virtually abolished, suggesting that either cyclin A2 or an E-type cyclin is necessary for S-phase.

The next question: what about the requirement for cyclin A2 in other cell types? In contrast to the ability of MEFs to upregulate cyclin E1 and compensate for loss of the A-type cyclins, both hematopoietic and embryonic stem cells could not proliferate in the absence of cyclin A2 (Kalaszczynska et al. 2009). It will be of great interest to use transgenic strains of mice that express Cre recombinase early in the male germ line, such as the Ngn3-Cre (Yoshida et al. 2004) or Stra8-Cre (Sadate-Ngatchou et al. 2008) mice, or GDF-9-iCre mice in the case of the female germ line (Lan et al. 2004), to elucidate the requirement for cyclin A2 function in these cells. These studies are already underway in our laboratory in collaboration with the Sicinski lab.

17.6.4 Is There a Role for Cyclin A1 in Human Infertility?

The specificity of the cyclin A1-deficient phenotype has led us and others to consider loss of function of the human *CCNA1* gene as both being involved in cases of infertility in otherwise healthy men and conversely serving as a potential target for male contraception. One such study proposed to use the expression of cyclin A1 as a new molecular diagnostic marker, noting that cyclin A1 expression was absent in the majority of cases of Sertoli cell-only syndrome and only very low levels were detected in specimens with spermatogonia only or where spermatogenesis arrest occurred at the level of primary spermatocytes (Schrader et al. 2002). This is, of course, what one would predict for any late male meiotic prophase-specific marker. A second study was undertaken to screen for mutations in the human *CCNA1* gene in 347 infertile men from a western Chinese cohort that might be causal for their infertility (Zhoucun et al. 2009) While 4 point mutations were identified in the exon-screening, none resulted in changes in amino acids, and not surprisingly, no association with impaired spermatogenesis could be detected. The authors suggested that mutations in the cyclin A1 gene are not likely to represent a frequent cause of male idiopathic infertility.

Several years ago, we undertook an analogous screen of DNAs from infertile men of predominantly European origin (Mandon-Pepin et al. 2002). We identified

a mutation in exon 6 in one of the alleles of human *CCNA1* in an infertile patient (Wolgemuth DJ et al., unpublished observations) which was not seen in any of the other 100 nor in DNAs from 35 ethnically matched fertile men. The change would putatively cause an alteration in the corresponding amino acid from arginine to glutamine. The changed amino acid has two interesting features that suggest functional importance. In cyclin A2 from frog, human, and mouse, and in cyclin A1 from frog, it is lysine, whereas in human and mouse cyclin A1, it is arginine. Both of these amino acids are highly basic and represent putatively conservative functional substitutions. In the infertile patient, it is glutamine, a nonconservative change. The crystal structure of the cyclin A2 protein has been solved, alone, in association with one of its catalytic partners, Cdk2, and also in the presence of an inhibitor of the cyclin A2/Cdk2 complex, p27 (Noble et al. 1997; Russo et al. 1996). In cyclin A2, the corresponding mutated amino acid would be present in an alpha helix that is involved in protein–protein interactions. We suggest that there is simply too little information available to assess the significance of mutations in human cyclin A1 in cases of idiopathic male sterility.

17.7 Conclusions

In summary, the cyclin A proteins of higher eukaryotes are unique at several levels. They function at two critical stages of the cell cycle, both during S-phase and at G2-M. Depending upon the physiological context, they can complex with both Cdk1 and Cdk2, although the full physiological context of this association remains to be determined. Unlike the other cyclin families, both cyclin A1 and cyclin A2 have been shown to be essential genes: loss of cyclin A2 in the mouse model is early embryonic lethal and cyclin A2 is absolutely required for spermatocytes to undergo the first meiotic division. A critical role for cyclin A2 has also been demonstrated in two stem cell systems (hematopoietic stem cells and embryonic stem cells) – will germ cell stem cells be added to this list?

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Chapter 18
Cell Cycle Adaptations and Maintenance of Genomic Integrity in Embryonic Stem Cells and Induced Pluripotent Stem Cells

Olga Momčilović, Christopher Navara, and Gerald Schatten

Abstract Pluripotent stem cells have the capability to undergo unlimited self-renewal and differentiation into all somatic cell types. They have acquired specific adjustments in the cell cycle structure that allow them to rapidly proliferate, including cell cycle independent expression of cell cycle regulators and lax G_1 to S phase transition. However, due to the developmental role of embryonic stem cells (ES) it is essential to maintain genomic integrity and prevent acquisition of mutations that would be transmitted to multiple cell lineages. Several modifications in DNA damage response of ES cells accommodate dynamic cycling and preservation of genetic information. The absence of a G_1/S cell cycle arrest promotes apoptotic response of damaged cells before DNA changes can be fixed in the form of mutation during the S phase, while G_2/M cell cycle arrest allows repair of damaged DNA following replication. Furthermore, ES cells express higher level of DNA repair proteins, and exhibit enhanced repair of multiple types of DNA damage. Similarly to ES cells, induced pluripotent stem (iPS) cells are poised to proliferate and exhibit lack of G_1/S cell cycle arrest, extreme sensitivity to DNA damage, and high level of expression of DNA repair genes. The fundamental mechanisms by which the cell cycle regulates genomic integrity in ES cells and iPS cells are similar, though not identical.

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18.1 Introduction

Preservation of pristine genetic information is of utmost importance for the survival of all organisms and tissue homeostasis. Yet, DNA is a metabolically active molecule and is continually subjected to modifications that may result in mutation. Alterations of DNA sequence need to be corrected before the next round of replication in order to prevent mutation becoming a part of the genome. Most mutations do not produce any measurable effect on phenotype. However, mutations occasionally lead to change in cell viability, function and proliferation, leading to development of new traits or disease, and are a major source of genetic diversity. Therefore, for organisms' most optimal survival, the majority of DNA changes need to be removed, while still allowing some to become part of the genome in order to maintain the balance between genetic diversity and disease.

Cellular genomes are constantly being exposed to genotoxic stresses from endogenous and exogenous sources. It is not surprising, then, that cells have evolved numerous protective mechanisms to counteract such challenges and maintain genomic integrity. Somatic cells have a plethora of responses to DNA damage, including DNA repair mechanisms adapted for particular types of DNA damage, and checkpoints that arrest the cell cycle until the damage is repaired (Jackson 2002; Khanna and Jackson 2001). Cells in which DNA damage overcomes DNA repair capacity, or in which damage cannot be repaired undergo cell death, before the mutation is passed in the next round of replication to daughter cells (Hirao et al. 2000; Jackson 2001, 2002; Norbury and Hickson 2001; Rich et al. 2000). Failure to repair DNA damage, or undergo apoptosis, can have detrimental consequences, leading to transmission of genetic changes to the daughter cells and mutagenesis. It is important for all cells to protect fidelity of genetic information in order to avoid mutagenesis that can lead to tumor formation, as well as to avoid extensive cell death and maintain tissue homeostasis.

In this chapter, we review DNA damage responses elicited by ionizing radiation in somatic cells and pluripotent stem cells, with focus on the adaptations of pluripotent stem cell's DNA damage responses in light of their developmental role. We review regulation of cell cycle progression in mouse and human ES cells, focusing on their mutual differences, as well as differences relative to somatic cells. We also contrast DNA damage responses, including cell cycle arrest, DNA repair, and apoptosis in somatic and ES cells. Finally, we discuss challenges in the induced pluripotent stem (iPS) cell field regarding iPS cell genomic stability and safety for potential clinical applications.

18.2 Pluripotent Stem Cells

Stem cells have two unique properties (1) capability to undergo self-renewal and replenish the stem cell pool, and (2) ability to differentiate into one or more cell types. In respect to their differentiation potential, or potency, stem cells can be

classified as pluripotent, multi/oligopotent, and unipotent. Pluripotent stem cells can differentiate into all cell types of an organism, whereas multipotent stem cells have restricted developmental potential and can differentiate into a limited number of cell types. Most adult stem cells, such as hematopoietic or neural stem cells, are multipotent and capable of differentiating to multiple lineages of a given system, such as hematopoietic, or central nervous system, respectively. Although pluripotent stem cells give rise to all tissue of the organism, they are not capable of producing extraembryonic tissues necessary for embryonic development. Only zygotes and 4–8 cell stage embryos have the property to contribute to both the embryo proper and supportive extraembryonic tissues and are hence referred to as totipotent. Thus, during ontogeny the differentiation potential of stem cells appears to become increasingly restricted (Fig. 18.1) (Larsen 2001; Mitalipov and Wolf 2009).

There are several types of pluripotent stem cells, including embryonic carcinoma cells, ES cells, epiblast stem cells, embryonic germ cells, as well as iPS cells. Historically, embryonic carcinoma (EC) cells were the first derived pluripotent stem cells, but human EC cells are genetically instable due to their origin from teratocarcinomas. These tumors are composed of differentiated cells of all three germ layers, as well as undifferentiated stem cell population that gives rise to differentiated cell types, and from which EC cells are derived (Atkin et al. 1974; Kahan and Ephrussi 1970). Embryonic germ cells are isolated from the primordial germ cells (PGC), and share numerous similarities with ES cells, but still have certain properties of PGC (Labosky et al. 1994; Matsui et al. 1992; Resnick et al. 1992). Epiblast stem cells are derived from the epiblast of early postimplantation embryo, and mouse epiblast stem cells were found to share numerous properties with human ES cells (Brons et al. 2007; Tesar et al. 2007). In this review we will focus on ES cells and iPS cells, which will be described in greater detail in the following sections.

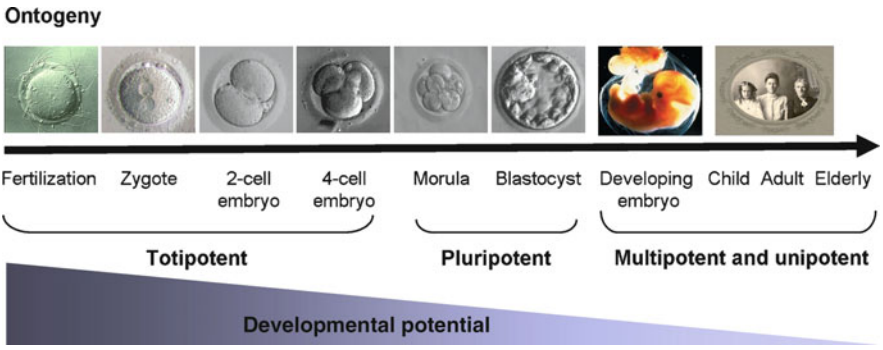

Fig. 18.1 Restriction of developmental potential during ontogeny

18.2.1 Embryonic Stem Cells

ES cells are isolated from the inner cell mass (ICM) of the blastocyst stage preimplantation embryos. ES cells have the capability of self-renewal and differentiation into all fetal and adult lineages. Under proper in vitro culture conditions ES cells can be indefinitely propagated while retaining their differentiation potential. Mouse ES cells transferred to blastocyst stage embryos contribute to all three germ layers of the recipient embryo, including the germ line, which is regarded as the definitive proof of the pluripotency (Evans and Kaufman 1981; Martin 1981). For ethical and technical reasons human ES cells have not been tested in this manner. As an alternative the gold standard for human ES pluripotency is formation of embryoid bodies composed of various differentiated cell types in vitro and development of teratomas in injected immunocompromised mice.

Mouse and human ES cells share numerous characteristics, of which the most important ones, self-renewal and differentiation were already described. However, they differ significantly in expression of cell surface proteins: human ES cells express SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, which are not present on mouse ES cells; instead, mouse ES cells express SSEA-1. In addition, mouse and human ES cells differ considerably in cell culture requirements. Self-renewal of mouse ES cells is supported by addition of leukemia inhibitory factor (LIF) and bone morphogenic proteins (BMP) (Evans and Kaufman 1981; Smith et al. 1988; Ying et al. 2003) to the cell culture medium, both of which induce differentiation of human ES cells. Instead, undifferentiated state of human ES cells is maintained by basic fibroblast growth factor (bFGF) and tumor growth factor β (TGFβ)/Activin/Nodal signaling (Amit et al. 2000; Thomson et al. 1998; Vallier et al. 2005; Xu et al. 2001).

LIF acts through a cell surface gp130 and LIF receptor (LIFR) functional heterodimer to activate STAT3/JAK pathway, ultimately leading to activation of c-Myc in mouse ES cells (Cartwright et al. 2005). BMP activate Inhibitor of Differentiation (Id) proteins via Smad1/5/8, and inhibit p38 which has a negative effect on self-renewal in mouse ES cells (Fig. 18.2a) (Qi et al. 2004; Ying et al. 2003);

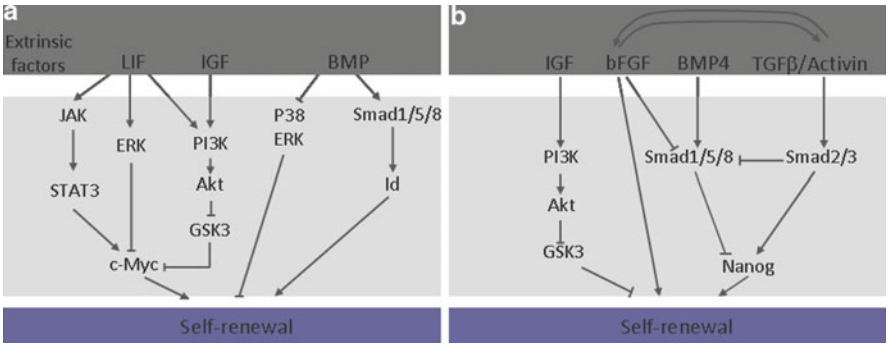

Fig. 18.2 Growth factor signaling and maintenance of self-renewal in mouse (**a**), and human (**b**) ES cells

in contrast, in human ES cells BMP and Smad1/5/8 act to inhibit self-renewal (Xu et al. 2002). bFGF and TGFβ/Activin have mutually stimulating effects, and act together to promote self-renewal of human ES cells by multiple mechanisms, including negative regulation of Smad1/5/8 activity and suppression of BMP signaling (Fig. 18.2b) (Greber et al. 2007; Vallier et al. 2005).

As mentioned above mouse epiblast stem cells are similar with human ES cells, including cell culture medium requirements, gene expression pattern and colony morphology (Brons et al. 2007; Tesar et al. 2007). Thus, mouse pluripotent stem cells can exist in two functionally different states: as LIF dependent "naïve" mouse ES cells and bFGF dependent "primed" epiblast stem cells. Since human ES and mouse epiblast stem cells share many similarities, it has been proposed that thus far derived human ES cells correspond to primed state in mouse pluripotent stem cells. Recently Hanna and colleagues reported "re-wiring" of primed human ES cells into more immature mouse ES cell-like state by ectopic induction of Oct-4, KLF4, and KLF2 coupled with treatment with LIF and GSK3β and ERK1/2 inhibitors. Therefore, it is possible that primed pluripotent stem cell state of human ES cells accounts for observed difference between human and mouse ES cells.

Several transcription factors are essential for maintenance of pluripotency in ES cells. POU5F1 (Oct-4), Nanog, and Sox2 are master regulators of the transcriptional network that controls early development and specifies the undifferentiated state in mouse and human ES cells (Chambers et al. 2003; Hart et al. 2004; Mitsui et al. 2003; Nichols et al. 1998). Together they bind to promoters of a number of genes and exert both activating and repressing effect on gene expression. They activate genes that encode transcription factors, signaling molecules, chromatin remodeling enzymes, and micro RNA that maintain self-renewal and pluripotency in ES cells. Oct-4, Nanog, and Sox2 also activate their own gene expression thereby creating a positive autoregulatory loop. On the other hand, the group of the repressed genes includes genes involved in differentiation into three germ layer lineages. Therefore, these master regulators activate genes that promote self-renewal and undifferentiated state, while repressing the genes involved in differentiation (Boyer et al. 2005).

18.2.2 Nuclear Reprogramming and Induced Pluripotent Stem Cells

The view that differentiation is a unidirectional process was challenged in the 1960s by experiments in which transfer of somatic cells into enucleated Xenopus oocytes resulted in production of fertile animal (Gurdon and Uehlinger 1966). This seminal work established that the somatic genome contains information necessary for the development of the organism, and that the oocyte provides environment in which specialized functions of the somatic cells are erased and the genome is "reprogrammed" into the totipotent state. However, mammalian somatic cell nuclear reprogramming was delayed 30 years until the first cloned mammal, sheep Dolly,

was born (Campbell et al. 1996). Somatic cell nuclear reprogramming in this fashion is still an extremely challenging procedure and is dependent on oocyte availability rendering it highly controversial in humans.

More recently, new technology emerged that allows reprogramming of cellular fate using a defined set of transcription factors instead of oocytes. Several groups achieved lineage conversion of fully differentiated cells by forced expression of key transcription factors, or repression of specifying regulators, demonstrating that it is possible to alter the cellular phenotype by interfering with gene expression (Davis et al. 1987; Niwa et al. 2005; Xie et al. 2004). Indeed, Yamanaka and his team successfully reprogrammed mouse somatic cells into a pluripotent like state using a defined set of transcription factors carried by retroviral integrating vectors (Takahashi and Yamanaka 2006). Since reprogrammed cells form ES-like colonies, express proteins found in ES cells endogenously, and more importantly, contribute to all germ layers in mouse chimera, these cells have been termed iPS cells. Within 1 year this success was extended by reprogramming human somatic cells into iPS cells by two independent teams using two different cocktails of transcription factors. Yamanaka and colleagues used the same set of transcription factors that they used to reprogram mouse somatic cells, c-Myc, Klf4, Oct-4, and Sox2 (Takahashi et al. 2007), whereas Thomson and his team reprogrammed human somatic cells with Lin28, Oct-4, Sox-2, and Nanog (Yu et al. 2007). As already discussed Oct-4, Nanog, and Sox-2 are central for the maintenance of pluripotency in both human and mouse ES cells. Lin28 is a negative regulator of micro RNA involved in differentiation, such as Let-7 (Viswanathan et al. 2008), whereas Klf4 is mainly activated by the Jak-Stat3 pathway and preferentially activates Sox2 (Niwa et al. 2009). C-Myc is a main target of the LIF signaling pathway in mouse ES cells and promotes expression of cyclin E and cellular proliferation (as will be described later). However, its overexpression in human ES cells can cause differentiation (Sumi et al. 2007), and mouse iPS chimera develop tumors and die. Since the first experiments, iPS cells have been derived without c-Myc (Nakagawa et al. 2008), from patients with various genetic diseases (Park et al. 2008), as well as with nonintegrating vectors (Yu et al. 2009).

18.3 Cell Cycle Regulation

18.3.1 Somatic Cells

The basic function of the cell cycle is to ensure accurate duplication of genetic information and equal segregation of copied DNA between two daughter cells. DNA replication occurs during the S phase of the cell cycle, after which copied DNA molecules are separated during the mitosis (M phase) into two daughter cells. S and M phases are separated by the gap phases, G_1 and G_2, during which cell prepares itself for DNA duplication and cell division. Progression through the cell cycle is tightly regulated by both extrinsic and intrinsic factors. Deregulation

of cell cycle control may have serious consequences and is part of malignant transformation.

The G_1 phase of the cell cycle is the time between exit from mitosis and the next round of DNA replication. During this period cells are particularly sensitive to intracellular and extracellular signals and decide their fate – whether they will go into S phase, pause in G_1, senesce or become quiescent. If the environment is favorable and signals for division are present, cell will traverse through the restriction point – a point in G_1 phase after which cell is committed to replicate DNA, even if the extracellular signals that stimulate cell division are removed afterwards.

At the molecular level, the passage through the restriction point and G_1 to S transition is regulated by a concerted series of protein phosphorylations and dephosphorylations, as well as timed expression of cell cycle regulators (Fig. 18.3). It is driven by cyclin dependent kinases (Cdk), which are kept inactive in the absence of proliferation signals (Gopinathan et al. 2011). Their activity is dependent upon the presence of their binding partners, cyclins, whose levels oscillate throughout the cell cycle and are tightly regulated. However, cyclin binding is not sufficient and Cdk activation involves removal of inhibitory phosphate groups by Cdc25 phosphatases, as well as phosphorylation at the activating sites by cyclin activating kinase (CAK). Finally, Cdk activity can be blocked by binding of Cdk inhibitory

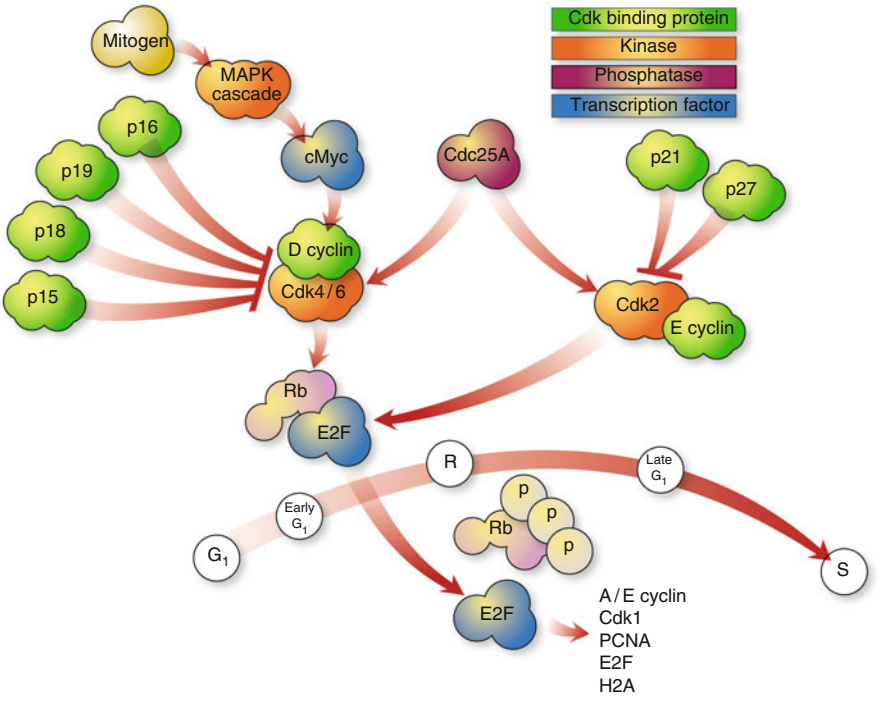

Fig. 18.3 Regulation of G_1 to S transition

proteins belonging to INK4 family (p15, p16, p18, p19) and CIP/KIP family (p21 and p27) that directly inhibit Cdk activity.

Mitogens act through cell surface receptors to activate the mitogen activated protein (MAP) kinase cascade resulting in increased levels of c-Myc transcription factor. C-Myc promotes expression of D cyclins that bind to and activate Cdk4 and Cdk6, triggering the phosphorylation of retinoblastoma (Rb) family members (p105, p107, and p130). Rb proteins are negative regulators of cell proliferation because they bind to and sequester E2F transcriptional factors that are required for the entry into S phase. Rb phosphorylation by cyclin D/Cdk4 and cyclin D/Cdk6 results in its partial inhibition and E2F release, followed by expression of E2F target genes, such as cyclin E, cyclin A, H2A, PCNA, as well as E2F, creating an amplifying loop. Cyclin E binds to Cdk2 which further phosphorylates Rb, resulting in its complete inactivation, and passage through the R point (Fig. 18.3). Besides the kinase dependent function in inactivating Rb, cyclin D/Cdk4, and cyclin D/Cdk6 assist in cyclin E/Cdk2 activation by titrating away their inhibitors. As cyclin E/Cdk2 activity peaks in late G_1, mitogen-dependent cyclin D/Cdk4 and cyclin D/Cdk6 activity is no longer necessary.

G_2 phase of the cell cycle is the period between the exit from the S phase and entry into mitosis. Transition from G_2 to mitosis is dependent on the activity of Cdk1, and is controlled by the availability of its binding partner, cyclin B, as well as posttranslational modifications of Cdk1 (Fig. 18.4). Cdk1 and cyclin B together form the maturation promoting factor (MPF) (Yamashita et al. 1992), which phosphorylates numerous substrates. The MPF targets include lamins whose phosphorylation leads to nuclear envelope breakdown (Peter et al. 1991), condensins that control chromosome condensation (Kimura et al. 1998), as well as microtubule associated proteins and motor proteins that are involved in centrosome separation and mitotic spindle formation (Blangy et al. 1995).

The level of cyclin B is set by balancing its gene transcription with protein degradation and peaks in late G_2 and early mitosis. Cyclin B gene expression is activated by transcription factors that are under control of the S phase specific cyclin A/Cdk2 complex in order to ensure its timely expression (Chae et al. 2004; Dynlacht et al. 1994; Ziebold et al. 1997). Cyclin B is rapidly degraded by anaphase promoting complex (APC), an E3 ubiquitin ligase that targets cyclin B for proteosomal degradation in early mitosis (Acquaviva and Pines 2006). Cdk1 is activated once the cyclin B protein level reaches a certain threshold, but full activation is achieved by posttranslational modifications of Cdk1. Fine tuning of Cdk1 function is regulated by opposing effects of Cdc25 phosphatase and Wee1/Myt1 kinases. Wee1 and Myt1 place inhibitory phosphate groups on tyrosine 14 and tyrosine 15 in Cdk1 (Booher et al. 1997; Liu et al. 1997). These negative marks are removed by Cdc25 phosphatases. In addition, Cdk1 has to be phosphorylated at threonine 161 by CAK for its full activation (Fig. 18.4).

Once activated, cyclin B/Cdk1 phosphorylates and thereby inactivates Wee1 and Myt1, further promoting its own activation (Watanabe et al. 2004, 2005). It can also help activate Cdc25 through phosphorylation, further enhancing the amplification of MPF signaling (Hoffmann et al. 1993).

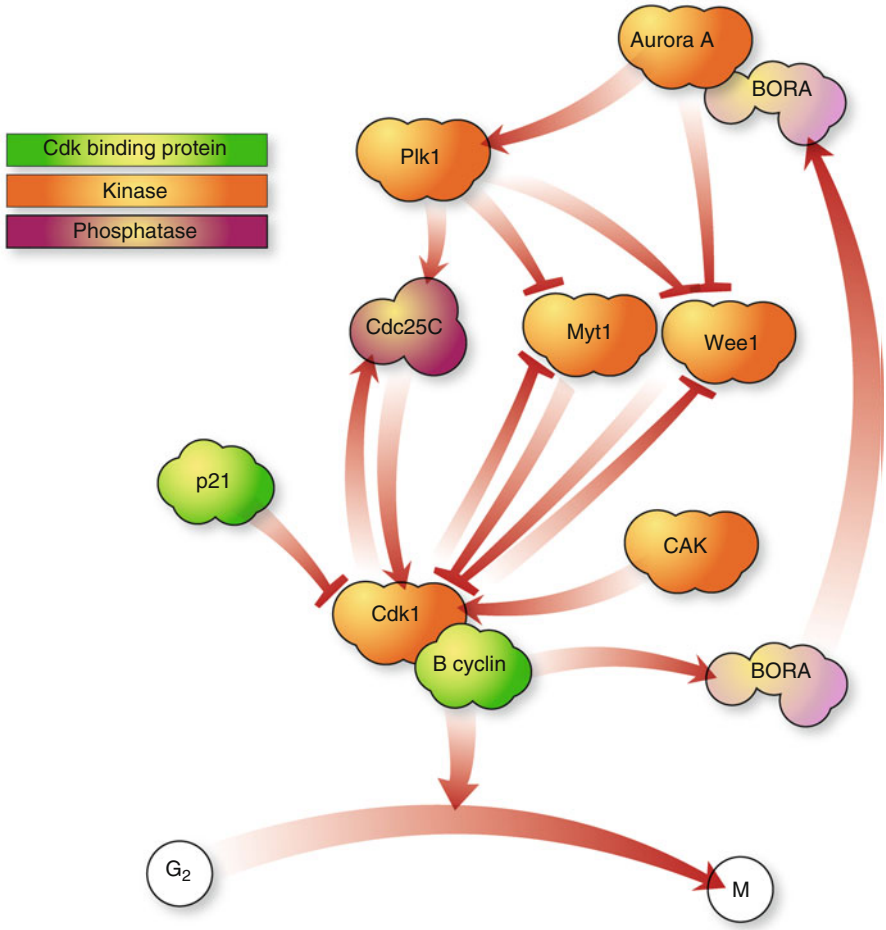

Fig. 18.4 Regulation of G_2 to M transition

There is a parallel regulatory pathway controlling the onset of mitosis that relies on activation of the Polo like kinase (Plk) 1 (Fig. 18.4). Plk1 is a positive regulator of mitotic entry and is involved in centrosome maturation, formation of a bipolar spindle, chromosome segregation, as well as cyclin B/Cdk1 activation (Alexandru et al. 2001; Yarm 2002). Plk1 is activated by Aurora A/BORA phosphorylation at threonine 210 (Seki et al. 2008). Plk1 phosphorylates Cdc25C at seine 198 resulting in Cdc25C activation (Roshak et al. 2000; Toyoshima-Morimoto et al. 2002), while phosphorylation of negative regulators of mitotic entry Wee1 and Myt1 leads to their inactivation (Inoue and Sagata 2005; Nakajima et al. 2003). Plk1 can also directly phosphorylate cyclin B (Toyoshima-Morimoto et al. 2001). Activated cyclin B/Cdk1 phosphorylate BORA, which enhances Aurora A/BORA binding to and activation of Plk1

(Chan et al. 2008; Hutterer et al. 2006), ultimately leading to further stimulation of cyclin B/Cdk1 activity and mitotic entry.

18.3.2 Early Embryos

Early stage mammalian embryos progress rapidly through the first several divisions with short or nonexistent G_1 and G_2 phases. For example, rodent embryonic epiblast cells expands from 20 to 25 cells to approximately 660 cells between 4.5 and 6.0 days post coitus (dpc), estimating an average cell cycle duration time of 10 h. The divisions are further accelerated between 6.5 and 7.0 dpc, when cell number increases to over 4,000, suggesting a cell cycle duration of merely 4.4 h (Power and Tam 1993; Solter et al. 1971; White and Dalton 2005). This is reminiscent of the rapid cell divisions in early embryonic development of *Xenopus* (Murray and Kirschner 1989) and *Danio* (Yarden and Geiger 1996), as well as nuclear divisions in cellular syncytium in *Drosophila* embryos (Edgar and Lehner 1996), where cell cycle consists of alternating rounds of DNA replication and chromosome segregation. Importantly, knock out studies demonstrated that most of the cyclins and Cdk, with the exception of cyclins A2 and B1, are functionally redundant in early mouse development (White and Dalton 2005).

The ICM, from which ES cells are isolated, exists for a very limited period of time before cells differentiate into the three germ layers. In addition to their transitory existence in vivo, the ICM is comprised of a small number of cells making it difficult to obtain sufficient material to perform biochemical studies. Working with human embryos adds an additional layer of technical and ethical complexity to studying early embryo development in vivo. In contrast, ES cells can be grown in culture for extended periods of time, without undergoing senescence or quiescence, and display characteristics of mammalian embryonic blastomeres, including shortened G phases. Therefore, human ES cells provide material for studying cell cycle regulation and DNA damage responses in early development.

18.3.3 Embryonic Stem Cells

18.3.3.1 Mouse Embryonic Stem Cells

ES cells have the remarkable property to self-renew which is intimately linked to the ability of cells to proliferate. Indeed, ES cells do not undergo senescence, quiescence, or contact inhibition in the culture, and appear less dependent of extracellular signals to divide suggesting specific adjustments to cell cycle regulation that allow them to rapidly proliferate.

Mouse ES cells are characterized by very short cell cycle (11–16 h), predominantly due to short G_1 phase that on average accounts for approximately 15% of the

cell cycle time (2 h) (Savatier et al. 1994). The benefit of having a shorter G_1 phase lies not only in achieving rapid proliferation, but also in avoiding differentiation signals that are active during early G_1. The tumor suppressor Retinoblastoma (Rb) is held inactive by hyperphosphorylation throughout the cell cycle, leading to constitutive E2F activation, and nonphasic transcription of E2F target genes (Stead et al. 2002). D-type cyclin expression is very low, whereas Cdk4 kinase activity is almost undetectable, providing evidence that mitogen-induced cyclin D/Cdk4 or cyclin D/Cdk6 activities do not have a role in regulating Rb/E2F and subsequently cyclin E/Cdk2 activity (Savatier et al. 1994, 1996). Cyclin E/Cdk2 activity is high and cell cycle independent (unlike in somatic cells where it peaks at the G_1 to S transition), and cyclin A/Cdk2 is constitutively active. The only cell cycle regulators expressed in a cell cycle dependent manner are cyclin B and Cdk1, which are expressed at the G_2 to mitosis transition (Stead et al. 2002). These findings are consistent with the observation that the restriction point is bypassed in mouse ES cells, resulting in facilitated G_1 to S transition and proliferation. Following differentiation, the restriction point is acquired; Rb/E2F and cyclin E become dependent on mitogen-induced activity of cyclin D/Cdk4 or cyclin D/Cdk6 complexes (Savatier et al. 1996; White et al. 2005).

More recent studies address the molecular mechanisms that govern expression of cell cycle regulators in ES cells. Micro RNA (miRNA) are small RNA molecules (18–24 nucleotides) that bind to both 3′ untranslated region (3′UTR) and coding regions of target mRNA, resulting in destabilization of mRNA, impeded mRNA translation, and repression of target gene expression (Wang and Blelloch 2009). Wang et al. (2007, 2008) reported that loss of one of the key proteins involved in biogenesis of miRNA, Dcgr8, results in accumulation of mouse ES cells in the G_1 phase of the cell cycle, suggesting that miRNA play an important role in the G_1 to S transition (also see Wang and Blelloch 2011). Numerous miRNA were demonstrated to promote cellular proliferation by modulating G_1 to S transition in cancer cells and germ line stem cells in *Drosophila melanogaster* (Fornari et al. 2008; Galardi et al. 2007; Hatfield et al. 2005; Ivanovska et al. 2008). In mouse ES cells, several miRNA belonging to the miR-290 cluster are specifically expressed in undifferentiated cells, rapidly downregulated during differentiation, and are able to rescue the proliferation defect in Dcgr8 deficient mouse ES cells. Among potential targets is p21, a Cdk-cyclin inhibitor, whose gene (*cdkn1a*) contains two 3′UTR binding sites for miR-291-3p, miR-294, and miR-295. Overexpression of p21 in mouse ES cells causes similar G_1 phase accumulation as observed in Dcgr8 deficient mouse ES cells. Micro RNA usually influence the level of mRNA of multiple genes, and members of miR-290 cluster were found to inhibit expression of other G_1/S negative regulators, such as p130 (retinoblastoma-like 2, Rbl2) and Lats2 in mouse ES cells (Wang et al. 2008).

The LIF/STAT3/c-Myc pathway has a major role in maintaining mouse ES cell self-renewal by promoting proliferation: c-Myc is a major target of LIF/STAT3 pathway and is a powerful inducer of cyclin E expression, which stimulates G_1 to S transition. In addition, Oct-4/Nanog/Sox2 master transcriptional regulators of pluripotency in mouse and human ES cells bind to and activate expression of several

miR clusters, including miR-290 cluster (Marson et al. 2008), providing a link between regulation of self-renewal and cell cycle control in ES cells.

18.3.3.2 Nonhuman Primate Embryonic Stem Cells

Nonhuman primate ES cells have slightly longer cell cycle duration than mouse ES cells: 12–21 h, with median cell cycle duration of 15 h, which is in accordance with a cell cycle time that is longer in primate embryos (approximately 20 h) in comparison to mouse embryos. Over 50% of cells are found in the S phase, and the expression of cyclin E and cyclin A is high. Following differentiation, the fraction of cells in S phase decreases, as do the levels of cyclins E and A. Similar to observations in mouse ES cells, cyclin E expression and Rb hyperphosphorylation are independent of the cell cycle stage, but unlike in mouse ES cells, cyclin A exhibits transient expression in cell cycle. In addition, nonhuman primate ES cells do not require serum in the medium, and MAPK activity does not affect the growth rate (Fluckiger et al. 2006).

18.3.3.3 Human Embryonic Stem Cells

Human ES cells' cell cycle is abbreviated similar to mouse and nonhuman primate ES cells, with median cell cycle duration of 15.8 h. Shortening of the G_1 phase is responsible for the abbreviated cell cycle in human ES cells. G_1 phase accounts for about 19% of the cell cycle, corresponding to 2.5–3 h. The vast majority of cells are in the S phase that lasts about 8 h. In healthy human ES cell cultures all cells are positive for the proliferation marker Ki-67, demonstrating the absence of quiescent cells (Becker et al. 2006). Unlike mouse ES cells, in human ES cells most of the cell cycle regulators including cyclins D2, E, and A exhibit phase-specific expression (Neganova et al. 2009); the comparison of expression of key cell cycle controllers between mouse and human ES cells is summarized in Table 18.1. Becker and colleagues demonstrated that the expression of Cdk4 is higher than that of Cdk6 in human ES cells, and that cyclin D2 expression is elevated in human ES cells in comparison to somatic cells. Furthermore, the expression of Cdk inhibitors p21, p27, and p57 is barely detectable. Thus, in comparison to mouse ES cells, human ES cells exhibit more prominent control of cell cycle progression, particularly through G_1 phase. Nevertheless, human ES cells still exhibit shortened G_1 phase and rapid proliferation, and data suggest that this is a result of elevated cyclin D2/Cdk4 activity (Table 18.1) (Becker et al. 2006).

Several miRNA were found to affect cell cycle progression in human ES cells. Similar to observations in mouse ES cells, knocking down members of the miRNA biogenesis pathway results in slower proliferation of human ES cells, due to an extended G_1 and shortened S phase of the cell cycle. This phenotype is at least

Table 18.1 Comparison of expression and activity of cell cycle controllers in mouse and human embryonic stem cells

	Mouse ES cells	Human ES cells
Cyclin D1	Almost undetectable	High in G_1
Cyclin D2	Very low	High in G_1
Cyclin D3	High	G_1
Cdk4	Almost undetectable	High activity in G_1
Cdk6	High	G_1
Cyclin E	Constitutively expressed	G_1–S
Cyclin A	Constitutively expressed	S–G
Cdk2	Constitutively active	Active in S
Cyclin B/Cdk1	Cell cycle dependent – G_2	Cell cycle dependent – G_2
p21	Undetectable	Very low

partially rescued by expression of miRNA belonging to several clusters which are specifically enriched in ES cells and downregulated during differentiation (Qi et al. 2009). In human ES cells miR-92b is abundantly expressed and is downregulated during differentiation, coinciding with extension of the cell cycle, G_1 phase in particular. Repression of miR-92b in human ES cells resulted in accumulation of cells in G_1 phase, and decrease of percentage of cells in S phase of the cell cycle, suggesting that this miRNA plays important role in regulating G_1 to S transition. Sengupta and colleagues (Sengupta et al. 2009) identified p57, a G_1/S cyclin/Cdk inhibitor protein as a direct target of miR-92b. In addition, miR-372, the human functional ortholog of mouse miR-290, affects the level of p21 in human ES cells, thereby promoting the G_1 to S transition (Qi et al. 2009). The miR-302 cluster is also involved in cell cycle regulation in human ES cells (Card et al. 2008). Four members, miR-302-a, -b, -c, and -d, are highly expressed in human ES cells, and their forced expression in primary and transformed cells increases the proportion of cells in the S phase and decreases the number of cells in the G_1 phase of the cell cycle. These four miRNA repress D1 cyclin mRNA, and inhibition of miR-302 in human ES cells leads to accumulation of cells in G_1, suggesting that members of the miR-302 cluster are involved in negative regulation of G_1, or promotion of S phase entry. Cdk4 is also a target of miR-302, and other potential targets include Rb, E2F1, p130 (Rbl2), Cdk2, and Cdk6, which are all engaged in regulation of the G_1 phase. Thus, miR-302 appears to posttranscriptionally control multiple G_1 phase regulators in human ES cells (Card et al. 2008). Transition from G_2 to M phase is also regulated by miRNA, and miR-195 was found to bind to the 3′UTR and repress the Wee1 kinase mRNA (Qi et al. 2009). Wee1 kinase is a negative regulator of cyclin B/Cdk1 complex, and its downregulation by miR-195 in wild type human ES cells results in an increase of the proportion of cells in the S phase. Both miR-195 and miR-372 are strongly expressed in pluripotent human ES cells and are downregulated following differentiation (Qi et al. 2009). Therefore, similar to observations in mouse ES cells and

cancer cells, miRNA have prominent role in regulating cell cycle progression in human ES cells.

As already mentioned, self-renewal is supported by rapid proliferation and since self-renewal is under control of Nanog, Oct-4, and Sox2, it is reasonable to speculate that there is a link between the transcriptional network that controls self-renewal and regulation of proliferation. Indeed, Nanog was found to accelerate G_1 to S transition, as well as S phase progression in human ES cells through direct transcriptional activation of *cdk6* and *cdc25A* gene expression. Overexpression of Nanog suppresses spontaneous differentiation and promotes human ES cells' expansion, without affecting apoptosis (Zhang et al. 2009). Furthermore, Oct-4, Sox2, and Nanog bind to the putative promoter region of the miR-302 cluster, and Oct-4 and Sox2 are required for miR-302 expression in human ES cells providing another link between key pluripotency transcriptional factors and cell cycle regulators (Card et al. 2008).

Link between cell cycle duration and differentiation has been established in cancer cells, somatic cells, and adult stem cells. As already described, cells in the G_1 are particularly responsive to intracellular and extracellular signals based on which they decide their fate. Therefore, it appears the G_1 stage of the cell cycle is a period during which ES cells are most sensitive to differentiation, and the shortening of the G_1 phase contributes to self-renewal. Indeed, it has been demonstrated in human ES cells that pharmacological activation of p53 followed by increased expression of p21 leads to accumulation of cells in G_1 phase of the cell cycle followed by differentiation (Maimets et al. 2008).

18.4 DNA Damage Responses

"DNA damage is an inescapable aspect of life" (Friedberg et al. 2006). DNA lesions occur constantly and can be divided into endogenous (or spontaneous) and exogenous (or induced). Endogenous DNA damage is a consequence of nature of metabolic processes that take place in the presence of water and reactive oxygen, conditions that allow hydrolytic and oxidative reactions to occur introducing changes in DNA bases, phosphodiester bonds and sugars. Oxidation and deamination of bases change their complimentary pairing, whereas the hydrolytic cleavage of the *N*-glycosyl bond between base and sugar moiety results in loss of base and creates an abasic site (AP; apurinic or apyrimidinic site). Additionally, replication enzymes introduce mismatched nucleotides in newly synthesized DNA strand, albeit at very low rates, but given the size of the genome, replication errors add a significant mutational burden. Some alkylating agents, such as *S*-adenosylmethionine, a methyl group donor in enzymatic methylation of DNA, are also normal products of metabolism and can react with many sites in DNA introducing methyl groups in

Table 18.2 Endogenous DNA damage frequencies (Friedberg et al. 2006)

Type of damage	Frequency (number of lesions per cells in 24 h)
Oxidation of guanosine (8-oxoG)	1,000–2,000
Cytosine deamination	100–500
Depurination	18,000
Depyrimidination	600
Methylation by *S*-adenosylmethionine (7-Methylguanine)	6,000
Methylation by *S*-adenosylmethionine (3-Methyladenine)	1,200

nonenzymatic reactions. Some of the most common types of endogenous DNA lesions and their frequencies are listed in Table 18.2.

Exogenous or environmental factors include ionizing and UV radiation, as well as a broad spectrum of chemicals that can react with multiple sites in DNA, including alkylating and cross-linking reagents. Major sources of ionizing radiation are cosmic rays and naturally occurring radioisotopes, as well as radiation exposure due to medical exams and treatments. However, the contribution of environmental sources to total DNA damage is small in most human populations in comparison to the endogenous DNA damage burden (with the exception of occupational exposure, or geographical proximity to radioisotope sources in the Earth's crust).

Metabolic products with affinity to damage DNA and certain environmental agents, such as UV and cosmic radiation, have been present since the origin of life, and have provided selective pressure for cells to evolve detection, repair, and tolerance mechanisms. The diversity of DNA lesions and the excess of undamaged, normal, DNA present a significant challenge to detection and repair proteins. Some DNA damage response factors are shared by multiple pathways, whereas others are specialized for a particular class of DNA damage. One of the first cellular responses includes cell cycle arrest that provides time for cells to repair damaged DNA. DNA repair mechanisms operate to restore normal DNA nucleotide sequence, chemistry, and structure. Sometimes damage is overabundant and cannot be repaired leading to cell death. However, excessive loss of cells can perturb tissue homeostasis and therefore cells have developed the ability to tolerate the presence of DNA damage as a more favorable response than cell death (particularly in case of postmitotic, fully differentiated cells). The tolerance mechanisms include translesion DNA synthesis, postreplicative fork filling, and replication fork progression, but these are not discussed in great detail in this chapter.

In this section we discuss checkpoint function and cell cycle arrest, as well as DNA repair mechanisms in somatic and ES cells. The discussion is limited to DNA double strand break damage responses, as double strand breaks frequently occur in replicating cells due to fork stalling and collapse and represent the most toxic form of DNA damage.

18.4.1 Checkpoint Activation

18.4.1.1 Somatic Cells

Ionizing radiation induces a variety of DNA lesions, including double and single strand breaks, as well as base damage (Goodhead 1989; Hutchinson 1985; Ward 1988), and, thus, evokes various DNA repair mechanisms. Double strand breaks (DSB) are particularly toxic for the cell and are more difficult to repair as they cause the loss of integrity of both DNA strands (Karagiannis and El-Osta 2004). Ionizing radiation-induced DNA damage activates checkpoint machinery in somatic cells and arrests the cell cycle allowing time for DNA repair to occur prior to proceeding to the next stage of the cell cycle.

The upstream checkpoint components, including damage sensors and transducers, are shared by the G_1/S, intra-S, and G_2/M checkpoints (Fig. 18.5). Two phosphoinositide 3-kinase like-kinase (PIKK) family members, ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and Rad3 related (ATR) protein kinases are central to initiation of DNA damage and replication checkpoints in response to various genotoxic stresses (Abraham 2001). Activated ATM and ATR kinases phosphorylate numerous protein targets and transduce signals generated by DNA damage sensors to cell cycle stage specific effector proteins. ATM exists as an inactive dimer that is

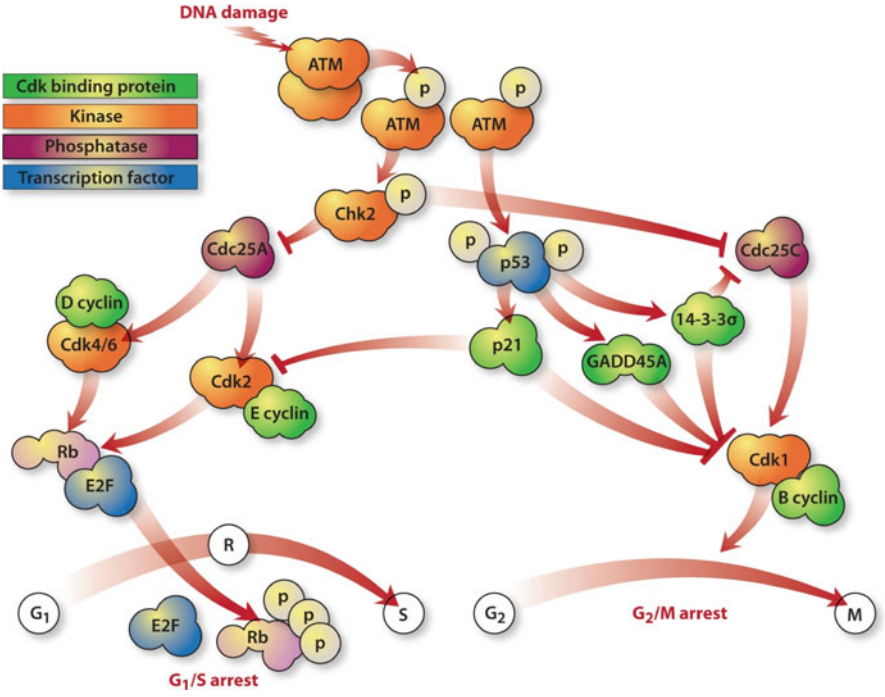

Fig. 18.5 Ionizing radiation induced checkpoint signaling and cell cycle arrest

autophosphorylated at serine 1981 in response to DNA double strand breaks (Bakkenist and Kastan 2003). Activated ATM monomers localize primarily at DSB, where they phosphorylate many substrates, including p53 (Banin et al. 1998; Canman et al. 1998), Chk2 (Matsuoka et al. 2000; Melchionna et al. 2000), H2AX (Lukas et al. 2004), Nbs1 (Gatei et al. 2000; Lim et al. 2000; Wu et al. 2000; Zhao et al. 2000), and BRCA1 (Cortez et al. 1999; Li et al. 2000).

The checkpoint signal generated by ATM and ATR is transmitted to checkpoint kinases, Chk1 and Chk2 (Fig. 18.5) that associate with DSB transiently and are released into the nucleus to transmit the signal throughout the nucleus (Lukas et al. 2003). Although the ATR-Chk1 pathway is predominantly activated in response to UV treatment (Guo et al. 2000), and ATM-Chk2 is predominant in response to ionizing radiation (Melchionna et al. 2000), there is significant crosstalk between these two pathways, particularly as they share multiple targets (Shiloh 2001; Zhou and Elledge 2000). Both Chk1 and Chk2 downregulate Cdc25 phosphatases (Falck et al. 2001; Furnari et al. 1999; Mailand et al. 2000) and activate p53 through phosphorylation (Hirao et al. 2000; Shieh et al. 2000).

The tumor suppressor p53 plays a major role in multiple cellular processes occurring in response to DNA damage, including cell cycle arrest, apoptosis, and senescence. It is constitutively expressed, but rapidly degraded due to Mdm2 ubiquitination and targeting for proteasome degradation (Chehab et al. 1999). ATM, ATR, Chk2, and Chk1, among other kinases, phosphorylate p53. ATM (Banin et al. 1998; Canman et al. 1998) and ATR (Tibbetts et al. 1999) phosphorylate p53 at serine 15 which impairs Mdm2 binding and enhances p53 accumulation and functional activation in response to DNA damage (Tibbetts et al. 1999). Chk2 and Chk1 phosphorylate p53 at serine 20 (Hirao et al. 2000; Shieh et al. 2000) promoting its tetramerization, stability, and activity (Hirao et al. 2000). Activated p53 promotes transcription of its target genes, such as p21 (el-Deiry et al. 1993), GADD45 and 14-3-3-σ (Hermeking et al. 1997), which play a role in the cell cycle arrest and other cellular responses to DNA damage, including apoptosis and feedback regulation.

Cell cycle progression is driven by temporal activation of cyclin/Cdk complexes, making them logical targets of the checkpoint response.

The main targets of the G_1/S cell checkpoint are cyclin/Cdk complexes that drive the transition from G_1 to the S phase of the cell cycle (Sherr 1994). Following ATM and Chk2 activation, Cdc25A phosphatase becomes phosphorylated and degraded (Arata et al. 2000; Mailand et al. 2000), rendering Cdk2 inactive. This event results in rapid initiation of the G_1/S arrest because cyclin E/Cdk2 complex is not activated and cannot phosphorylate Rb in order to promote E2F release (Arata et al. 2000). The G_1/S arrest is sustained by p53 dependent p21 expression and inactivation of Cdk2 (Harper et al. 1993; Nyberg et al. 2002). Therefore, ATM- and Chk2-dependent p53 activation is not required for cell cycle arrest initiation but is required for the maintenance of this arrest.

The main target of the G_2/M cell cycle arrest is the cyclin B/Cdk1 complex that is essential for mitotic entry (Fig. 18.5). This complex is kept inactive by Cdk1 phosphorylation at tyrosine 14 and 15, and depends on Cdc25 phosphatases to

remove these inhibitory phosphate groups. Cdc25C is phosphorylated by Chk2 at serine 216 which creates a binding site for an adaptor protein 14-3-3-σ, resulting in nuclear exclusion and degradation of Cdc25C-serine 216 (Furnari et al. 1999; Peng et al. 1997). In addition, 14-3-3-σ affects nuclear localization of cyclin B/Cdk1 (Giono and Manfredi 2006). Cyclin B/Cdk1 complex has also been reported to be inhibited by p21 and GADD45, aiding the maintenance of G_2/M arrest (Bunz et al. 1998; Zhan et al. 1999). Therefore, at least three p53 dependent genes, p21, 14-3-3-σ, and GADD45, are involved in initiation and maintenance of G_2/M cell cycle arrest.

18.4.1.2 Early Embryos

Early mouse development is characterized by extreme sensitivity to radiation induced DNA damage (Goldstein et al. 1975; Heyer et al. 2000; Matsuda et al. 1989), which is likely due to atypical DNA damage response in early embryonic development of multicellular organisms in general. For example, inhibition of DNA replication by aphidicolin treatment in *Drosophila*, zebrafish, and *Xenopus* embryos resulted in reduced DNA content, but cleavage continued without delay, suggesting that checkpoint function is anomalous in early embryos (Clute and Masui 1997; Ikegami et al. 1997; Raff and Glover 1988). The apoptotic response does not take place in one or two cell embryos, and is confined to ICM cells of blastocyst stage embryos (Adiga et al. 2007a). In addition the apoptotic function of p53 is also absent from early cleavage mouse embryos (Jurisicova et al. 1998).

Irradiation of one-cell mouse embryos in the G_2 stage of the cell cycle with 10 Grays of γ-radiation induces 22.5 h delay in cleavage relative to nonirradiated embryos, whereas irradiation of two cell stage embryos in the G_2 phase induces 2.5 h delay until next cellular division. These data suggest that early mouse embryos arrest in G_2/M. However, most of the irradiated one and two cell stage embryos cease development following G_2/M cell cycle arrest prior to reaching the blastocyst stage (Yukawa et al. 2007). Absence of G_1/S cell cycle arrest was also documented following fertilization of oocytes with irradiated sperm (Shimura et al. 2002). Sperm-irradiated embryos enter the S phase of the cell cycle but demonstrate suppression of DNA synthesis. However, mouse p53 deficient embryos do not exhibit S-phase delay, suggesting the existence of p53 dependent S-phase checkpoint in one cell stage embryos (Shimura et al. 2002). In spite of the S-phase checkpoint, wild type embryos formed by fertilization of oocytes with irradiated sperm continue cleavage until blastocyst stage, with half of them displaying less than 2n DNA content. At the blastocyst stage sperm-irradiated embryos show a delay in development and arrest in G_2/M stage of cell cycle (Adiga et al. 2007b). In contrast, p53 deficient embryos fertilized with irradiated sperm do not exhibit S phase delay, undergo abnormal chromosome segregation, and form numerous micronuclei. Cleavage is halted, and p53 knock-out embryos degenerate before reaching the blastocyst stage (Toyoshima 2009). Therefore, it appears that p53-dependant S phase checkpoint contributes to survival of early embryo, but does not prevent eventual suppression of embryonic development. As discussed earlier, one of the

p53 transcriptional targets is p21. Mouse p21 knock-out zygotes demonstrate S-phase delay, similar to wild type sperm-irradiated embryos, suggesting that the p53-dependent S phase checkpoint is independent of p53 transcriptional function (Toyoshima et al. 2005). Sperm-irradiated p21 deficient embryos develop morphologically normally until blastocyst stage, but apoptosis is more prominent. In addition, p21 activation can be readily detected at the blastocyst stage concomitant with G_2/M arrest in wild type sperm-irradiated embryos, suggesting that p21 has a role in inhibition of apoptosis, as well as acquiring of G_2/M cell cycle arrest at blastocyst stage (Toyoshima 2009).

18.4.1.3 Embryonic Stem Cells

Several uncharacteristic phenotypes associated with response of ES cells to DNA damage include (1) lack of a functional DNA damage induced G_1/S cell cycle arrest (Aladjem et al. 1998; Fluckiger et al. 2006; Hong and Stambrook 2004; Momcilovic et al. 2009), (2) hypersensitivity to DNA damaging agents, and high levels of apoptosis in response to DNA damage (Aladjem et al. 1998; Hong and Stambrook 2004; Momcilovic et al. 2009; Qin et al. 2007), (3) uncharacteristic localization and expression of checkpoint control proteins, at least in mouse ES cells (Aladjem et al. 1998; Chuykin et al. 2008; Hong and Stambrook 2004), and (4) reduction in expression of pluripotency factors in mouse ES cells (Lin et al. 2005).

The DNA damage signaling factor Chk2 has been reported to localize aberrantly to the centrosomes in mouse ES cells and failed to translocate to the nucleus following irradiation (Hong and Stambrook 2004). In human cells we (Momcilovic et al. 2009) documented activation of the ATM signaling cascade within 15 min of DNA damage. Phosphorylated ATM colocalized with the marker of DNA double strand breaks (DSB), γ-H2AX, suggesting that activated ATM is recruited to the DSB. Phosphorylated Chk2 is localized to the poles of mitotic spindles in nonirradiated human ES cells, but in contrast to mouse ES cells, phosphorylated Chk2 is diffusely distributed in the nuclei of irradiated human ES cells. Interestingly, Chk2, as well as several other proteins involved in DNA damage response, have been described to associate with centrosomes during mitosis in the absence of the DNA damage, and to get mobilized to the nucleus in response to DNA damage. On the basis of the emerging data it has been proposed that centrosomes may have a functional role in DNA damage response, serving as either "command centers" (Doxsey 2001), where DNA damage response proteins come in close proximity and/or are sequestered from unfavorable interactions, or as a subject of DNA damage response (Loffler et al. 2006).

Conflicting reports of p53 localization and activity have been described in mouse ES cells in response to DNA damage. Aladjem et al. (1998) reported that mouse ES cells do not activate p53-dependent DNA damage responses and undergo p53-independent apoptosis in response to ionizing radiation. Several groups reported that p53 inefficiently translocates to the nucleus after DNA damage in these cells

(Aladjem et al. 1998; Chuykin et al. 2008; Hong and Stambrook 2004). In contrast, others have described that treatment with DNA damaging agents results in p53 dependent repression of *nanog* promoter and differentiation of mouse (Lin et al. 2005) ES cells, implying that p53 does successfully translocate to the nucleus after DNA damage. In human ES cells, phosphorylated p53 localizes to the nuclei of irradiated cells (Momcilovic et al. 2009).

Activation of the ATM-dependent checkpoint signaling cascade in irradiated human ES cells results in induction of temporary G_2/M but not G_1/S cell cycle arrest. Sixteen hours following irradiation, human ES cells return to the cell cycle, and mitotic index is restored to the preirradiation level. Furthermore, ATM has an essential role in induction of this G_2/M cell cycle arrest in human ES cells, as inhibition of ATM kinase function with a specific competitive small molecule inhibitor KU55933 abolishes G_2/M arrest and results in an increased percentage of mitotic cells following irradiation relative to KU55933 untreated irradiated cells (Momcilovic et al. 2009).

It has also been suggested that differentiation is an alternative DNA damage response of ES cells relative to somatic cells (Lin et al. 2005; Qin et al. 2007). In these studies, a rapid decrease in *nanog* gene expression is observed following UV irradiation or exposure to the radiomimetic drug doxorubicin. We, however, demonstrated that protein levels of Oct-4 and Nanog do not change for 1 week period after irradiation. We observed a reduction in *nanog* and *oct-4* gene expression by quantitative PCR within first 6 h of irradiation, similar to the report in mouse (Lin et al. 2005) and earlier report in human (Qin et al. 2007) ES cells. However, 24 h postirradiation the levels of Nanog and Oct-4 mRNA return to near that of controls, and remain close to the control levels for up to 48 h after irradiation. In addition to the quantitative PCR and western blot analysis, immunocytochemical analysis confirmed nuclear retention of Oct-4 and Nanog 24 h after γ-irradiation, suggesting that human ES cells retain expression of pluripotency markers after DNA damage.

Interestingly, mouse and human ES cells show uncoupling of the spindle assembly checkpoint (SAC) and apoptosis, allowing them to tolerate polyploid or aneuploid states. Following pharmacological disruption of microtubules, mouse ES cells transiently activate SAC, but enter the next cell cycle with 4C DNA content without undergoing apoptosis. After differentiation, SAC activation resulted in a strong apoptotic response, similar to MEF and other somatic cells. The uncoupling of SAC and apoptosis appears to be an intrinsic property of ES cells that confers unusual tolerance for polyploidy that is lost during differentiation, and explains why mouse ES cells undergo tetraploid cell division in culture (Mantel et al. 2007).

Wang et al. (2009), examined S and G_2/M checkpoint function in human EC cells. Human EC cells exhibit an S phase delay in response to irradiation, which has not been detected in mouse, primate or human ES cells. ES cells of different species all show tremendous sensitivity to DNA damage and undergo extensive cell death within hours of DNA damage. Cell death of human ES cells is apparent within hours of exposure to two Grays of γ-irradiation. For example, cleavage of caspase-3 occurs 4 h after irradiation, and cell loss can be microscopically visualized 6 h after irradiation (Momcilovic et al. 2009). However, human EC cells display higher

survival following irradiation when compared to their differentiated counterparts. It is interesting to note these several differences between ES and EC cells given their numerous similarities and it will be illuminating to examine these two cell types further to better understand their similarities and differences.

18.4.2 Double Strand Break Repair

Double strands break are regarded as highly toxic for the cell because even one DSB can be potentially lethal (Rich et al. 2000). Pathological DSB are generated by both exogenous agents (ionizing radiation, radiomimetic drugs), as well as endogenous sources (reactive oxygen species (ROS) formed during metabolism, stalled replication forks, incorrect resolution during metabolic processing of DNA). There are also physiological (intentional) DSB created during meiotic recombination or V(D)J recombination that would be equally toxic to pathological DSB if not removed. However, the predominant cause of DSB in proliferating cells is errors in DNA replication. Given the toxicity of DSB for the cell, several repair pathways exist to remove them. The two main pathways are nonhomologous end joining (NHEJ) and homologous recombination (HR).

Double strand breaks occur in various forms, such as blunt ends, 5′ and/or 3′ overhangs, and gaps, and may contain nonligatable groups such as 3′-phosphate or 3′-phosphoglycolate groups, requiring processing before ligation (Friedberg et al. 2006). NHEJ is an error-prone process that involves processing of DNA ends which may lead to loss of genetic information (Lees-Miller and Meek 2003; Lieber et al. 2003). Since it does not require the presence of the sister chromatid in the cell, it is present in all phases of the cell cycle, but is predominant during G_1 and early S phases (Lee et al. 1997; Takata et al. 1998). HR, however, depends on the presence of the sister chromatid and consequently is predominant in the late S and in G_2 phases of the cell cycle when the duplicated chromatids are present (Haber 2000; Rothkamm et al. 2003).

The choice of the pathway depends on several factors, such as kinetics of repair, cell cycle stage, or cell type. NHEJ and HR have rather different kinetics, with NHEJ being responsible for the fast mode of repair. HR depends on the presence of sister chromatid in the cell. Consequently, HR is confined to late S and G_2, whereas NHEJ is preferred in G_1 and early S (Takata et al. 1998). Finally, the ability of a particular cell type to tolerate mutations greatly affects the choice of DSB repair pathway. Somatic cells may be more tolerant to inaccurate repair system such as NHEJ, which may be highly disadvantageous for the germ cells [during certain stages of spermatogenesis NHEJ factor Ku is not expressed (Goedecke et al. 1999)].

18.4.2.1 Nonhomologous End Joining

NHEJ is error-prone because it involves processing of DNA ends which might lead to loss of nucleotides on both sides of the break (Mahaney et al. 2009). Since it does

not require template, it is the predominant way to repair DSB prior to S phase and is a major repair pathway in vertebrates (Branzei and Foiani 2008; Rothkamm et al. 2003). In addition, it has a physiological role and is essential for V(D)J recombination in immunoglobulin genes; hence, mutations in genes that encode NHEJ proteins lead to severe combined immunodeficiency phenotype (SCID) and increased radiosensitivity.

The first step of NHEJ is recognition of and binding to DNA ends by the Ku70/Ku80 heterodimer (Ku70/80) which occurs within seconds of break formation. Ku70/80 represents the most abundant end-binding factor in eukaryotes. The complex can accommodate a variety of end structures, such as blunt ends, 5′ and 3′ overhangs, or covalently closed hairpins in a sequence-independent manner. Ku70/80 is essential for recruitment of other NHEJ proteins to the DSB in vivo (Mahajan et al. 2002; Mari et al. 2006; Uematsu et al. 2007; Yano et al. 2008). The DNA dependent protein kinase catalytic subunit (DNA-PKcs) is a member of the PIKK family (together with ATM and ATR), and is the first to be recruited to DSB by Ku70/80 (Uematsu et al. 2007). DNA structure is an important factor for DNA-PK activation, with DSB being the most effective activators irrespective of DNA sequence. Without DNA, the interaction between Ku70/80 and DNA-PKcs is not strong (Gottlieb and Jackson 1993; Suwa et al. 1994). Together DNA-PKcs and Ku70/80 form a DNA-PK complex which tethers the broken DNA ends and protects them from nuclease attack. Among the DNA-PK targets are NHEJ proteins Ku70, Ku80 (Douglas et al. 2005), XRCC4 (Yu et al. 2003), Artemis (Ma et al. 2005a), and Ligase IV (Wang et al. 2004), but their phosphorylation does not appear to be required for NHEJ in vivo. The best candidate for DNA-PK appears to be DNA-PKcs which is autophosphorylated at numerous sites. Autophosphorylation appears to have a role in regulating DNA-PKcs enzymatic function and dissociation of phosphorylated DNA-PKcs from DNA bound Ku70/80 (Chan and Lees-Miller 1996; Ding et al. 2003; Merkle et al. 2002).

The next step in NHEJ is processing of DNA ends. Irradiation induced DSB often contain nonligatable end groups and/or other DNA lesions and can be very complex requiring end processing. As mentioned earlier, NHEJ operates in the absence of the sister chromatid or regions of microhomology that could serve as template, and can lead to a loss of genetic information (Budman and Chu 2005). The main candidate for end processing is Artemis. Artemis is a $5' \rightarrow 3'$ exonuclease that in the presence of DNA-PKcs and ATP attains endonuclease activity toward double strand to single strand DNA transitions and DNA hairpins (Ma et al. 2002, 2005b). Artemis can process DNA ends until they are compatible (removal of 5′ and trimming of 3′ overhangs), so at least for some NHEJ events polymerase may not be necessary. However, because radiation induced breaks can be very complex and require further processing leading to formation of DNA gaps, the following steps may include extension by terminal deoxyribonucleotidetransferase (TdT) (Chappell et al. 2002; Karimi-Busheri et al. 2007), or polymerase λ or μ (Bertocci et al. 2006; Davis et al. 2008; Paull 2005). Lastly, Ku70/80 recruits and loads XRCC4-ligase IV functional complex that ligates DNA termini (Grawunder et al. 1997, 1998; Mari et al. 2006).

18.4.2.2 Homologous Recombination

HR serves several biological functions including, crossover generation during meiosis, telomerase maintenance, preservation of stalled replication forks, as well as DSB repair (San Filippo et al. 2008). There are several distinct mechanisms of HR that participate in the above mentioned processes (Table 18.3), but here we will focus on double strand break repair and synthesis dependent strand annealing, as these pathways are typically associated with DSB repair.

DSB repair using HR is critically dependent on the presence of extensive regions of homology and repairs DSB using the information present on the undamaged sister chromatid. Use of the homologous chromosome as a template (such as in G_1 when the sister chromatid is not present) can lead to loss of heterozygosity, which may be more deleterious than error-prone NHEJ; thus, HR mediated DSB repair occurs when an identical DNA molecule is present in the cell (late S and G_2 phase) (San Filippo et al. 2008).

The first step of HR repair is conversion of broken DNA ends into HR substrates, which involves degradation of 5′ ends leaving 3′ overhang several hundred bases long (Fig. 18.6). The candidate for exonucleolytic cleavage is the Mre11/Rad50/Nbs1 (MRN) complex (Paull and Gellert 1998, 1999). The MRN complex participates in virtually all aspects of DNA end metabolism – DSB detection (Mirzoeva and Petrini 2001), DSB processing (Lewis et al. 2004), HR and meiosis (Bressan et al. 1999), telomere maintenance (Boulton and Jackson 1998; Kironmai and Muniyappa 1997), and cell cycle checkpoint activation (Carney et al. 1998; Zhao et al. 2000). Upon resection, single-stranded DNA (ssDNA) ends are bound by replication protein A (RPA), which assists in removal of secondary structures that would inhibit the subsequent steps. However, precoating of ssDNA with RPA prevents binding of the Rad51 recombinase, which is absolutely required for HR. Rad52, a DNA-binding protein, facilitates interaction of Rad51 with ssDNA in the presence of RPA, and counteracts an otherwise inhibitory effect of RPA (Sung 1997).

Table 18.3 Homologous recombination pathways (San Filippo et al. 2008)

Pathway	Biological role	Products
Double strand break repair (DSBR)	Meiotic recombination Double strand break repair	Crossovers and noncrossovers
Synthesis dependent strand annealing (SDSA)	Double strand break repair Meiotic recombination	Only noncrossovers
Single strand annealing (SSA)	Repair of breaks within repetitive sequences	Deletion of one direct repeat and nucleotide sequence between repeats
Break induced replication (BIR)	Repair when there is only one DSB end – telomere elongation, replication fork restart	Accurate repair if sister chromatid or homologous chromosome are used, nonreciprocal translocation if repeat sequence on nonhomologous chromosomes is used

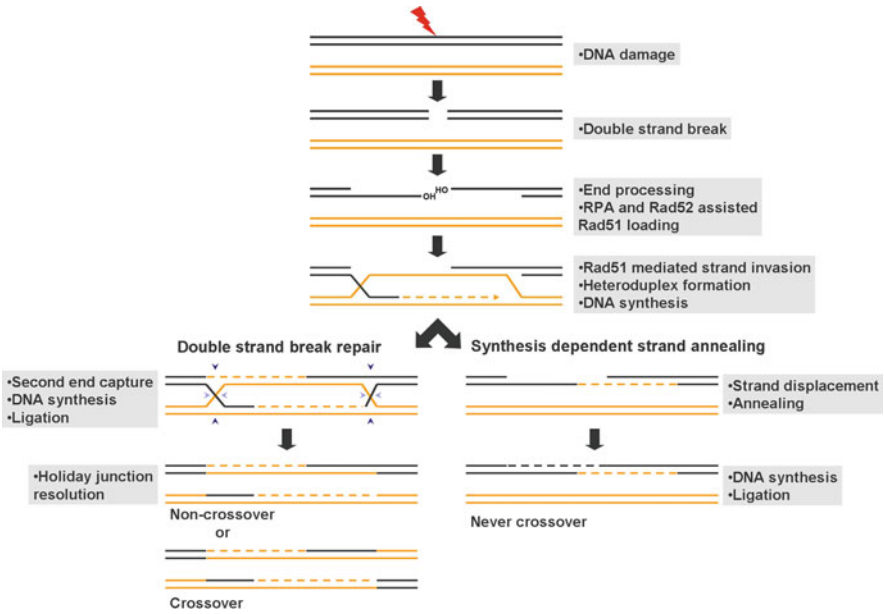

Fig. 18.6 Homologous recombination

Rad51 binding to DNA induces extensive stretching and unwinding of B-DNA leading to presynapting nucleoprotein filament formation that assists homologous strand pairing (Sung and Robberson 1995). Interestingly, Rad51 closely related homologues in *Escherichia coli* RecA, and *Saccharomyces cerevisiae* Rad51 form similar nucleoprotein filaments as well, and its structure is evolutionarily more highly conserved that the protein sequence itself (West 2003). During synapsis, heteroduplex DNA is formed between the invading strand coated with Rad51 and the template DNA molecule by homologous pairing leading to displacement of a single strand from the DNA template molecule. Rad54 stimulates the formation of this joint molecule by introducing negative supercoils into the template DNA, which favors invasion of the incoming single strand (Sigurdsson et al. 2002; Tan et al. 1999).

Once the heteroduplex is formed it can be extended by branch migration. RPA coats the displaced strand preventing reannealing and thus accelerates branch migration. Branch migration is inefficient with Rad51 alone, but is greatly improved by cofactors, such as RPA and Rad52 (Baumann et al. 1996; Baumann and West 1999; Benson et al. 1998; Gupta et al. 1997; Sung and Robberson 1995; Van Dyck et al. 1998). The 3′ end of the invading strand primes DNA synthesis using the sister chromatid as a template. Capture of the second ssDNA tail and Rad52 promoted annealing of the second end with displaced strand lead to formation of an intermediate with two Holliday junctions (cruciform DNA structure formed by crossing of the invading strand and displaced strand, and that links two recombining DNA molecules). Finally, the Holliday junctions are resolved by

specialized endonucleases giving rise to crossover or noncrossover depending on whether all four strands are cut (two dark and two light blue arrows; crossover) or the invading and displaced strand (light blue arrows; noncrossover) (Sugawara et al. 2003; Szostak et al. 1983; Wolner et al. 2003).

Unlike meiotic HR, homologous recombination DSB repair rarely leads to formation of crossovers and hence another model, synthesis dependent strand annealing, was proposed to account for data on mitotic HR (Ferguson and Holloman 1996; Nassif et al. 1994; Strathern et al. 1982). In this model, following strand invasion and repair DNA synthesis, the second DSB end is not captured, and an intermediate with two Holiday junctions is not formed. Instead, the branch migration proceeds until complete displacement of the invading strand is achieved, so that it anneals with the second resected DSB end. Now, the DNA synthesis fills in the gap on the second resected DSB end, whereas the displaced strand reanneals with its complimentary strand forming an intact template DNA molecule.

HR may be enhanced when NHEJ is defective, suggesting that Ku70/80 binding to DNA ends may interfere with HR (Fukushima et al. 2001; Pierce et al. 2001), and some even suggest that in mammalian cells Ku70/80 and Rad52 compete for binding to broken DNA ends. However, experiments with XRCC4/Ligase IV mutants demonstrated that in the absence of functional NHEJ homologous recombination is stimulated, suggesting that (1) NHEJ might be the primary pathway for DSB repair, and (2) if NHEJ fails, HR might take over (Delacote et al. 2002; Frank-Vaillant and Marcand 2002).

18.4.2.3 Early Embryos

Early embryonic cells have a much shorter cell cycle in comparison to somatic cells in mammals as well as other animals, and the integrity of the genome is at great risk from errors in replication. DNA repair in a newly formed zygote is dependent upon maternal mRNA and proteins deposited in the oocyte (Vinson and Hales 2002). These stored transcripts and proteins are also important in chromatin remodeling which is necessary for the onset of embryonic gene expression, as well as in protection of genomic integrity until embryonic gene activation. Expression of embryonic genes starts in one to two cell stage mouse embryos (Schultz 2002), whereas in humans it occurs at the four to eight cell stage (Telford et al. 1990). Therefore, if the DNA repair proteins are not stored in the oocyte, or if there is a DNA repair defect in oocyte and embryonic gene expression does not commence in time, the embryo will die (Jurisicova et al. 1998).

Phosphorylation of histone H2AX at serine 139 by ATM, ATR or DNA-PK kinases serves as a biological marker for detection of DSB. Phosphorylated H2AX (γ-H2AX) covers several megabases surrounding a DSB and can be microscopically visualized, providing a tool for monitoring DSB formation and repair (Rogakou et al. 1998; 1999). Early mouse embryos display stage specific phosphorylation of H2AX (Adiga et al. 2007a; Yukawa et al. 2007). One and two cell stage embryos exposed to 3, 5, or 10 Grays of γ-irradiation do not form γ-H2AX

foci 30 min after irradiation, which in contrast can be readily observed in oocytes, the second polar body, and four-cell stage embryos (Adiga et al. 2007b; Yukawa et al. 2007). Time course analysis of γ-H2AX foci formation revealed that 64 h following exposure of one cell embryos to irradiation a fraction of blastomeres formed γ-H2AX foci. Similar results were obtained in irradiated two cell mouse embryos. In contrast, γ-H2AX foci formed in six to eight cell stage embryos within 30 min of irradiation, persisted for 12 h, and disappeared 24 h postirradiation. In early blastocyst stage embryos visible γ-H2AX foci formed within 30 min of irradiation, and began to fade 6 h later (Adiga et al. 2007a). Thus, it appears that one and two cell stage mouse embryos exhibit delayed phosphorylation of H2AX, and that DNA damage persist until morula and blastocyst stage when γ-H2AX foci can form. H2AX is, however, present in one and two cell stage embryos, and both ATM and DNA-PKcs kinases are phosphorylated and presumably activated in response to γ-irradiation (Adiga et al. 2007a; Yukawa et al. 2007). Therefore, it is possible that there is some unknown mechanism that inhibits phosphorylation of H2AX, or that some other factors required for H2AX phosphorylation by ATM and DNA-PK are absent from one and two cell stage mouse embryos (Adiga et al. 2007a; Yukawa et al. 2007). Alternatively, since chromatin undergoes extensive remodeling following fertilization, the modified chromatin state may be responsible for the absence of γ-H2AX staining in one and two cell stage embryos. Nevertheless, the absence of γ-H2AX focus formation may have adverse effects on DNA damage response in preimplantation embryos, as it normally serves as a platform for repair protein complex assembly (Adiga et al. 2007a). Absence of γ-H2AX may confer increased sensitivity to DNA damage and eventually lead to loss of damaged cells.

18.4.2.4 Embryonic Stem Cells

It has been suggested that mouse ES cells rely mainly on HR to fix DSB because they spend most of their cell cycle time in S phase of the cell cycle when the sister chromatid is present, as well as in order to prevent accumulation and subsequent propagation of genetic changes. In support of this hypothesis, Rad51 knock out mouse embryos exhibit profound radiosensitivity and die early in development due to decreased proliferation and massive chromosome loss (Tsuzuki et al. 1996). Rad51 null ES cells cannot be isolated from knock out mouse embryos, nor generated in vitro (Lim and Hasty 1996). A p53 null background only partially improves the phenotype of Rad51 deficient mouse embryos, as double knock-outs exhibit extended development, but still die once accumulated DNA damage interferes with normal cellular function and mitosis (Lim and Hasty 1996). Similarly, complete loss of Rad50, Nbs1, and Mre11 is early embryonic lethal, suggesting that the MRN complex is required for normal cellular function, proliferation, and growth. Mre11 knock out ES cells cannot be obtained (Xiao and Weaver 1997), Nbs1 deficient ICM fail to proliferate and die in vitro (Zhu et al. 2001), whereas early Rad50 null mouse ES cells are hypersensitive to ionizing radiation and cannot

be propagated in cell culture (Luo et al. 1999), indicating that mutations in these genes are toxic at the cellular level.

Rad52 knock-out mice are viable and show no impaired viability, fertility, or immune system deficiency. Mouse Rad52 nullizygous ES cells do not show signs of radiosensitivity, and exhibit 30–40% reduction in HR, unlike yeast mutant Scrad52 cells which are not viable (Rijkers et al. 1998). This suggests that there are functionally related genes in mammalian cells that can compensate the absence of Rad52. Rad54 inactivation in mouse ES cells leads to decreased HR and increased radiosensitivity, but Rad54 null mice are viable and fertile (Essers et al. 1997), suggesting that Rad54 is not essential for normal mouse development.

Mice missing any of the genes involved in NHEJ are viable, or die only late in embryonic development. Nullizygous ligase IV and XRCC4 mice are late embryonic lethal due to massive p53 dependent neuronal apoptosis, arrested lymphogenesis, and other cellular defects (Barnes et al. 1998; Frank et al. 2000; Li et al. 1995). Ku70 or Ku80 inactivation in mice results in growth retardation, profound immunodeficiency, as well as marked radiosensitivity and inability to perform end-joining at the cellular level (Gao et al. 1998; Gu et al. 1997; Jin et al. 1998; Nussenzweig et al. 1997). Mice lacking DNA-PKcs are immunodeficient, but do not exhibit growth retardation (Gao et al. 1998).

Mouse ES cells that lack DNA-PKcs do not exhibit increased radiosensitivity or sensitivity to etoposide treatment, unlike DNA-PKcs knock out fibroblasts (Gao et al. 1998; Jin et al. 1998), suggesting that NHEJ is not a predominant DSB repair pathway in ES cells. In addition, wild type mouse ES cells express low levels of DNA-PKcs, corresponding to lower activity of DNA-PK in these cells in comparison to mouse embryonic fibroblasts (MEF). Furthermore, mouse ES cells express a 20-fold higher level of Rad51 in comparison to MEF, and about the same level of XRCC4 (Tichy and Stambrook 2008).

Important insight into role of HR and NHEJ came from studies by Banuelos et al. (2008) in wild type and ATM, H2AX, and DNA-PKcs mutant mouse ES cells. H2AX knockout mice exhibit similarities to ATM null mice, including radiosensitivity, growth retardation, and chromosomal abnormalities (Celeste et al. 2002; Xu et al. 1996). Mouse ES cells that lack H2AX are highly sensitive to ionizing radiation and radiomimetic drug treatments, show increased spontaneous and irradiation induced chromosomal abnormalities, and a subtle reduction in size of Rad51 foci following ionizing radiation exposure (Bassing et al. 2002). Mouse ES cells defective in ATM or H2AX exhibit faster DSB repair in comparison to wild type mouse ES cells, decreased survival, and inability to form foci of phosphorylated ATM (although ATM is autophosphorylated). In addition, cells that do not have ATM or H2AX express higher level of DNA-PKcs than wild type mouse ES cells. Inhibition of DNA-PK activity resulted in reduced DSB rejoining in H2AX knock-out, but not wild type mouse ES cells, suggesting that DNA-PK has an important role in DSB repair in H2AX deficient mouse ES cells. However, in wild type mouse ES cells DNA-PKcs activity still contributes to their survival following irradiation, because inhibition of DNA-PKcs with its specific inhibitor NU7026 reduces survival of wild type and H2AX mutant mouse ES cells. Taken together, it

appears that DNA-PK dependent DNA repair is not the main pathway for repair of DSB, but still contributes to survival of mouse ES cells following DNA damage. In instances when HR is impaired, mouse ES cells can redirect DSB repair toward NHEJ.

Both mouse (Saretzki et al. 2004) and human (Maynard et al. 2008) ES cells exhibit increased capacity for DNA damage removal following various genotoxic treatments, including DSB removal after ionizing radiation in comparison to differentiated cells. During differentiation of mouse (Saretzki et al. 2004) and human (Saretzki et al. 2008) ES cells expression of antioxidant and DNA repair genes is reduced, while the frequency of γ-H2AX positive cells increases. Wild type human ES cells express higher level of DNA-PKcs and KU70 relative to mouse ES cells and more similar level to differentiated human cells (Banuelos et al. 2008). Interestingly, there is a general difference between mouse and human somatic cells in their dependence on NHEJ. For example, DNA-PK activity (Finnie et al. 1995) and Ku70 expression (Anderson and Lees-Miller 1992) are higher in human relative to mouse somatic cells, and Ku80 is an essential protein in human, but not mouse cells (Li et al. 2002). In addition, human ES cells rejoin DSB faster than mouse ES cells (Banuelos et al. 2008) suggesting there might be difference between mouse and human ES cells' choice or repair pathway.

18.4.3 Maintenance of Genomic Integrity in ES Cells

We have already discussed sources of DNA lesions, and some DNA damage response pathways, including cell cycle arrest and DNA repair. In this section, we will briefly summarize other mechanisms that contribute to the maintenance of genomic integrity in ES cells under steady state conditions, i.e., in the absence of exogenously induced DNA damage.

Two main strategies employed by cells for limiting DNA changes are (1) mutation suppression and (2) elimination of cells that acquire mutations. Indeed, mouse ES cells have a significantly lower rate of spontaneous mutation relative to their differentiated counterparts (Cervantes et al. 2002). ES cells are actively proliferating cells that go through numerous rounds of replication that would lead to telomere attrition and cellular senescence. However, in ES cells telomere length does not decrease due to expression of TERT (reverse transcriptase of telomerase) and TR (telomerase template RNA) subunits of telomerase, enzyme that maintains telomeres. During differentiation of both mouse and human ES cells telomerase activity declines due to epigenetic changes in the *tert* and *tr* promoters (Saretzki et al. 2004, 2008).

Under physiological conditions ROS produced as by-product of oxidative phosphorylation are a major source of endogenous DNA damage. ES cells maintain lower level of oxidative stress in comparison to somatic cells by reduced oxygen consumption, low mitochondrial biogenesis, high expression of certain antioxidant genes, and they shift their ATP production toward glycolysis instead of oxidative

phosphorylation (Saretzki et al. 2004, 2008; St John et al. 2006). It should be mentioned that ROS also serve as important signaling molecules in the cell, and low levels are necessary for the maintenance of self-renewal in adult stem cells, whereas high levels induce differentiation (Ito et al. 2006). During differentiation of ES cells oxygen consumption and mitochondrial mass increase, whereas expression of certain antioxidant genes decreases creating ROS rich environment (Saretzki et al. 2004, 2008). In addition, mouse ES cells have high activity of efflux pumps, such as P-glycoprotein that eliminate toxic chemicals from the cell. The activity of P-glycoprotein was found to decrease with differentiation of mouse ES cells (Saretzki et al. 2004). Finally, as already discussed, ES cells appear to have efficient DNA repair mechanisms, and that expression of DNA repair genes reduces with differentiation (our unpublished results).

Elimination of cells with DNA lesions is a very efficient approach to keeping the pristine cellular population, but would lead to cellular depletion as all cells would be eventually eliminated. However, different cell types exhibit different sensitivity to DNA damage, and ES cells in general appear to be very sensitive, most likely due to absent G_1/S arrest (Momcilovic et al. 2009). DNA damage induced differentiation has also been proposed as an effective means of elimination of damaged pluripotent stem cells (Lin et al. 2005; Qin et al. 2007). However, this hypothesis is still being tested. It is possible that radiomimetic drugs may elicit differentiation due to continuous infliction of DNA damage and persistent DNA damage signaling.

18.4.4 Induced Pluripotent Stem Cells and Genomic Stability

Reprogramming of somatic cells into the pluripotent state by transcription factors opens doors to more ethically accessible and patient specific cell replacement therapies, but also provides tools for studying how differentiation can be reversed and what is it that enables cells to change their function, morphology and gene expression so drastically. Thus, how does reprogramming work? This is of course very complex and still under investigation, but studies performed thus far strongly indicate that the key is chromatin remodeling and change in the epigenetic landscape (Hochedlinger and Plath 2009). Epigenetic changes refer to changes in DNA methylation and histone modifications (acetylation, methylation, and phosphorylation) that are inheritable and alter chromatin organization. The chromatin structure affects DNA accessibility to DNA and RNA polymerases, as well as transcription factors, so epigenetic changes modify gene expression. In general, in pluripotent stem cells chromatin is more open and accessible to transcriptional machinery; upon differentiation chromatin becomes condensed and transcription repressive and each cell type acquires a specific epigenetic profile (Keenen and de la Serna 2009). Transcription factors used in the reprogramming cocktail (Oct4, Sox2, Klf4, c-Myc, or Oct-4, Nanog, Sox2, Lin28) can be envisioned to act in several manners (1) activate expression of genes involved in pluripotency maintenance, including their own genes and chromatin remodeling enzymes (Boyer et al. 2005), (2) repress

genes involved in differentiation, and hence, strip somatic cells of their differentiated gene expression profile (Boyer et al. 2005), (3) activate expression of other genes that contribute to the pluripotent state, such as those involved in metabolism and proliferation (Hochedlinger and Plath 2009), and (4) sequester somatic cell specific transcription factors in inactive complexes (Hochedlinger and Plath 2009).

Reprogramming is still a very inefficient process with 0.01–0.1% efficiency (Okita et al. 2007; Takahashi and Yamanaka 2006). Each iPS clone carries 10–20 viral transgenes integrated at various genomic sites (Takahashi and Yamanaka 2006). It is possible that due to random viral integration essential endogenous genes are disrupted or oncogenes activated, affecting cellular viability. Furthermore, it is necessary for somatic cells to receive the correct relative expression of the transgenes, and the probability that a somatic cell will have four transgenes integrated with optimal stoichiometry is similar to the efficiency or reprogramming with four retrovirally carried transgenes. In addition, c-Myc, Lin28, and Klf4 are known oncogenes that may activate a tumor suppression response, including *Ink4/Arf* locus and p53–p21 axis that induce cell cycle arrest, senescence and apoptosis (Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009). *Ink4/Arf* locus encodes p15, p16, and p19 tumor suppressors that are in somatic cells expressed at basal level and upregulated in the presence of aberrant proliferative signals (such as those induced by oncogenes). Tumor suppressors p15 and p16 bind to and inhibit Cdk4 and Cdk6, rendering them unable to phosphorylate Rb and promote passage through the restriction point; p19 binds to and inactivate Mdm2 thereby promoting p53 stabilization and activation of p53 dependent gene expression (Collado et al. 2007; Serrano et al. 1997; Sharpless 2005). In ES and iPS cells *Arf4/Ink* locus is inactive. However, during reprogramming the cell culture conditions enhance expression of this locus and reduce the efficiency of reprogramming, emphasizing the importance of silencing of the *Arf4/Ink* locus to allow proliferation and reprogramming (Li et al. 2009). Mouse p53-deficient fibroblasts can be reprogrammed with increased efficiency using only three transgenes (Oct-4, Sox2, and Klf4) in comparison to wild type fibroblasts (Hong et al. 2009), and decreasing the p53 or p21 levels enables production of germline capable iPS using only Oct-4 and Sox2 (Kawamura et al. 2009), further highlighting the role of p53–p21 in suppression of reprogramming. The mechanism by which oncogenes block reprogramming include cell cycle arrest (activation of p53–p21 and Rb pathways), as well as apoptosis (p53 pathway), and senescence (Arf).

Collectively, these studies raise questions of genomic stability and identity of iPS cells. Which are the cells that actually get reprogrammed? Are those the cells in which tumor suppressor loci are already repressed? How do they respond to the DNA damage? Are they prone to induce tumors after differentiation and transplantation into patients? Induced pluripotent stem cells can induce malignancies in mice (Okita et al. 2007), and injection of iPS generated by transfection of p53 deficient T-lymphocytes with Oct-4, Sox2, Klf4, and c-Myc into recipient blastocyst resulted in production of chimeric mice that developed tumors and died within 7 weeks, clearly demonstrating the safety concerns resulting from permanent p53 suppression (Hong et al. 2009). In order for iPS to have real therapeutic potential their genomic stability must be maintained, more efficient methods for iPS production

need to be established, and strict quality and safety protocols put in place. The use of integrating viral transgenes can be potentially dangerous due to insertional mutagenesis and risk of transgene reactivation. However, nonintegrating vectors and reprogramming using chemicals, although potentially safer, are even less efficient in inducing pluripotency than integrating retroviruses. Perhaps transient suppression of p53 could increase efficiency without a long term negative effect on genomic stability, but this still needs to be tested.

Our unpublished results demonstrate that human iPS cells are extremely radiosensitive and undergo excessive cell death similar to human ES cells. However, iPS cells activate checkpoint signaling cascade, phosphorylate ATM, Chk2, and p53, and arrest in G_2/M stage of the cell cycle. Human ES and iPS cells form Rad51 foci following ionizing irradiation and display sister chromatid exchanges after treatment with topoisomerase I inhibitor camptothecin, suggesting that both pluripotent stem cell types utilize HR to repair DSB. Human ES and iPS cells both show higher level of expression of double strand break, mismatch, base excision, and nucleotide excision repair genes relative to their differentiated counterparts in the steady state condition. Thus, it appears that DNA damage responses in iPS cells are very similar to the ones in human ES cells, and that during reprogramming of somatic cells' DNA damage response acquires certain adjustments that accommodate specific need of pluripotent stem cells in balancing self-renewal with maintenance of genomic stability.

18.5 Conclusions

ES cells are cells with the capability to undergo unlimited self-renewal and differentiation into any somatic cell type. Since proliferation supports self-renewal, it is not surprising to discover that ES cells are poised to proliferate. The loss of strict G_1 to S transition, and ability to divide in the absence of mitogenic signals (Savatier et al. 1994, 1996; Stead et al. 2002; White et al. 2005) directly reflect the need of early embryonic cells to divide and increase their numbers in order to reach certain developmental milestones. However, due to their developmental potential it is essential that these cells maintain genomic stability in conditions that promote genetic changes (rapid proliferation and absence of G_1/S arrest). It is possible that the absence of G_1/S arrest contribute to high rates of spontaneous and induced apoptosis that could eliminate damaged cells. ES cells appear to be able to very efficiently repair DNA damage (Maynard et al. 2008), thereby, actively suppressing endogenous and induced DNA lesions from becoming part of the genome. It is still not clear if ES cells are more prone to apoptosis in certain cell cycle stages, or throughout the cell cycle. Since ES cells arrest in G_2/M stage of the cell cycle, it could be possible that cells that are in G_1 at the time of DNA damage undergo cell death, whereas cells that are in late S or G_2 arrest and repair DNA. This hypothesis still needs to be tested.

The generation of iPS cells provides hope for patient-specific therapies, but this field is still nascent, and has to move from proof of principle to understanding of iPS cells identity, genomic stability, and tumorigenicity. Preliminary studies show that iPS share many similarities with ES cells, but we are only beginning to understand their differences and potentials. The differences in cell cycle regulation between ES and iPS cells might also reflect on genomic maintenance, which ultimately would greatly affect the way we utilize iPS in various applications for fundamental biology as well as medical approaches.

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Chapter 19
Cell Cycle Regulation by microRNAs in Stem Cells

Yangming Wang and Robert Blelloch

Abstract The ability to self-renew and to differentiate into at least one-cell lineage defines a stem cell. Self-renewal is a process by which stem cells proliferate without differentiation. Proliferation is achieved through a series of highly regulated events of the cell cycle. MicroRNAs (miRNAs) are a class of short noncoding RNAs whose importance in these events is becoming increasingly appreciated. In this chapter, we discuss the role of miRNAs in regulating the cell cycle in various stem cells with a focus on embryonic stem cells. We also present the evidence indicating that cell cycle-regulating miRNAs are incorporated into a large regulatory network to control the self-renewal of stem cells by inducing or inhibiting differentiation. In addition, we discuss the function of cell cycle-regulating miRNAs in cancer.

19.1 Introduction: Self-Renewal Process of Stem Cells

Self-renewal defines a process by which a stem cell generates one (asymmetric division) or two (symmetric division) daughter cells that have similar developmental potential as the mother cell. Different stem cells have different developmental potential, which are restricted by specific epigenetic programs, but generally a stem cell should have the ability to differentiate into at least one cell type. The rate of self-renewal strictly depends on the particular type of stem cell. Embryonic stem (ES) cells are cell lines derived from the inner cell mass of a developing blastocyst

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(Rossant 2008). Like their counterparts in the inner cell mass, ES cells are pluripotent stem cells. In other words, they can differentiate into all derivatives of three primary germ layers as well as germ cells. ES cells can self-renew rapidly and indefinitely; therefore, they can provide large amount of tissues for tissue replacement therapy. Late in embryonic development, transient fetal stem cells such as fetal hematopoietic and neural stem cells are generated, which have limited developmental potential but retain a high self-renewal rate to fulfill the needs of fetal growth (Mikkola and Orkin 2006; Kriegstein and Alvarez-Buylla 2009; He et al. 2009).

Eventually in a developed organism, adult stem cells replace fetal stem cells to maintain tissue homeostasis. Adult stem cells have limited developmental potential but many still have extensive self-renewal potential evidenced by their long-term maintenance both within their niche and even following serial transplantation experiments (Osawa et al. 1996; Li and Clevers 2010). However, the self-renewal rate of adult stem cells is generally slower than ES and fetal stem cells. In fact, many adult stem cells spend most of their life in quiescent state, which is a reversible cell cycle arrest stage. It is thought that quiescence is important for maintaining tissue homeostasis and preventing cancer growth. Cancer is a class of diseases in which a group of cells display unlimited growth, invasion, and eventually metastasis. How and in what cells cancer is initiated are not well understood. However, several features of somatic stem cells make them plausible sites for cancer initiation. Preserved replicative capacity may enable them to accumulate mutations over a prolonged life-span. In addition, the developmental potency of somatic stem cells may underlie the cellular heterogeneity of many human cancers (Reya et al. 2001; Harmes and DiRenzo 2009). Therefore, abnormalities in the self-renewal program of adult stem cells may lead to cancer initiation and progression. Understanding self-renewal mechanisms will provide fundamental insights into development and tumorigenesis, in turn leading to efficient methods to control stem cell self-renewal for advancing regenerative medicine and cancer therapy.

Self-renewal is accomplished by cell division. Cell division is achieved through a series of highly regulated events making up the cell cycle, including the alternating activities of various cyclin-dependent kinases (Cdks), which are activated only upon binding to specific cyclins. Higher organisms incorporate many protein modulators of Cdk activity such as Cdk inhibitors to differentially regulate cell cycle progression in different cells or in response to different environmental conditions. As in many other processes, these modulators are themselves regulated often at the posttranscriptional level. microRNAs (miRNAs) provide one important means of posttranscriptional regulation (Bartel 2009). Accumulating evidence shows that miRNAs are new players regulating many protein-coding genes and specific pathways in the cell cycle (Wang and Blelloch 2009). In this chapter, we summarize the data supporting the cell cycle regulating roles of miRNAs in ES cells, adult stem cells, and cancer cells. Furthermore, we describe how these cell cycle-regulating miRNAs affect self-renewal through their influence not only on proliferation but also on differentiation.

19.2 Cell Cycle Regulation

A cell division cycle usually consists of four major phases: the G1 phase in which cells prepare for DNA replication and respond to external signals by either remaining or exiting the cell cycle; the S phase in which cells replicate their DNA; the G2 phase in which cells prepare for division; and the M phase in which one cell divides into two cells. When cells exit the cell cycle at G1, they enter a nondividing state termed G0. Some cells such as neurons and muscle cells permanently exit cell cycle and become terminally differentiated. Others such as fibroblasts, hepatocytes, and adult stem cells usually stay in quiescence, a state in which cells stop proliferation but retain the ability to reenter cell cycle when needed. The duration of cell cycle time is highly variable among different cells (Dalton 2009). Mouse ES cells in culture have a cycle time as short as ~10 h. In contrast, stem cells in resting mouse skin may have a cycle time of more than 700 h (Bickenbach 1981). This difference is largely due to the varying length of G1, which is the most variable phase of the cell cycle.

During the G1 phase, a cell senses its environment for the presence of growth factors and nutrients as well as evaluates the integrity of its genome. These tasks are accomplished through a restriction or check point at the G1/S transition (Massagué 2004). Following the restriction point, a cell can pass through S phase and mitosis independent of mitogens and growth factors. The G1 restriction point requires the sequential activation of the Cdk4/6 and the Cdk2 kinases, which are expressed throughout the cell cycle but only activated upon binding to their specific cyclins. During the early G1 phase, the mitogenic factors stimulate the expression of the D-type cyclins. The Cdk4/6–Cyclin D complex then phosphorylates proteins of the retinoblastoma (pRb) family. This event leads to a partial inhibition of Rb and release of the E2F transcription factors, increasing the transcription of the E2F targets. Among the E2F targets, there are the E-type cyclins which activate Cdk2 further phosphorylating Rb. This feed-forward loop fully releases E2F, leading to the transcription of genes required for progression through S phase. In addition, the Cdk2–Cyclin E also phosphorylates several other targets important in the progression through S phase (Stein et al. 2006). Upstream inhibitors including members of the INK (p15, p16, and p18) and CIP families (p21, p27, and p57) modulate the activity of the Cdk–cyclin complexes. Some of these inhibitors are induced upon stresses such as nucleotide depletion and DNA damage. For example, the DNA damage checkpoint pathway activates the transcription of p21 through the posttranslational modification of p53, which arrests cells in the G1 phase until the attenuation of DNA damage signaling by the DNA repair machinery. Differential expression of the cell cycle regulatory factors shapes the G1/S transition kinetics in different cell types. Aberrations in the expression of these regulatory factors can lead to uncontrolled proliferation, the hallmark of cancer. Therefore, it is not surprising that most cancer cells have a faster G1/S transition compared to their normal counterparts.

19.3 miRNA Biogenesis

MiRNAs are ~22-nucleotide short RNAs that are produced by most eukaryotic cells to control gene expression. Despite their small size, they play important functions in a variety of developmental and physiological processes. The first miRNA was discovered in 1993 by Rosalind Lee, Rhonda Feinbaum, and Victor Ambros. In studying the function of the gene *lin-14* in *Caenorhabditis elegans* development, they discovered that the LIN-14 protein level was regulated by a short RNA product encoded by the *lin-4* gene (Lee et al. 1993). The lin-4 gene gives rise to a 22-nucleotide RNA containing sequences partially complementary to multiple sites at the 3′ untranslated region (UTR) of the *lin-14* mRNA. This complementarity was necessary and sufficient to inhibit the translation of *lin-14* mRNA into LIN-14 protein. At the time, this small RNA was thought to be an idiosyncrasy in the worm. However, in 2000 another miRNA, let-7, was identified (Reinhart et al. 2000). The let-7 represses *lin-41*, *lin-14*, *lin-28*, *lin-42*, and *daf-12* expression during developmental stage transitions in *C. elegans*. Interestingly, let-7 was found to be conserved in many species, indicating the existence of a wider phenomenon (Pasquinelli et al. 2000). Since then, thousands of miRNAs have been cloned and identified in various species. Around 700 miRNAs have been confirmed in humans, while more than 1,000 miRNAs are predicted to be encoded by human genomes (Creighton et al. 2010).

Mature miRNAs are generated through two sequential cleavages by RNase III enzymes (Kim et al. 2009a). They are usually first transcribed as part of a long RNA transcript (pri-miRNA) by RNA polymerase II (Fig. 19.1). The first cleavage is conducted in the nucleus by the microprocessor complex (Gregory et al. 2004; Han et al. 2004, 2006) comprising the RNaseIII enzyme Drosha and its RNA binding partner Dgcr8. The cleavage generates a short hairpin (pre-miRNA) around 60–75 nucleotides. The pre-miRNA is then exported into the cytoplasm by Exportin 5 in a Ran-GTP dependent manner (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004). Another RNase III enzyme Dicer along with its partner TRBP conducts the second cleavage on the pre-miRNA to generate the mature miRNA duplex (Hutvágneret al. 2001). The duplex enters a third protein complex called the RNA induced silencing complex (RISC), which produces and directs the mature miRNA to its targets. miRNAs typically bind to the UTR of their mRNA targets and mediate the repression of translation and/or destabilization of these mRNA targets. The interaction between miRNAs and their mRNA targets is largely dependent on base-pairing between only a small fraction of the miRNA sequence (second to eighth position at the 5′ end, also known as seed sequence) and the 3′ UTR of their mRNA targets (Bartel 2009). Interestingly, recent studies suggest that miRNAs can also regulate mRNA targets by binding to the coding region and the 5′ UTR (Baek et al. 2008; Selbach et al. 2008; Forman and Coller 2010). The relatively loose requirement for the interaction between miRNAs and their mRNA targets enables a single miRNA to simultaneously target hundreds of mRNAs. In fact, around 60% of human genes are predicted to be regulated by miRNAs (Friedman et al. 2009).

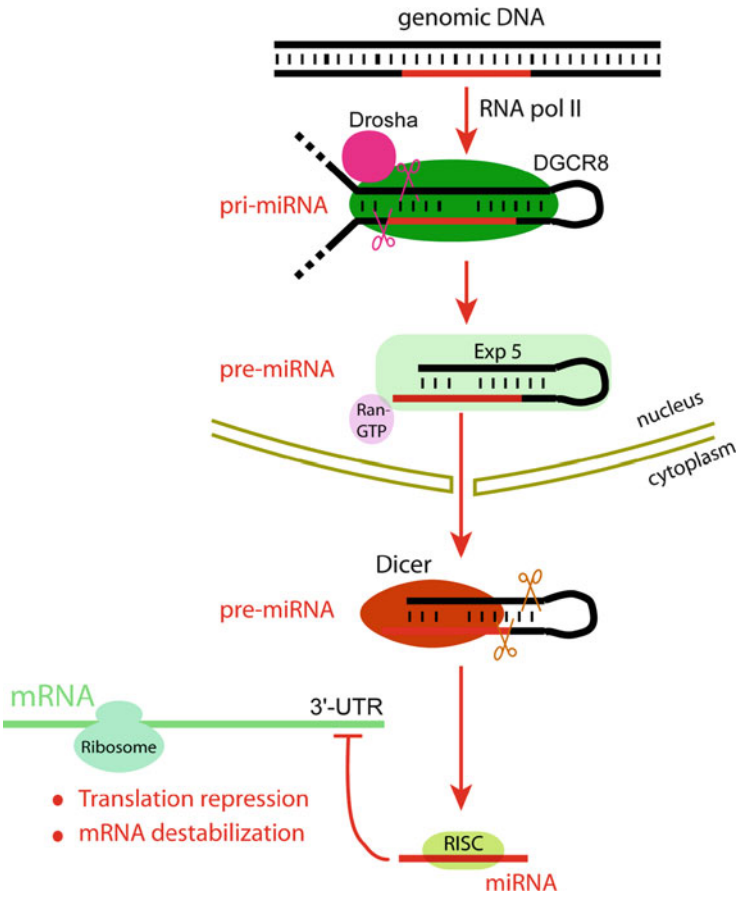

Fig. 19.1 miRNA biogenesis pathway. In the nucleus, the Drosha/Dgcr8 complex recognizes a stem loop structure of approximately 33 base pairs in length and cleaves the hairpin 11 base pairs from the stem-single strand RNA junction leaving a characteristic 2-nucleotide 3′ overhang. In the cytoplasm, the Dicer containing complex cleaves the pre-miRNA at the base of the hairpin loop to form a 2-nucleotide 3′ overhang and generates an approximately 22 nucleotide mature miRNA duplex. A single strand of the duplex is then loaded into the RISC

For these reasons, miRNAs are ideal candidates for regulating complicated processes such as cell proliferation that involves a large number of genes.

19.4 miRNAs Regulate the G1/S Transition in Mouse Embryonic Stem Cells

ES cells can self-renew rapidly and indefinitely (Dalton 2009). The proliferation rate of mouse ES cells is extremely fast with a cycling time of ~10 h. The length of cycling time is increased to more than 18 h in differentiated cells. The

rapid self-renewal of ES cells is largely due to their unique cell cycle structure. ES cells have an unusually short G1 phase (~2 h) with most (~70%) cells in S phase. In addition, unlike differentiated cells, ES cells lack a G1 restriction point or checkpoint, therefore can proliferate even in the absence of growth factors or mitogens (Jirmanova et al. 2002). This property is reminiscent of many cancer cells (Pardee 1974; Cherington et al. 1979; Berthet and Kaldis 2007), suggesting that cancer cells may hijack molecular factors used by ES cells for their proliferative advantages. In mouse ES cells, the Cdk4/Cdk6–Cyclin D complex is not present or active, while the Cdk2–Cyclin E complex is constitutively active throughout the cell cycle. During differentiation, the Cdk2–Cyclin E activity is decreased and becomes cell cycle dependent (Stead et al. 2002; Savatier et al. 1996). The decrease of Cdk2 activity is at least in part due to the upregulation of multiple G1/S transition inhibitors (e.g., p21, p27). The result is an elongated G1 phase and slower proliferation in the differentiated cells.

The molecular details resulting in high Cdk2 activity and, therefore, rapid G1/S transition in ES cells are not well understood. Studies in miRNA-deficient mouse ES cells suggest a central role for miRNAs (Murchison et al. 2005; Wang et al. 2007). Knockout of Dicer or Dgcr8, two essential proteins for miRNA biogenesis, results in slower proliferation in ES cells. Furthermore, both Dicer and Dgcr8 knockout ES cells accumulate in G1 phase, suggesting an important role of miRNAs for the G1/S transition in these cells (Fig. 19.2a). To identify specific miRNAs that promote the G1/S transition in ES cells, a screening strategy (Fig. 19.2b) was designed where chemically synthesized miRNA duplexes, called miRNA mimics, were individually transfected into the Dgcr8 knockout cells (Wang et al. 2008). The transfected cells were then evaluated for changes in their rate of cell proliferation. This unbiased screening approach identified multiple miRNAs that partially rescued the proliferation defect. These miRNAs include members of the miR-290 cluster (miR-291a-3p, miR-291b-3p, miR-294, and mir-295) and the miR-302a-d, and those with the slightly different seed sequence "AAAGUGC" including miR-20, miR-93, and miR-106 belonging to the miR-17/20/106 family. These results are consistent with the notion that members of miRNA families (defined by their common seed sequence) will often have overlapping roles in physiological processes (Miska et al. 2007; Ventura et al. 2008; Alvarez-Saavedra and Horvitz 2010). All these miRNAs are expressed in wild type ES cells with members of the miR-290 cluster being the highest. The mir-290 cluster alone makes up greater than 70% of the total quantity of miRNAs in ES cells (Marson et al. 2008). Importantly, expression of this cluster is rapidly downregulated upon differentiation, coincident with the elongation of cell cycle (Melton et al. 2010; Houbaviy et al. 2003).

Transfection of miR-291a-3p, miR-291b-3p, miR-294, and miR-295 individually fully rescues the G1 accumulation phenotype along with enhanced proliferation suggesting that they are acting to promote the G1/S transition. Because of their function in regulating the cell cycle, these miRNAs were then termed the ESCC miRNAs for *E*mbryonic *S*tem cell enriched *C*ell *C*ycle-Regulating miRNAs (Wang et al. 2008). It was hypothesized that ESCC miRNAs promote the G1/S transition by suppressing inhibitors along the Cdk2–Cyclin E pathway as this is the key G1/S

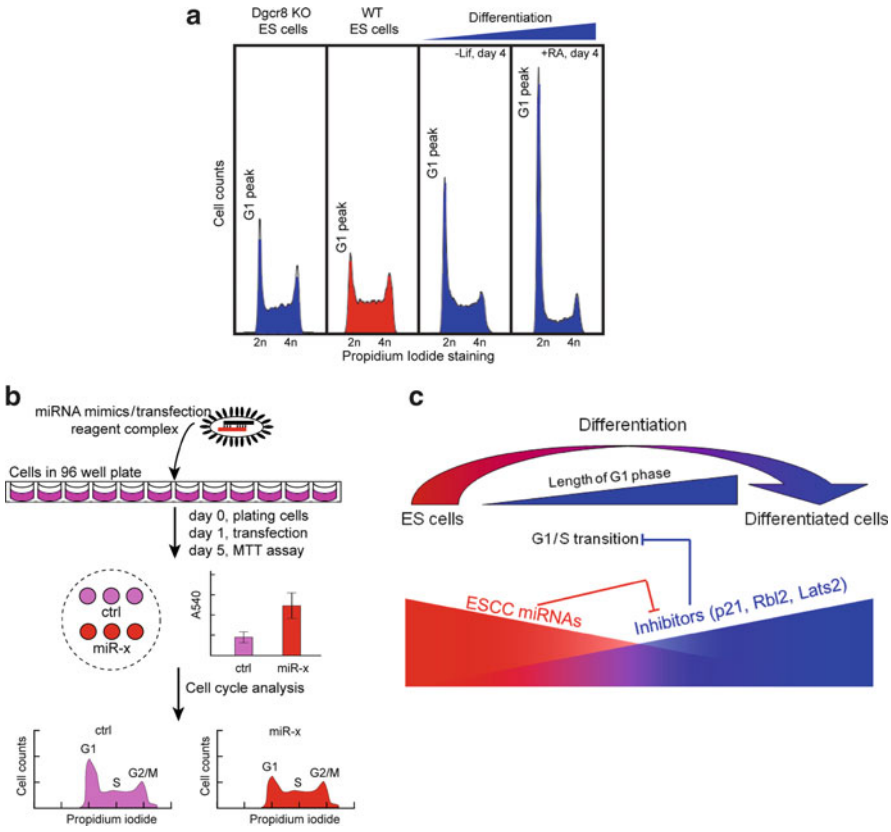

Fig. 19.2 miRNAs regulate the G1/S transition in mouse ES cells. (**a**) Cell cycle profiles of ES cells, differentiated ES cells, and miRNA-deficient ES cells. More cells accumulate in G1 phase in differentiated cells and miRNA-deficient ES cells than undifferentiated ES cells. (**b**) Screening strategy to identify cell cycle-regulating miRNAs in miRNA-deficient ES cell model (adapted from Wang et al. 2008). (**c**) ESCC miRNAs promote the G1/S transition in ES cells by repressing multiple inhibitors along the Cdk2-Cyclin E pathway (adapted from Wang and Blelloch 2009)

regulating pathway in ES cells (Fig. 19.2c). Indeed, careful analysis identified p21, Rbl2, and Lats2 as targets of ESCC miRNAs. mRNA profiling experiments suggest that dozens more cell cycle regulating genes are direct targets of ESCC miRNAs, since these genes are downregulated by the transfection of ESCC miRNAs and harbor miRNA binding sites at their 3′ UTRs (Yangming Wang and Robert Blelloch, unpublished data). p21 was confirmed as a functional target because ectopic expression of p21 without its 3′ UTR in wild-type ES cells lead to an increase in p21 protein levels and accumulation of cells in the G1 phase. However, it only partially phenocopies the G1 accumulation seen in the Dgcr8 knockout cells, indicating that ESCC miRNAs regulate G1/S transition through multiple targets. These findings show that miRNAs work through multiple targets reinforcing specific phenotypic

outcomes. That is, miRNAs act at a global scale to regulate a particular biological process.

ES cell differentiation is accompanied by an elongation of the G1 phase. In addition, the G1 regulatory molecules, such as p21 and p27, are exquisitely regulated during differentiation and in many models appear to play a pivotal role in differentiation (Savatier et al. 1996; Sabapathy et al. 1997; Parker et al. 1995; Ohnuma et al. 1999; Fujii-Yamamoto et al. 2005; Ullah et al. 2008). Therefore, it is expected that G1/S transition-promoting ESCC miRNAs may also play a role in promoting ES cell self-renewal by preventing differentiation. Indeed, a recent report (Melton et al. 2010) showed that ESCC miRNAs can prevent ES cell differentiation induced by another miRNA, let-7. let-7 preferentially regulates transcripts that are enriched in ES cells, including many transcripts that are regulated by the pluripotency transcription factors Oct4, Sox2, Nanog, and Tcf3. Interestingly, a number of the direct targets of let-7 are indirectly upregulated by the ESCC miRNAs, which may explain how the ESCC miRNAs antagonize let-7 induced differentiation. The targets oppositely regulated by let-7 and the ESCC miRNAs include known pluripotency regulators such as the Myc proteins, Sall4, and Lin28. At this point, how ESCC miRNAs upregulate these genes is still not clear. It will be interesting to figure out whether the upregulation is through direct regulation of cell cycle-regulating genes by ESCC miRNAs. Furthermore, it will be important to dissect the function of ES cell cycle structure in preventing differentiation.

19.5 Cell Cycle Regulation by miRNAs in Human Embryonic Stem Cells

Human ES cells were established by Thomson et al. in 1998 and hold great value for regenerative medicine and studying early human development (Thomson et al. 1998). There are significant differences between human and mouse ES cells in terms of morphology of cell colony, surface antigens, and growth factor requirements. Some of these differences may be species-specific. Alternatively, these differences could reflect different development stages of these ES cells. This hypothesis is supported by the derivation of mouse epiblast stem (EpiS) cells that share similar features with human ES cells (Tesar et al. 2007; Brons et al. 2007). Despite these differences, human and mouse ES cells share core similarities including unique cell cycle structure characterized by a short G1 phase, self-reinforcing transcriptional network, and a poised epigenetic state. Similarly, miRNAs have shared roles as regulators of the G1/S transition in human ES cells. A study by Deborah et al. found that miRNAs from the miR-302 cluster promote the G1/S transition in human ES cells (Card et al. 2008). This cluster is regulated by pluripotent transcriptional factors Oct4/Sox2 and is highly and specifically expressed in human ES cells. Moreover, they showed that miR-302 inhibits expression of Cyclin D1 in human ES cells. However, whether the inhibition of Cyclin D1 promotes the G1/S transition is not clear and needs more detailed investigation.

In another study by Qi et al., knocking down Dicer or Drosha leads to the accumulation of human ES cells in G1 phase (Qi et al. 2009), reminiscent of the phenotype observed in Dicer or Dgcr8 knockout mouse ES cells. In addition, this study shows that transfection of miR-372, an ortholog of ESCC miRNAs in human, rescued the G1 accumulation defect. p21 was also identified as a target of miR-372, suggesting that ESCC miRNAs-p21 axis promoting G1/S transition is conserved in both human and mouse ES cells (Fig. 19.3). Interestingly, the authors showed that another two miRNAs with similar seed sequence as miR-372, miR-106a, and miR-302c did not rescue the G1 accumulation defect. In contrast, miR-106a and miR-302c promotes the G1/S transition in the Dgcr8 knockout model (Wang et al. 2008). It should be interesting to investigate the molecular mechanisms that cause this difference in different species and how miRNAs with similar seed sequences may lead to different functional outcomes. This is particularly important for miR-302 as the previous study showed that miR-302 can promote G1/S transition in human ES cells. A simple explanation could be that synthetic RNA oligos in Qi et al.'s study do not guarantee the incorporation of only sense miRNA strand into the RISC. The incorporation of antisense strand may complicate the phenotypic outcome.

Another important finding in Qi et al.'s study is that miRNAs are also important for the G2/M transition of human ES cells. Knocking down Dicer or Drosha leads to significant accumulation of cells in the G2 phase. Further analysis showed that miR-195 can rescue this defect by regulating WEE1 kinase. WEE1 kinase is an inhibitor for the Cdk1–Cyclin B complex, which is important for the G2/M transition. This regulation may be specific for human ES cells because no significant accumulation of Dicer or Dgcr8 knockout mouse ES cells in G2 is observed, and miR-195 does not promote proliferation of Dgcr8 knockout mouse ES cells and is

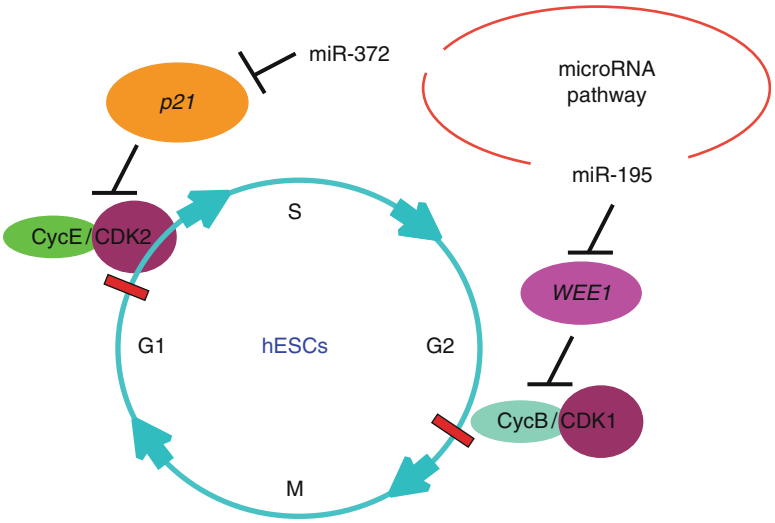

Fig. 19.3 miRNAs regulate the G1/S and G2/M transition in human ES cells. This figure was previously published in Qi et al. (2009)

not highly expressed in mouse ES cells (Yangming Wang and Robert Blelloch, unpublished data). Since mouse EpiS cells share more similarities and may be developmentally closer with human ES cells (Tesar et al. 2007; Brons et al. 2007), it would be interesting to investigate whether miR-195 can also regulate G2/M transition in mouse EpiS cells.

19.6 Cell Cycle Regulation by miRNAs in Somatic Stem Cells

Compared to their role in ES cells, miRNA function in somatic stem cells is less clear. However, analysis of tissue specific knockouts of Dgcr8 or Dicer suggests that miRNAs play essential roles in the proliferation, survival, and differentiation of somatic stem cells. For example, Dicer knockout in the epidermis increases the number of BrdU-positive and phosphohistone-H3-positive mitotic suprabasal cells relative to wild type control (Yi et al. 2008). Further analysis by flow cytometry revealed an approximately threefold increase in the number of G2/M suprabasal cells. This response may be secondary to an increasing need to replace lost cells. Alternatively, it may indicate that miRNAs regulate the cell cycle exit in skin stem cells. Indeed, miR-203 was found to induce cell cycle exit in skin stem cells by inhibiting expression of p63, an essential regulator of stem-cell maintenance in stratified epithelial tissues.

In adult neural stem cells, let-7b and miR-9 were found to inhibit proliferation and induce differentiation (Zhao et al. 2009, 2010). Both miRNAs target Tlx, an important nuclear receptor maintaining self-renewal of neural stem cells (Qu et al. 2010). Let-7b was also found to inhibit expression of Cyclin D1 which is important for the G1/S transition. In a recent study, miR-184 was shown to positively regulate neural stem cell proliferation (Liu et al. 2010). Interestingly, miR-184 is directly repressed by methyl-CpG binding protein 1 (Mbd1) which inhibits gene expression via a DNA methylation-mediated epigenetic mechanism. Further analysis revealed that Mbd1, miR-184, and Numbl, a target of miR-184, formed a regulatory network controlling the balance between proliferation and differentiation of neural stem cells. This study supports the hypothesis that factors promoting proliferation of stem cells may also help prevent stem cell differentiation and vice-versa.

19.7 Cell Cycle Regulation by miRNAs in Cancer

miRNA profiling experiments in normal and tumor tissues revealed that a large number of miRNAs are dysregulated in tumors (Lu et al. 2005), suggesting potential roles of miRNAs in tumor progression including tumor initiation, proliferation, and metastasis. Some cancers share very similar molecular characteristics as ES cells (Ben-Porath et al. 2008); therefore, it is not surprising that molecular mechanisms promoting ES cell proliferation are also used by cancer cells to achieve

growth advantages over normal tissues. The miR-372 and miR-373, two orthologs of ESCC miRNAs in human, can promote tumor formation in human primary fibroblasts in cooperation with oncogenic Ras (Voorhoeve et al. 2006). Interestingly, the two miRNAs are also highly expressed in human germ cell tumors. These miRNAs target Lats2, an inhibitor of Cdk2–Cyclin E complex that is important for the G1/S transition. Tumors also use other miRNAs with similar seed sequences as ESCC miRNAs to promote proliferation. For example, members of the miR-106b family promote the G1/S transition in breast cancer cell lines by targeting p21 (Ivanovska et al. 2008); in gastric cancer, miR-93 and miR-106b were shown to promote cell cycle progression by targeting p21, in addition to their role in preventing apoptosis by targeting E2F1 and Bim (Petrocca et al. 2008; Kim et al. 2009b). In addition, miR-221/2 can promote cell proliferation in various cancers by targeting G1/S transition inhibitors such as p27 and p57 (le Sage et al. 2007; Galardi et al. 2007; Fornari et al. 2008). Together, these data suggest that miRNAs can serve as potent oncogenes to promote cancer cell proliferation by targeting various cell cycle inhibitors.

19.8 Conclusion

The data summarized in this chapter support essential roles of miRNAs in cell cycle regulation in ES, somatic stem, and cancer cells. In addition, cell cycle-regulating miRNAs in stem cells serve as mediators that couple the self-renewal maintenance and cell cycle regulation. As miRNA knockout mouse projects are carrying on in many research laboratories, we expect the discovery of more cell cycle-regulating miRNAs in different stem cells and cancers. The exciting challenge will be how to manipulate these tiny molecules to control cell proliferation for advancing regenerative medicine and cancer therapy.

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Chapter 20
Cell Cycle Regulation During Proliferation and Differentiation of Mammalian Muscle Precursor Cells

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Abstract Proliferation and differentiation of muscle precursor cells are intensively studied not only in the developing mouse embryo but also using models of skeletal muscle regeneration or analyzing in vitro cultured cells. These analyses allowed to show the universality of the cell cycle regulation and also uncovered tissue-specific interplay between major cell cycle regulators and factors crucial for the myogenic differentiation. Examination of the events accompanying proliferation and differentiation leading to the formation of functional skeletal muscle fibers allows understanding the molecular basis not only of myogenesis but also of skeletal muscle regeneration. This chapter presents the basis of the cell cycle regulation in proliferating and differentiating muscle precursor cells during development and after muscle injury. It focuses at major cell cycle regulators, myogenic factors, and extracellular environment impacting on the skeletal muscle.

20.1 Introduction

Precise control of the cellular proliferation, differentiation, and also cell death is vital for the proper embryonic and postembryonic development. Despite a great variety of cell types, they utilize the same molecular machinery governing both the cell cycle progression and withdrawal. However, in each cell type analyzed cell cycle can be specifically tuned and its machinery is influenced by tissue-specific factors. Reciprocally, timing and the expression of these factors are effectively controlled by the cell cycle regulators. These mutual interactions can be studied in rapidly proliferating and differentiating embryonic tissues and organs and also in those of adult organisms. Mechanisms governing proliferation and differentiation of skeletal muscle cells can be analyzed at the level of developing embryo, and

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importantly can also be pinpointed in adult organisms, by studying activation, proliferation, and differentiation of satellite cells. These cells can be forced to reentry cell cycle either after muscle injury or after their isolation and in vitro culture. Analyses of these adult cells allowed deciphering molecular mechanism governing the quiescence, proliferation, and differentiation of muscle precursor cells. Some of them are common for both embryonic and adult cells localized within the muscle. In this chapter, we focus on the processes governing the cell cycles and differentiation of mammalian cells. However, it has to be emphasized that vast amount of data originated from the experiments first performed using model organisms such as amphibian and birds, or even invertebrates, and then confirmed in mammals.

20.2 Molecular Basis of Cell Cycle Regulation

The ascent of the cell cycle studies dates to the time of discovery of the activity controlling meiotic maturation of Rana pipiens oocytes, termed as maturation promoting factor (MPF) or M-phase promoting factor (Masui and Markert 1971; Smith and Ecker 1971; Masui 2001). Soon it was demonstrated that MPF operates not only in amphibian, but also in murine oocytes (Balakier and Czolowska 1977), and controls meiotic as well as mitotic divisions (Rao et al. 1977; Sunkara et al. 1980). Biochemical nature of MPF has been exposed after cloning and characterization of yeast kinases cdc2 and CDC28, i.e., CDK1 (cyclin-dependent kinase 1; Hartwell et al. 1974; Hindley and Phear 1984; Simanis and Nurse 1986; Lee and Nurse 1987; Hartwell 1991) and discovering the first cyclin (Evans et al. 1983). In the years to follow, several other CDKs, cyclins, and their positive and negative regulators were identified, and their role in the staging of the cell cycle of various organisms and cell types was revealed (for a review see e.g., Wong 1996; Ciemerych and Sicinski 2005; Malumbres and Barbacid 2005; Umeda et al. 2005; Gubbels et al. 2008; Doonan and Kitsios 2009). In addition, it has been proved that homologues or orthologues of these crucial factors were present and operational in plant, fungal, and animal cells. As a result, the Sèvres standard[1] of the cell cycle was designed, and the role of the cell cycle regulators has been assigned.

20.2.1 Sèvres Standard of Mammalian Cell Cycle Regulation[1]

The majority of mammalian cell types, except the blastomeres that build the preimplantation embryo, are under the influence of extracellular signals, such as

[1]International Bureau of Weights and Measures, located in Sèvres (France), kept the measurement standards of the International System of Units (SI): the standard kilogram, atomic clocks, and other metrological devices. The phase "Sèvres standard" can be used to emphasize that the described phenomena can be considered as a typical ones.

hormones and growth factors, which are commonly described as mitogens. These factors determine whether the cell will continue to proliferate, differentiate, undergo apoptosis, become senescent, or withdraw from the cell cycle, i.e., become quiescent. Highly specialized cellular mechanisms control and impact on the cellular reactions.

Decisive role in the regulation of both the quiescent state and cell cycle initiation is played by the members of the pocket family proteins, i.e., retinoblastoma protein (pRb), p107, and p130 (Grana et al. 1998; Adams 2001). pRb binds and inactivates the members of E2F transcription factors family preventing the expression of genes encoding cell cycle factors responsible for the progression through and beyond G1 phase (Classon and Dyson 2001; Blais and Dynlacht 2004). The E2F transcription factor family includes the transcriptional activators such as E2F1, E2F2, and E2F3a and repressors, i.e., E2F4, E2F5, and E2F6 (De Falco et al. 2006). E2F7 and E2F8 are also described as inhibitory ones (Moon and Dyson 2008). Interestingly, E2F3 locus encodes not only E2F3a but also E2F3b transcription factor (Leone et al. 2000). Each of the E2Fs is regulated by different pocket protein family members, i.e., E2F1, E2F2, E2F3a, and E2F3b are associated with pRb; E2F4 and E2F5 preferentially bind p107 and p130. E2F6 and E2F7 form transcriptional repressor complexes, but they are not associated with any pocket proteins (Cobrinik 2005). To fulfill their function E2Fs have to interact with their specific cofactors, i.e., DP proteins (DP1 and DP2).

In consistence with its anti-proliferative role, pRb protein was also shown to be a major player in the regulation of cellular differentiation (e.g., Korenjak and Brehm 2005; De Falco et al. 2006). Changes in the extracellular environment that stimulate the cell to resume or continue the cell cycle result in the pRb inactivation (Fig. 20.1). Mitogens acting on the cell can activate intracellular pathways, e.g., mitogen-activated protein kinase (MAPK) pathway, which in turn may lead to the synthesis of D-type cyclins that bind and activate G1-phase-specific CDKs – CDK4 and CDK6 (CDK4/6) (Sherr et al. 1992; Xiong et al. 1992). It has to be remembered, however, that activation of CDKs requires not only their interaction with appropriate cyclin but also removal of inhibitory phosphorylation, which is mediated by Cdc25 phosphatase (Russell and Nurse 1987; Gabrielli et al. 1992), and additional phosphorylation catalyzed by CDK-activating kinase (CAK, Kaldis 1999). CDKs activation also requires the removal/destruction of specific inhibitors. Activity of G1 controlling CDK4/6 is negatively controlled by INK4 family members – $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ (Serrano et al. 1993; Hannon and Beach 1994; Quelle et al. 1995; Guan et al. 1996, Fig. 20.1). Other CDKs, such as those that control S and G2/M-phase, i.e., CDK2 and CDK1, are blocked by the CIP/KIP inhibitors – $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ (Harper et al. 1993; Polyak et al. 1994; Lee et al. 1995, Fig. 20.1). Interestingly, cyclin D–CDK4/6 complexes also interact with members of CIP/KIP family. However, in such configuration CDK4/6 complexes not only remain active but also, by titrating CIP/KIP inhibitors, positively influence activity of CDK2 (La Baer et al. 1997; Cheng et al. 1999; Sherr and Roberts 1999; Geng et al. 2001; Tong and Pollard 2001). Upregulation of CDK inhibitors can be induced by cellular stress, e.g., $p21^{CIP1}$ expression is induced by

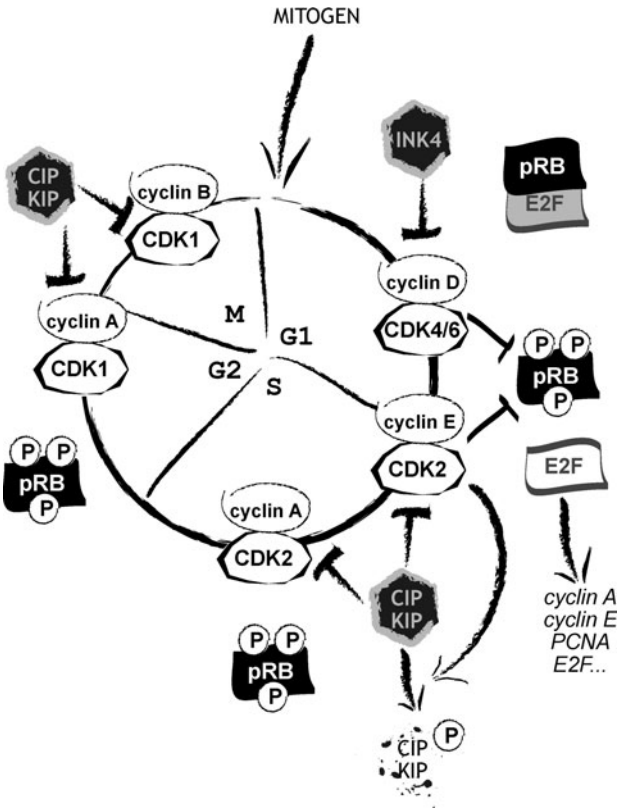

Fig. 20.1 Cell cycle regulation. Cell cycle progression is under control of periodically active cyclin–CDK complexes. During G0–G1 transition, in response to the mitogen stimulation, D-type cyclins become synthesized and activate CDK4/6, which phosphorylate and inactivate pRb. As a result, pRb releases E2F transcription factors and allows the transcription of cell cycle progression genes such as E- and A-type cyclin. In G1–S, E-type cyclins activate CDK2, further phosphorylate pRb, and also phosphorylate its own inhibitors CIP/KIP proteins, directing them for degradation and securing the S-phase progression. Further stages of S phase remain under control of cyclin A–CDK2 complexes, and initiation of M-phase requires the action of cyclin A-CDK1 and then cyclin B-CDK1. Upon degradation of A- and B-type cyclins, mitosis can be completed and the cell can either reenter the next cell cycle, or differentiate or become quiescent. In quiescent cells, CDK activity is negatively regulated not only by CIP/KIP inhibitors but also by the INK4 that inhibits CDK4/6

p53 transcription factor (Sharpless and DePinho 2002). Most importantly, however, expression of these inhibitors is characteristic for cell cycle withdrawal that accompanies cellular differentiation and can be mediated by the tissue-specific transcription factors.

Formation of active cyclin D–CDK4/6 complexes, which takes place during cell cycle reentry, leads to the phosphorylation and inactivation of pRb (Zarkowska and

Mittnacht 1997). Phosphorylated pRb is no longer able to interact and block E2F transcription factors (Fig. 20.1). As a result, E2F-dependent transcription of various cell cycle regulators, such as E-and A-type cyclins, is induced (Lees et al. 1993; Dimova and Dyson 2005). E-type cyclins interact and activate CDK2, which hyperphosphorylates pRb, further preventing its interaction with E2Fs (Lundberg and Weinberg 1998; Harbour et al. 1999; Fig. 20.1). Cyclin E-CDK2 also influences the function of its own regulators, i.e., it phosphorylates and activates Cdc25 phosphatase (Hoffmann et al. 1994), or marks its own inhibitor – $p27^{KIP1}$ for degradation (Sheaff et al. 1997; Furstenthal et al. 2001b). Among the targets phosphorylated by CDK2 are factors involved in the initiation of DNA replication (Krude et al. 1997; Arata et al. 2000; Zou and Stillman 2000; Furstenthal et al. 2001a; Geng et al. 2003, 2007), centrosome duplication (Okuda et al. 2000; Chen et al. 2002; Tarapore et al. 2002), or proteins required for histone biosynthesis, such as $p220^{NPAT}$ (Ma et al. 2000). During the further stages of the S-phase, CDK2 interacts with A-type cyclins (Fig. 20.1; Girard et al. 1991; Pagano et al. 1992; see also Fisher 2011), and by phosphorylating proteins involved in DNA replication, e.g., by Cdc6, these complexes play a crucial role in the regulation of their stability (Petersen et al. 1999; Coverley et al. 2000). At the G2–M transition cyclin A switches from CDK2 to CDK1 and controls the initiation of prophase (Furuno et al. 1999, Fig. 20.1). Notably, cyclin A degradation is a prerequisite step for the completion of the cellular division (Minshull et al. 1990; den Elzen and Pines 2001). Lastly, MPF, i.e., cyclin B-CDK1, directs various M-phase-associated processes such as reorganization of cellular cytoskeleton, cellular organella (e.g., Golgi cysterns), depolymerization of nuclear envelope lamina, formation of mitotic or meiotic spindle, or even chromatin condensation. Cyclin B degradation, leading to CDK1 inactivation, is required for the M-phase exit (see e.g., Gotoh et al. 2011). Importantly, not only timely activation and inactivation of CDKs, but all processes governing the cell cycle stages are precisely controlled by the checkpoint mechanisms ensuring proper completion of each of them and correction of possible mistakes (see de Medina Redondo and Meraldi 2011).

The real outbreak of the cell cycle studies, which also focused at cell cycle withdrawal during cellular differentiation, took place in the last two decades of the twentieth century. It resulted in a multitude of in vivo and also in vitro experiments involving analyses of various cells, including mammalian cell lines and tissues. Results of these projects led to the conclusion that many of major cell cycle regulators play indispensable role in the cell cycle progression. However, by the end of the twentieth century, due to the rapid development of molecular biology techniques allowing generation of genetically modified mice, crucial role of the vast majority of cell cycle regulators has been questioned (for a review, see e.g., Aleem et al. 2004; Sherr and Roberts 2004; Ciemerych and Sicinski 2005). Moreover, not only the redundancy of cell cycle regulators, but also the cell cycle modifications distinctive for specific cell types, such as embryonic cells, stem cells, or differentiating cells, have been revealed (see Momčilović et al. 2011).

20.2.2 Embryonic and Stem Cell Cycle Variants

"Sèvres standard" of the cell cycle described above *does* exist, but one has to be aware of the fact that each cell type can be portrayed by its custom tuned cell cycles. The cell cycles of early mammalian embryos can serve as one of the examples of the specific cell cycle machinery adjustment. During oogenesis maternal "information" (mRNAs and proteins) is synthesized and accumulated within the oocytes. However, majority of these factors become rapidly degraded at the two-cell stage of mouse embryo (Flach et al. 1982; Latham 1999). Thus, almost entire embryogenesis, except the one-cell stage, is governed by the products of embryonic genome. Interestingly, the maternal influence on the progression of the cell cycle of one-cell embryo seems to manifest in the specific regulation of the first mitotic division of such embryo. This particular mitosis, for yet unknown reasons, is as twice long as the second and the following ones (Ciemerych et al. 1999; Sikora-Polaczek et al. 2006; Kubiak et al. 2008), illustrating the notion that the tuning of the cell cycle regulation starts very early during mammalian development. Interestingly, preimplantation mammalian development occurs regardless of the presence of extracellular mitogens. Starting from the one-cell stage (zygote) up to blastocyst stage embryo can be cultured in the serum-free medium. The growth factor independency of cell cycles was shown to be possible due to the lack of the expression of pRb. Maternal stores of this protein become degraded at the early two-cell stage, and then its expression is resurrected at the blastocyst stage, i.e., at the time when embryo is about to implant and start differentiation processes (Iwamori et al. 2002). Moreover, these early cell cycles seem to be independent of p53 and $p21^{CIP1}$, what might be a part of the mechanism ensuring the unperturbed and environment-independent cleavage divisions of preimplantation embryo. Again, p53 and $p21^{CIP1}$ become important regulatory factors at the time of blastocyst implantation (Adiga et al. 2007a, b; Artus and Cohen-Tannoudji 2008; Houlard et al. 2009).

Preimplantation mouse embryo is not the only example of the cell cycle independency from pRb. It was also documented by the analyses of the embryonic stem cells (ES cells), which originate from inner cell mass, i.e., pluripotent cells, of blastocyst. These cells are characterized by very rapid cell cycles that take approximately 10 h (see Wang and Blelloch 2011). Similarly to preimplantation mouse embryo, also ES cell cycles are deficient of pRb activity. However, in contrast to embryos that cells fail to express pRb, this protein was shown to be synthesized by ES cells, but kept in the hyperphosphorylated, i.e., inactive, state (Savatier et al. 1994; Conklin and Sage 2009). Interestingly, lack of all three members of pocket proteins family, i.e., pRb, p107, and p130, does not influence the ES cell cycle progression (Dannenberg et al. 2000; Sage et al. 2000), strongly suggesting that this axis of cell cycle regulation is dispensable. In consequence, E2F-dependent genes, such as cyclins A and E, are stably expressed, which results in the lack of CDK2 activity oscillations (Stead et al. 2002; White and Dalton 2005). Moreover, ES cell cycles were suggested to occur either independently of cyclin D–CDK4/6 complexes (Savatier et al. 1996), or at least to be regulated by only one unique kinase, i.e., cyclin D3-CDK6, which in addition was shown to be resistant to $p16^{INK4a}$ inhibition (Faast et al. 2004). It seems highly

possible that these "special" cellular machinery modifications adapt ES cells to efficient propagation. Importantly, induction of ES cell differentiation "reverses" the cell cycle regulation to the canonical one, i.e., cyclin D expression rises and CDK4 activity becomes resurrected (Savatier et al. 1996), pRb status changes, and the expression of E2F-dependent targets, and their activity, resume oscillatory pattern (White and Dalton 2005; White et al. 2005).

In contrast to rapidly proliferating pluripotent ES cells, majority of the cells that underwent differentiation remain quiescent. This state is sustained by the high levels of hypophosphorylated pRb, INK4, and CIP/KIP inhibitors. However, along with quiescent terminally differentiated cells almost every mammalian tissue was shown to contain the subpopulation of so-called somatic stem cells. These cells are long living, retaining ability to self-renew, and thus, are essential for the renewal of the tissues in adult organisms. In contrast to embryonic cells and ES cells, somatic stem cells divide rarely and are predominantly found in the quiescent state (Cotsarelis et al. 1990; Potten et al. 1997; Li and Clevers 2010). Well-studied examples of adult stem cells are hematopoietic stem cells (HSCs). Different subpopulations of HSCs were reported to divide with various frequencies, e.g., very rarely every 4–5 months (Wilson et al. 2008; Foudi et al. 2009) or more frequently (Cheshier et al. 1999; Kiel et al. 2007). Skin stem cells divide every 200 h, and intestine epithelium, as being one of the "fastest" adult stem cells, every 9–10 h (Fuchs 2009; Li and Clevers 2010). Besides being able to constantly self-renew, adult stem cells are also ready to respond to the environmental cues and to differentiate. To avoid the abnormal development of tissues they reside in, the interplay between stem cell "machinery" controlling the cell cycle progression and differentiation has to be finely tuned. The well-studied examples of such tuning are processes associated with cellular proliferation and differentiation during embryonic and adult myogenesis.

20.3 Cell Cycle and the Developing Skeletal Muscle

Strict control of cell proliferation is particularly crucial for proper development of tissues and organs during embryogenesis. Formation of functionally and morphologically correct structures depends on the balance between sufficient propagation of precursor cells and their differentiation. During muscle development, first muscle precursor cells are singled out, and then satellite cells that can be considered as muscle stem cells are generated from them. Due to their ability to persist, self-renew within the adult muscle, and activate in response to the environmental changes, satellite cells are one of the excellent examples of the precisely tuned balance between quiescence, proliferation, and differentiation.

20.3.1 Outline of Mouse Embryo Myogenesis

In vertebrates nearly all body muscles are derived from epithelial structures, i.e., somites that are formed by segmentation of paraxial mesoderm localized along

axial structures of a developing embryo. During mouse embryogenesis, this process starts at approximately 8th day postcoitum (dpc) and is followed by gradual differentiation of somites into compartments containing precursor cells for several tissues and organs. Dorsal part of the somite develops into dermomyotome that will give rise to skeletal muscles, connective tissue of the skin, and to endothelium and smooth muscles of some blood vessels (Kardon et al. 2002; Ben-Yair and Kalcheim 2005). In many different groups of vertebrates, from amphibians to birds and mammals, specification and then determination of skeletal muscle cells are induced by signals released by structures adjacent to somites, i.e., notochord, neural tube, and embryonic ectoderm (Bryson-Richardson and Currie 2008). In response to Sonic Hedhehog (Shh) synthesized by notochord, or Wingless proteins (Wnts) secreted by neural tube and dorsal ectoderm, dermomyotome cells start to express the first markers of muscle precursor cells, i.e., Pax3 and Pax7 transcription factors. Shh itself is one of the very few factors that have been shown to influence proliferation of myogenic cells in mammals and other vertebrates such as birds. Experiments performed by Duprez et al. revealed that Shh increases proliferation rate of myogenic cells leading to hypertrophy of muscle in chick embryos (Duprez et al. 1998). The possible role of Shh as a regulator of cell divisions was further indicated by the phenotype of Shh-deficient mice. These mutants were characterized by the lack of both epaxial (associated with the vertebrae, ribs, and base of the skull) and hypaxial (abdominal and limb) muscles (Chiang et al. 1996). During unperturbed development Shh was shown to be involved exclusively in the formation of epaxial muscles; thus, ablation of hypaxial ones might result from decreased proliferation of precursor cells, as suggested by Parker et al. (2003). It has also been shown that Shh promotes cell division of muscle cells in adults (Koleva et al. 2005; Elia et al. 2007). Administration of cyclopamine, a specific chemical inhibitor of the Shh pathway, influences proliferation of primary cultures of satellite cells and myoblasts isolated from both chick and mouse. In regenerating muscles, inhibition of Shh pathway leads to reduced number of satellite cells at injury site, impaired activation of MyoD and Myf-5, and decreased level of IGF-I (Straface et al. 2009).

Various Pax proteins control development of many lineages during embryogenesis (Buckingham and Relaix 2007). Two of them, Pax3 and Pax7, are the key regulators of skeletal muscle formation, acting as the master regulators of this process. Despite that their myogenic role was analyzed in many studies (Maroto et al. 1997; Tajbakhsh et al. 1997; Borycki et al. 1999; Seale et al. 2000; Ridgeway and Skerjanc 2001; Relaix et al. 2004; Bajard et al. 2006; Buckingham and Relaix 2007), Pax3 and Pax7 target genes have only started to be identified (McKinnell et al. 2008; White and Ziman 2008; Sato et al. 2010). Among Pax3 and Pax7 direct or indirect targets are transcription factors known as early myogenic regulatory factors, e.g., Myf-5, MyoD (Tajbakhsh et al. 1997; McKinnell et al. 2008; Collins et al. 2009; Sato et al. 2010). In the mouse embryo, Pax3 is initially expressed in newly formed somites and then become restricted to dermomyotome, while Pax7 appears later in the central part of this compartment (Jostes et al. 1990; Goulding et al. 1994; Williams and Ordahl 1994; Buckingham and Relaix 2007). Expression of Pax genes is subsequently followed by the synthesis of factors responsible for

determination of the cell fate and the onset of myogenesis, i.e., MyoD and/or Myf-5 (Sassoon et al. 1989; Ott et al. 1991), which together with myogenin and MRF4 (Myf-6) belong to the MRFs. The common feature of MRFs is the presence of bHLH (basic helix-loop-helix) domain enabling their heterodimerization with E proteins (Olson and Klein 1994). In general, complexes of MRF and E protein regulate myogenic differentiation by recognizing and binding to E-box consensus sequences (CANNTG) present within the promoters of genes encoding muscle-specific proteins, such as myosin heavy chains (MyHCs) or muscle creatinin kinase (MCK) (Olson 1992; Olson and Klein 1994). Experimental overexpression of MRFs was shown to induce myogenic differentiation program and convert various types of cells into myogenic cells, including differentiated ones, such as neurons, chondroblasts, and fibroblasts (Braun et al. 1989; Edmondson and Olson 1989; Weintraub et al. 1989, 1991; Choi et al. 1990; Miner and Wold 1990; Lattanzi et al. 1998). Myotubes derived from MyoD-converted chondroblasts and fibroblasts were identical if compared to myotubes obtained from cells isolated from breast muscles, including localization of muscle sarcomeric proteins such as alpha-actinin, titin, MyHCs, and others (Choi et al. 1990). However, overexpression of MyoD, Myf-5, MRF4, and myogenin in NIH3T3 fibroblasts did not result in the full conversion into multinucleated myotubes (Russo et al. 1998). Thus, exogenous expression of MRFs can induce myogenic conversion of different cell lines, however, with variable success (see Sect. 20.4).

In dermomyotome, expression of Myf-5 has been shown to be induced by Pax3 via engagement of Dmrt2 transcription factor (Sato et al. 2010). Besides Myf-5, MyoD has also been identified as a target for both Pax3 and Pax7, emphasizing the role of Pax genes in orchestrating the progression from undifferentiated toward determined and then differentiating cell (Bajard et al. 2006; Hu et al. 2008; McKinnell et al. 2008). Expression of MyoD or Myf-5 induces the cells localized within dermomyotome to migrate downward and create the cellular layer called myotome (Parker et al. 2003). Once they become localized within the myotome, they stop to divide. Myotome, however, continues to extend gradually due to proliferation of cells localized in the medial part of dermomyotome, which do not activate expression of MRFs, but instead maintain synthesis of Pax3 and Pax7, and as a result of it continue dividing (Kassar-Duchossoy et al. 2005; Relaix et al. 2005). Besides Pax3- and Pax7-positive cells, precursor cells are also present in the hypaxial part of dermomyotome, localized at the level of forming limb buds. These cells retain ability to proliferate due to inhibition of Pax3-mediated expression of MyoD (Bendall et al. 1999). Divisions of these precursor cells enable their sufficient propagation during migration and homing within limb buds, where they undergo differentiation (Birchmeier and Brohmann 2000).

As it was signalized above, Pax3 influences proliferative state of muscle precursor cells. So far this factor is the best recognized regulator of muscle cell proliferation. Downregulation of Pax3 expression in dermomyotome of chick embryos resulted in the cell cycle arrest of muscle precursors (Amthor et al. 1999) and also led to the reduction of precursor cells population in dermomyotomes of mice lacking Pax3 (Franz and Kothary 1993). Importantly, Pax3 activity is crucial for the

survival of precursor cells migrating from dermomyotome toward limb buds (Bober et al. 1994; Goulding et al. 1994; Williams and Ordahl 1994). Linkage between Pax3 activity and cell proliferation was further supported by the fact that chromosomal translocation resulting in fusion of DNA binding domain of Pax3 or Pax7 genes with transcriptional activation domain of FOXO1a (FKHR) leads to uncontrolled division of the muscle cells and development of rhabdomyosarcomas (Barr 2001). Moreover, constitutive expression of Pax3 or Pax7 in in vitro cultured myoblasts results in their extensive proliferation; however, the mechanism of this phenomenon remains unknown (Collins et al. 2009). Identification of Pax3/Pax7 targets by microarray analyses (Khan et al. 1999; Mayanil et al. 2001; White and Ziman 2008) will undoubtedly help to understand how these proteins influence the cell cycle machinery.

Muscle precursor cells that reach the limb buds start to express MyoD approximately at 10.5 dpc, while Myf-5 is expressed between 10th and 12th dpc (Ott et al. 1991). In the meantime, cells present within the myotome undergo final differentiation manifested by their fusion and formation of multinuclear myotubes, and subsequently skeletal muscle fibers. During mouse embryo development, first wave of fiber formation is observed between 11th and 14th dpc and is followed by the formation of secondary fibers taking place between 14th and 16th dpc. Fiber formation remains under control of so-called late MRFs, i.e., myogenin and MRF4, as, for example, myogenin-null mice are characterized by the lack of secondary fibers, which is a consequence of myoblasts' inability to differentiate and fuse to each other (Venuti et al. 1995). The population of Pax3- and Pax7-positive cells, initially localized in the middle part of dermomyotome, which enters the myotome around 10th day of development, will serve as a source of skeletal muscle progenitor cells (Kassar-Duchossoy et al. 2005; Relaix et al. 2005). During the later stages of myogenesis progeny of these cells, characterized by the expression of Pax7, localize between muscle fibers and surrounding basal lamina (Kassar-Duchossoy et al. 2005; Relaix et al. 2005), and become satellite cells, i.e., so-called muscle stem cells, responsible for their growth and regeneration in adult organisms.

20.3.2 Some Lessons on Myogenesis from the Cell Cycle Mouse Mutants

Successful progression of myogenesis depends, to a great extent, on the unperturbed proliferation on muscle precursor cells, securing the generation of the sufficient pool of cells that will next differentiate into muscle fibers. Not surprisingly, interplay between cell cycle regulators, Pax proteins, and/or MRFs has to be perfectly tuned. Important lessons on the role of the cell cycle regulators in myogenesis were learned from experiments involving manipulation of the levels of cell cycle regulators in in vitro growing cells (see Sect. 20.4) or analyses of the mutant mice lacking genes encoding crucial elements of cell cycle machinery.

The role of pocket protein family member pRb in the differentiation of skeletal muscles has been documented by many studies, including analyses of knockout mice. Initial experiments leading to the generation of pRb-null mice showed that deletion of this gene causes embryonic lethality between 12th and 15th dpc, i.e., prior to the stage of terminal muscle differentiation (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Morgenbesser et al. 1994). Two approaches allowed uncovering the role of pRb in muscle development. First one based on the observation that pRb-null embryos die due to severe anemia that might by caused by the placental abnormalities. Experiments involving "tetraploid complementation" technique allowed to generate mutant embryos developing within wild-type, i.e., "healthy," placentas (Wu et al. 2003). Second approach is based on the "construction" of conditional pRb knockout mice (de Bruin et al. 2003). Both experiments lead to the full-term development of pRb-deficient mice, i.e., long enough to allow the manifestation of the defects in skeletal muscle development. The rescued embryos were characterized by the presence of hypoplastic myofibers in several muscle types analyzed (intercostal and limb muscles, diaphragm, and tongue; de Bruin et al. 2003). Moreover, analyses of mice carrying pRb hypomorphic minigene, i.e., expressing low levels of pRb, uncovered similar phenotypes – dispersed myotubes within intercostal muscles (Zacksenhaus et al. 1996). In developing muscles of these mutant mice, myoblasts were undergoing apoptosis or aberrant DNA replication before they were able to initiate terminal differentiation. Myotubes were shorter, less abundant, contained fewer myofibrilles, and fail to express MRF4 (Zacksenhaus et al. 1996). Thus, on the basis of their own results, and experiments conducted by others, the authors concluded that in the absence of pRb terminal withdrawal from the cell cycle is obstructed, resulting in apoptosis or abnormal DNA endoreduplication (Zacksenhaus et al. 1996). However, they also noted that even in the absence of pRb some myogenic precursor cells (MPCs) are able to undergo activation and differentiate into myotubes. Moreover, myoblasts derived from pRb-null ES cells failed to preserve their terminal differentiation, i.e., nuclei present within the myotubes were able to resume DNA synthesis after mitogen stimulation (Clarke et al. 1992; Schneider et al. 1994).

Also p130 was suggested to play a role in the regulation of myogenesis. Histological analyses of 10.5 dpc p130-null embryos showed that they were characterized by the reduced number of myocytes within the differentiating myotome. However, this phenotype was only manifested when mutant mice were generated in Balb/cJ genetic background. C57BL/6J p130-deficient mice were viable with no detectable defects in myogenesis (LeCouter et al. 1998). Thus, it is likely that lack of pRb can be compensated by p107 and p130. However, possibility of such "replacement" could not be tested using mouse model due to the fact that double knockout mice, i.e., lacking pRb and p107, or pRb and p130, die at 11.5 dpc (Lee et al. 1996). However, experiments on triple knockout ES cells underscored the crucial role of pRb, p107, and p130 in the cellular differentiation (Dannenberg et al. 2000; Sage et al. 2000). Unlike wild-type ES cells that are introduced subcutaneously into nude mice differentiate into variety of cell lines including myoblasts, $pRb^{-/-}p107^{-/-}p130^{-/-}$ ES cells were able to differentiate only into primitive neuronal cells and

never gave rise to myoblasts (Dannenberg et al. 2000). Defects in muscle development were also manifested in mice lacking pRb and E2F1 (Tsai et al. 1998), or pRb and E2F3 (Ziebold et al. 2001). Concomitant deletion of E2Fs and their repressor, i.e., pRb, led to the prolonged survival of such double mutant embryos, and again allowed the demonstration of "muscle phenotype" of pRb-null mice.

Other players that were shown to be involved in the regulation of myogenic differentiation are the members of the Id family. They also carry bHLH domain and inhibit E proteins or MyoD–E protein complexes from binding DNA (Benezra et al. 1990a; Christy et al. 1991; Sun et al. 1991; Melnikova and Christy 1996). In particular, Id1 was shown to be a potent negative regulator of MyoD (Benezra et al. 1990b; Jen et al. 1992; Katagiri et al. 2002) and also to prevent the expression of $p21^{CIP1}$ (Prabhu et al. 1997). Besides Id1, Id2 was also shown to be synthesized in myogenic cells and to regulate their proliferation (Zhao and Hoffman 2004). Id2 ablation rescued the pRb phenotype, i.e., prolonged survival of mutant mice and influenced myogenesis, once again revealing crucial role of pRb in muscle development (Lasorella et al. 2000). It should be also mentioned that Id2 plays a role in the placental development (Janatpour et al. 2000); thus, it is possible that observed rescue can be attributed to the genetic interactions between pRb and Id2 within this organ.

The crucial role of pRb revealed during the analyses of various mice mutants has been emphasized by number of other studies, which documented that participation of pRb in myogenesis relies on its ability to bind crucial MRFs, i.e., myogenin and MyoD, as well as other transcription factor, MEF2 (myocyte enhancer factor; Gu et al. 1993; Novitch et al. 1996, 1999; Huh et al. 2004). Interestingly, reciprocal interactions between pRb and MRFs were also documented. MyoD was shown to induce the expression of pRb, and also of $p21^{CIP1}$, which ensures the effective cell cycle exit of the differentiating myoblasts (Martelli et al. 1994; Halevy et al. 1995; Rao and Kohtz 1995; de la Serna et al. 2001; Magenta et al. 2003). However, as mice lacking $p21^{CIP1}$ do not reveal any malfunctions in myogenesis, it is possible that $p21^{CIP1}$ can be replaced by other CDK inhibitors, i.e., $p27^{KIP1}$ or $p57^{KIP2}$ (Zabludoff et al. 1998; Zhang et al. 1999b). It has to be stressed that interaction between MyoD and pRb is crucial for proper expression of genes controlling formation of myotubes and fibers during final stages of differentiation (Gu et al. 1993; Novitch et al. 1996). However, more importantly, pRb displaces histone deacetylases (HDACs) from MyoD, enabling MyoD-dependent transcription and progress of differentiation in muscle cells (Puri et al. 2001; see Sect. 20.3.3). Differentiation promoting function of pRb was also shown to be associated with its ability to negatively regulate Ras activity (Lee et al. 1999; Takahashi et al. 2003). Importantly, the activity of MyoD was shown to be influenced by the Ras-family members, e.g., N-ras (Kong et al. 1995; Ramocki et al. 1997, 1998), and the interplay of N-ras, MyoD, and pRb was emphasized during the analysis of yet another mutant mice. Loss of N-ras resulted in the significant rescue of skeletal muscle development of pRb-null embryos (Takahashi et al. 2003).

The other cell cycle regulators that impact on the myogenic differentiation are D-type cyclins, which in addition to their growth promoting function play unique

roles in differentiation of specific cell types. The best studied so far is cyclin D1, the role of which in the regulation of transcription and differentiation of various tissues, e.g., retinas or mammary glands, is very well documented (Bienvenu et al. 2001, 2010). As far as differentiation of myoblasts is concerned, several lines of evidence engage D-type cyclins in this process. Differentiation of muscle cells is accompanied by the decrease in cyclin D1 level (Skapek et al. 1995; Guo and Walsh 1997). Analysis of mouse embryos documented that developing muscles express almost exclusively cyclin D3, but not D1 or D2 (Ciemerych et al. 2008). Expression of this cyclin is dramatically induced when myoblasts exit the cell cycle and fuse into myotubes (Kiess et al. 1995; Cenciarelli et al. 1999). Interestingly, cyclin D3 fails to accumulate in pRb-null myoblasts, in which it is subjected to rapid degradation (Cenciarelli et al. 1999). In wild-type mice, however, it was shown to associate with hypophosphorylated form of pRb and as a result to escape GSK3β-mediated phosphorylation, which is a prerequisite step for its degradation in proteosome (De Santa et al. 2007). pRb and cyclin D3 interaction was also proven to be crucial for the proper regulation of myogenic differentiation (Mariappan and Parnaik 2005). However, there is no hard data coming from the analyses of cyclin D-null mice proving their indispensability for the proper progression of myogenesis. Nevertheless, multiple lines of evidence coming from in vitro studies prove importance of this protein in the myoblast proliferation and differentiation (see Sect. 20.4).

20.3.3 Epigenetic Regulation of Proliferation and Differentiation of Muscle Precursor Cells

During myogenic differentiation, MyoD activates expression of several muscle-specific genes necessary for the myoblasts fusion and myotubes formation, such as myogenin or M-cadherin, and those responsible for generating force by skeletal muscles, such as MyHCs (Berkes and Tapscott 2005). For that reason, to prevent premature and uncontrolled differentiation of myoblasts, activity of MyoD must be inhibited in proliferating cells. The exact mechanisms leading to inhibition of MyoD activity still remain unclear. It is assumed, however, that inductive molecules/environmental cues released during embryogenesis, or serum-derived growth factors present in in vitro culture, activate some crucial cell cycle regulators (see Sect. 20.5). However, reciprocal interactions between MyoD and cell cycle machinery are much more complicated than presented so far. It is clear that the central role of MyoD in controlling these processes is possible only by its cooperation with many additional modulators. Despite a great gap in our knowledge, some of them have been already identified and characterized.

Activation or repression of any of the genes depends on chromatin conformation within these genes and surrounding sequences. Chromatin relaxation enabling gene expression is achieved inter alia by histone acetyltransferases (HATs) and chromatin remodeling factors such as SWI/SNF complex. While transferring acetyl groups

to lysine residues of histones increases transcription factors' access to DNA, activity of HDACs leads to the suppression of chromatin structure and repression of transcription (Strahl and Allis 2000). Modification of histones by methylation/demethylation also influences chromatin conformation and modulates its interactions with transcriptional machinery. Histone modifying enzymes, together with chromatin remodeling complexes, are engaged in the regulation of expression of genes encoding both cell cycle regulators and muscle-specific factors. Results of in vitro experiments documented that in proliferating C2C12 myoblasts, MyoD remains bound to HDACs and hence is unable to heterodimerize with E proteins and to drive transcription of muscle-specific genes such as MyHCs (Mal et al. 2001, Fig. 20.2). HDACs also inhibit the activity of MEF2 transcription factors that serve as MyoD cofactors and are crucial for the induction of MyoD-dependent transcription (Gossett et al. 1989; Molkentin et al. 1995; Dressel et al. 2001; McKinsey et al. 2001). In addition, in proliferating myoblasts, dimers of MyoD and HDACs are found within promoters of some genes normally expressed during myogenic differentiation, such as genes encoding myogenin or acetylcholine receptor (Liu et al. 2000; Mal and Harter 2003; Fig. 20.2). It is believed that expression of such genes is inhibited by HDACs activity before the onset of differentiation by deacetylation of histones within their regulatory regions and conversion of chromatin to transcriptionally silent form (Sartorelli and Caretti 2005).

Apart from HDACs chromatin repression is also sustained by the activity of histone methyltransferases (Fig. 20.2), such as Ezh2 (Enhancer of zeste homologue 2), of Polycomb family that methylates lysine 27 of histone H3 (H3K27) (Caretti et al. 2004). However, in proliferating myoblasts, unique pattern of deacetylation and methylation of repressed genes does not exist, as for example, promoter of repressed myogenin gene is characterized by methylation of lysine 9, while methylation of lysine 27 is detected within promoters of transcriptionally inactive MCK and MyHCs genes (Zhang et al. 2002; Caretti et al. 2004). As suggested by Forcales and Puri, distinct pattern of modifications within regions encoding genes and/or regulatory sequences repressed in proliferating myogenic cells may serve as an "indicator" for their proper expression during subsequent differentiation. In other words, genes that will be expressed earlier in differentiating myogenic cells (such as myogenin) are "marked" differently than those expressed at later stages of differentiation (such as MyHCs or MCK; Forcales and Puri 2005).

During myogenic differentiation, activation of many genes crucial for proper progression of this process is possible only after MyoD-mediated recruitment of chromatin-relaxing factors such as HATs, and chromatin remodeling complexes, for example SWI/SNF (Puri et al. 1997a, b; Sartorelli et al. 1999; de la Serna et al. 2001; Simone et al. 2004; Fig. 20.2). In differentiating myoblasts and myotubes, MyoD is found in a complex with p300 and PCAF acetylases being stably associated with muscle-specific genes. It is considered that recruitment of HATs by MyoD to its target genes leads to histone acetylation within such regions and subsequent expression of modified genes (Sartorelli and Caretti 2005). Presence of p300 and PCAF acetylases together with SWI/SNF complex is also necessary for the expression of pRb gene driven by MyoD in differentiating muscle cells

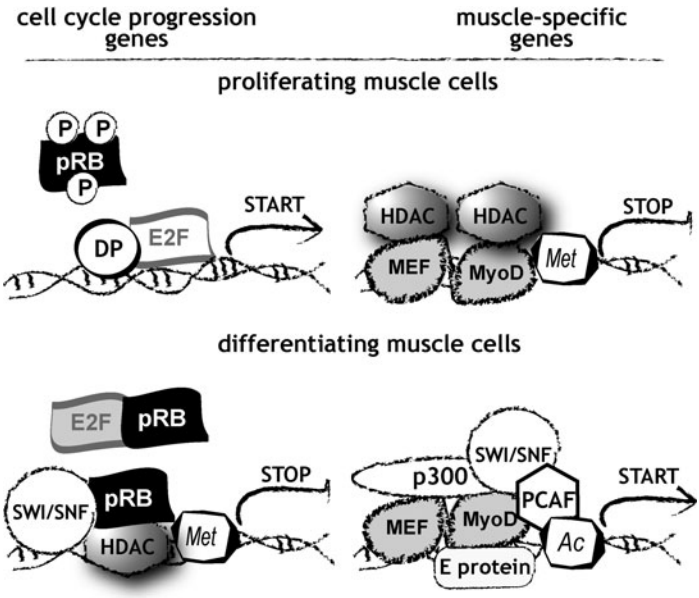

Fig. 20.2 Epigenetic regulation of gene expression in proliferating and differentiating muscle cells. In proliferating muscle cells, activation of muscle-specific genes is inhibited, while cell cycle progression genes are expressed. MyoD and MEF, cofactors necessary for MyoD-dependent transcription, stay bound to HDAC, and as a result remain inactive. Activity of HDAC leads to deacetylation of histones within muscle-specific genes preventing transcription of such modified DNA regions. Expression of muscle-specific genes is also repressed by the activity of histone methyltransferases (Met) converting chromatin into transcriptionally silent form. As a result of CDKs' action, pRb is hyperphosphorylated, and thus, becomes inactive and unable to bind E2F transcription factors. Active E2F/DP complexes promote expression of cell cycle progression genes. In differentiating muscle cells, activated pRb binds to E2F preventing transcription of cell cycle regulators. pRb also induces repression of these genes by recruitment of HDAC and SWI/SNF complex that together convert chromatin into transcriptionally inactive form. In contrast, chromatin within muscle-specific genes is relaxed due to activity of histone acetylases (HATs), such as p300 and PCAF, and chromatin remodeling complexes, such as SWI/SNF, all recruited by MyoD. Binding of MyoD–E protein dimers, together with MEF transcription factors, leads to the expression of muscle-specific genes

(Martelli et al. 1994; Magenta et al. 2003). Modulation of the action of p300, PCAF, or components of SWI/SNF complex by specific antibodies was performed in differentiating myoblasts or MyoD-overexpressing fibroblasts. It resulted in inhibition of MyoD-dependent expression of both pRb and muscle-specific genes, such as myogenin, MCK, and MyHCs (Eckner et al. 1996; Simone et al. 2004). Besides histones, MyoD was also shown to be a substrate of PCAF acetylase. Modification of MyoD by PCAF increases its transcriptional activity (Puri et al. 1997a, b; Sartorelli et al. 1999; Fig. 20.2).

As mentioned above, MyoD induces expression of pRb gene during myogenic differentiation. In return, active pRb releases MyoD from HDAC inhibition and

facilitates its interactions with MEF2, thereby promoting MyoD-dependent transcription (Gu et al. 1993; Novitch et al. 1999; Puri et al. 2001). However, the most important role of pRb is played through its interactions with E2F transcription factors (Macaluso et al. 2006), as described above. pRb was also shown to inhibit the expression of E2F target genes by the relocation of HDACs to such sequences, induction of histone deacetylation, and repressive chromatin conformation (Shin et al. 1995, Fig. 20.2). Repression of cell cycle genes is also achieved by pRb via recruitment of Ezh2 methyltransferase and/or SWI/SNF complexes to their promoters (Dunaief et al. 1994; Strober et al. 1996; Blais et al. 2007, Fig. 20.2). Activity of Ezh2 was shown to prevent expression of genes encoding CDK2 as well as MCM3 and MCM5, i.e., proteins that are crucial for proper DNA synthesis (Blais et al. 2007). Inhibition of transcription is achieved via Ezh2-processed trimethylation of lysine 27 of histone 3 (H3K27Me3) leading to repression of chromatin within modified region (Blais et al. 2007). Depletion of pRb in terminally differentiated myotubes by siRNA results in erasure of H3K27Me3 modifications, followed by reexpression of cell cycle genes such as CDK2, MCM3, MCM5, or BRCA1 and cell cycle reentry (Blais et al. 2007). Therefore, pRb plays a dual role during myogenic differentiation progression acting as both a repressor of cell cycle gene transcription and a coactivator of the muscle-specific gene expression, as it was shown that the lack of pRb results in perturbed expression of MyHCs and MCK genes, and impaired myoblasts fusion (Gu et al. 1993; Novitch et al. 1996).

Presented examples indicate interplay between cell cycle machinery, genes regulating differentiation of muscle cells, and epigenetic factors involved in the regulation of both these processes. Undoubtedly, the central role in triggering both cell cycle arrest and myogenic differentiation is played by MyoD. Surprisingly, mice devoid of this factor do not reveal any serious malfunctions of embryonic myogenesis, probably due to compensation by Myf-5 (Rudnicki et al. 1992; Kablar et al. 1999). However, lack of MyoD is manifested in adults, as satellite cells isolated from such mice are characterized by abnormalities in both the cell cycle withdrawal and myogenic differentiation (Sabourin et al. 1999; Yablonka-Reuveni et al. 1999).

20.4 Quiescence, Cell Cycle Reentry, and Differentiation of Adult Skeletal Muscle Precursor Cells

20.4.1 Satellite Cells and Their Function in Muscle Regeneration

Satellite cells were first identified by Mauro and Katz in 1961 (Katz 1961; Mauro 1961). In adult skeletal muscle, they remain quiescent until appropriate signals, e.g., injury or disease caused degeneration, induce them to reenter the cell cycle (Charge and Rudnicki 2004). Satellite cells, localized between myofiber and basal

lamina (Schultz et al. 1978), consist 3–8% of muscle nuclei (Schmalbruch and Hellhammer 1976, 1977; Gibson and Schultz 1982). After muscle injury they undergo activation, the first manifestation of which is the cell cycle reentry. At this stage, they are usually described as MPCs or myoblasts. Satellite cells number decreases with age, however, usually remains sufficient to support effective regeneration of muscles of healthy individuals (Renault et al. 2002; Shefer et al. 2006; Day et al. 2010). The decline in the cell number and their proliferative capacity is drastically enhanced in certain pathologies, such as muscular dystrophies. One of the intensively studied example of dystrophies is Duchenne muscular dystrophy (DMD) (Decary et al. 2000), which is caused by mutation in the gene coding dystrophin, i.e., protein responsible for linking myofiber cytoskeleton with basal lamina (Ryder-Cook et al. 1988; Sicinski et al. 1989; Emery 2002). Analyses of satellite cell number in the best-studied animal model of DMD, i.e., mdx mice, carrying mutation in dystrophin gene, revealed dramatic decrease in injured and regenerating skeletal muscles (Reimann et al. 2000). Thus, in the case of dystrophies, the population of satellite cells might be prematurely exhausted causing inability to regenerate the skeletal muscle and premature death (Chamberlain et al. 2007).

Our current knowledge on the progression of skeletal muscle regeneration allows clear description of the MPCs behavior during subsequent stages of this process. Briefly, skeletal muscle reaction to the injury covers satellite cell activation, intensive proliferation, differentiation, i.e., formation of first myotubes, and subsequently muscle fibers. Myoblasts' differentiation and fusion into myotubes, i.e., their terminal differentiation, are associated with the cell cycle withdrawal. Throughout the process of muscle regeneration, some of the proliferating myoblasts are "left aside," i.e., do not fuse, but localize within the specific niches, become quiescent, and as a result renew the satellite cells pool. The mechanisms controlling the choice between being cells that regenerate the muscle and that "regenerate" the satellite cell population are not precisely described yet. Also, the factors that control the satellite cell activation and influence the temporal expression of cell cycle regulators responsible either for cell cycle reentry or withdrawal are only partially understood.

Since the identification of satellite cells, many different approaches of analysis of their quiescence, cell cycle reentry, and differentiation have been designed. Among the "materials" that are intensively studied are in vitro cultured single myofibers (Rosenblatt et al. 1995; Rossi et al. 2010), satellite cells/MPCs isolated from skeletal muscles (Foucrier et al. 1999), and various cell lines, such as L8 (Yaffe and Saxel 1977a) and C2C12 (Yaffe and Saxel 1977b; Blau et al. 1985). Another approach covers the in vivo analyses involving skeletal muscle injury leading to satellite cell activation (Zimowska et al. 2001; Moraczewski et al. 2008; Brzoska et al. 2009), or various mouse models (e.g., mdx and SMN) (Banks and Chamberlain 2008; Park et al. 2010). It has to be noted, however, that behavior of in vitro cultured cells only partially "reflects" their differentiation accompanying regeneration of skeletal muscle. Nevertheless, isolated satellite cells and cultured MPCs are considered as a useful tool in the studies involving

biochemical and cytological analysis of the processes accompanying cellular proliferation and differentiation.

First studies focusing at the satellite cell population suggested relative homogeneity of their population. However, currently it is accepted that satellite cell niche is occupied by more than one population of cells. Characterization of the subpopulation of various satellite cells was possible, thanks to many research projects that proposed specific markers. Detection of the cells expressing these proteins allows to precisely subdivide the population of the satellite cells and to mark off the ones that are the most "potent" in supporting the regeneration. One has to remember, however, that the expression of some markers varies as the cells react to the changing environment, and as the "decisions" about the self-renewal vs. differentiation are made.

The cells that become predestined to form a pool of satellite cells during embryonic development express Pax7 transcription factor (Zammit et al. 2006). Pax7 and Myf-5 are among the proteins that are most frequently used as satellite cell markers (Seale et al. 2000). However, some satellite cells were shown to lack Myf-5 expression (Beauchamp et al. 2000). As a rule, satellite cells, i.e., quiescent ones, should not express early myogenic differentiation marker, i.e., MyoD (Fig. 20.5). Thus, the cells that express MyoD are usually excluded from the pool of satellite cells (Cornelison and Wold 1997; Kitzmann et al. 1998). Among the factors characterizing the quiescent satellite cells are also proteins involved in cell adhesion, such as M-cadherin mediating adhesion between these cells and the sarcolemma of adjacent muscle fiber (Irintchev et al. 1994), or integrin α7 subunit (LaBarge and Blau 2002; Gnocchi et al. 2009). The latter one is coexpressed with other markers, i.e., Myf-5 and hepatocyte growth factor (HGF) receptor – c-met (Cornelison and Wold 1997; LaBarge and Blau 2002). The list of satellite cell markers was also extended with syndecan-3 and syndecan-4 (Cornelison et al. 2001). However, it has to be noted that M-cadherin, integrin α7, syndecan-3, and syndecan-4 cannot be considered as perfect markers since their expression continues after the induction of satellite cell activation and differentiation (e.g., Brzoska et al. 2003; Xiao et al. 2003; Wrobel et al. 2007; Tanaka et al. 2009). Importantly, these proteins are widely accepted to be involved not only in the cell adhesion but also in the signal transduction. Last but not least, expression of HSC antigen, i.e., CD34, however, always together with other markers, e.g., Myf-5, is also employed to determine the population of quiescent satellite cells (Beauchamp et al. 2000; Ieronimakis et al. 2010). Presence of almost none of these markers is unique for satellite cells, although combined analyses of such markers as Pax7, integrin α7 subunit, and CD34, supplemented by the examination of cell localization, allow identification of satellite cells. Molecules listed above cannot be considered as "definitive" marker list as almost every year satellite cell population further loses its homogeneity and undergoes extensive "deconstruction".

The most wanted among the satellite cell population is the one that has the highest "regenerative" potential. In search for such cells, Tanaka et al. focused at so-called side population (SP) (Tanaka et al. 2009). These cells, present in many adult tissues and organs, including bone marrow (Goodell et al. 1996) or other adult tissues (Asakura and Rudnicki 2002), are characterized by the ability to exclude

vital dyes, e.g., Hoechst 33342. Analyses of satellite cells isolated from murine hindlimb revealed the presence of rare SP consisting of 0.05–0.5% cells, described by them as "satellite-SP cells." These cells express syndecan-4, Pax7, HSCs marker Sca-1, and also ABCG2 transporter responsible for the exclusion of Hoechst 33342 dye, and are characterized by high potential to participate in muscle regeneration (Tanaka et al. 2009). Other studies supported the idea of the satellite cell heterogeneity by defining two coexisting subpopulations of satellite cells, i.e., so-called low and high proliferating cells. Interestingly, both types of cells demonstrate diverse myogenic potential (Rossi et al. 2010). These findings strongly suggest that cells localized within the satellite stem cell niche differ not only in the proteins that they express, but also in their ability to resume the cell cycle in response to the changing niche, i.e., the environment that can force them to remain quiescent, self-renew, or activate.

20.4.2 Satellite Cells' Niche and Self-Renewal

The niche, an important "factor" influencing the behavior and fate of the satellite cell progeny, secures proper cellular environment protecting the balance between satellite cell renewal and differentiation. Unevenness of these processes may lead either to the premature exhaustion of satellite cells or to their uncontrolled, and thus possibly dangerous, overproliferation. The influence of the niche on satellite cells is based on their physical interaction with muscle fiber and basal lamina, presence of extracellular signals (e.g., Dahlqvist et al. 2003; Le Grand et al. 2009), or mechanical properties of the niche (e.g., Boonen et al. 2009).

Two different mechanisms controlling niche-residing satellite self-renewal have been proposed. One of them suggests that equal divisions of satellite cells generate identical progeny. Resulting daughter cells randomly choose their fate, i.e., some of them differentiate, others retain satellite cell characteristics (Kuang et al. 2008). Other scenario proposes that the presence of the cellular asymmetry results in the generation of two cells of various fates. One daughter cell will be determined to renew satellite cell population, the other will proliferate and differentiate into myotubes (Kuang et al. 2008). Indeed, the existence of the cellular asymmetry was supported by several lines of evidence. One of the tested theories was the "immortal strand hypothesis." It proposes that during stem cell division, segregation of DNA strands is nonrandom, i.e., one daughter cell, presumably the self-renewing stem cell, inherits sister chromatid built of the "older" DNA template strands. The "younger" DNA strand is passed to the cell that is committed to differentiate (Herreros and Giannelli 1967; Cairns 1975). The stem cell retains the old, i.e., "immortal," DNA strand that does not contain possibly dangerous replication errors. Such segregation was shown to accompany the satellite cell divisions. Self-renewing Pax7 and Sca-1 expressing cells inherit "older" DNA strand (Shinin et al. 2006, 2009; Conboy et al. 2007), and the cell that will differentiate, as predicted by the expression of muscle-specific protein – desmin, inherits the newly synthesized,

i.e., younger strand (Conboy et al. 2007). However, it has to be noted that only a subpopulation of satellite cells divides asymmetrically (Shinin et al. 2006; Conboy et al. 2007), which can be another illustration of the fact that population initially described as satellite cells is not homogenous.

Another manifestation of the satellite cells asymmetry is the localization of Numb protein. Numb protein can be inherited either by both "types" of daughter cells, i.e., the one that retains the ability to self renew and the one that will differentiate, i.e., myoblast (Conboy and Rando 2002; Shinin et al. 2006; Jory et al. 2009), or by only one daughter cell. Interestingly, in the case of asymmetric localization, the cell that takes over the Numb also inherits an old DNA strand and is considered a self-renewing one (Shinin et al. 2006; Jory et al. 2009). In the differentiating myoblasts, Numb action is crucial for the repression of Notch, i.e., the factor that was shown to be engaged in the stem-cell renewal via maintaining expression of Pax7 (Conboy and Rando 2002; Dahlqvist et al. 2003; Sun et al. 2008). Interestingly, the Notch ligand, delta-like 1 (DLL1), the expression of which leads to the increase in the expression of Pax7, is also asymmetrically distributed in dividing myoblasts. The cell that will differentiate inherits more DLL1, initiates Notch signaling pathway on the sister cell, and, via this action, promotes its self-renewal (Kuang et al. 2007). Interfering with proper DLL1 processing, i.e., its shedding, results in elevation of Notch signaling and expansion of Pax7-positive cells (Sun et al. 2008). Moreover, mutant mice carrying DLL1 hypomorph, and as a result expressing lower levels of this protein, are characterized by the premature differentiation of myoblasts and loss of muscle precursor cells (Schuster-Gossler et al. 2007). Notch activation was also documented to cause rapid induction of members of Hes family of transcription factors that function as transcriptional repressors, and MyoD was shown to be among the targets of Hes1 (Jarriault et al. 1998; Kuroda et al. 1999). Another Hes protein, i.e., Hes 6 was suggested to block the expression of $p21^{CIP1}$, and thus, support myoblast proliferation (Cossins et al. 2002). However, it has to be noted that Hes6 was also reported to promote the differentiation (Gao et al. 2001; Cossins et al. 2002). The switch from myoblast proliferation to differentiation correlates with transition from Notch to Wnt signaling (Brack et al. 2008). As it was described above, during embryogenesis, Wnt proteins produced by dorsal neural tube and surface ectoderm influence the somite differentiation and thus myogenesis. In adult organisms, Wnts are secreted by skeletal muscle fibers and are also able to influence the behavior of satellite cells. Besides Wnt proteins, other extracellular signals such as growth factors (e.g., IGF-I, HGF, epidermal growth factor [EGF], and TGFβ) or cytokines (e.g., IL-1) that penetrate to the satellite cell niche can also impact on their fate (see Sect. 20.5).

20.4.3 Maintaining Quiescence and Inducing Activation of MPCs

Quiescence is a state common for many cells within organism. However, in case of satellite cells, any in vitro studies of this process are highly difficult to conduct.

At the very moment, the satellite cells are isolated from myofibers; they become activated and reenter the cell cycle. Although, culture of whole myofibers gives a chance to follow quiescent satellite cells for few days and also to have an insight into their niche (Wozniak and Anderson 2005). Another approach allowing the analysis of satellite cells in G0 phase of the cell cycle includes the short and mild trypsinization of in vitro cultured myoblasts that already formed myotubes. Such treatment removes most myotubes, leaving only undifferentiated residual, reserve cells that can be considered as an "equivalent" of satellite cells. After proper stimulation, these cells can reenter the cell cycle (Kitzmann et al. 1998; Carnac et al. 2000). Moreover, the analyses can be conducted using various myogenic cells that were forced to withdraw from the cell cycle, such as cultured in serum-free conditions (e.g., Coller et al. 2006) or in that various signaling pathways were manipulated (e.g., Reed et al. 2007).

Elevated activity of Raf kinase was shown to induce the cell cycle arrest in several cell types studied. Thus, the mouse myoblasts expressing inducible Raf kinase can be used to study the cell cycle regulation during myogenic cells proliferation and differentiation. Importantly, they were shown to express Pax7 (Reed et al. 2007). Using such model system, Reed et al. showed that induction of G0 in Raf expressing mouse myoblasts was accompanied by the translocation of pRb and E2F5 into the nucleus. However, other pocket proteins – p107 and p130 retained their cytoplasmic localization (Reed et al. 2007). Analyses of the status of pocket proteins family members in in vitro cultured C2.7 myoblasts revealed that, similarly as in other cell types, pRb was active in nonproliferating reserve cells and myoblasts, and inactive only in proliferating cell (Carnac et al. 2000). In subpopulation of reserve cells, p130 was expressed and complexed with E2F transcription factors, while p107 was not detectable (Carnac et al. 2000). In agreement, overexpression of p130 in cycling C2 myoblasts led to the suppression of S-phase. It also led to the reduction of MyoD expression. Similar observation was made when p130 and MyoD were coexpressed in 10T1/2 fibroblasts; again the presence of this pocket protein resulted in reduction of MyoD level (Carnac et al. 2000). Thus, p130 inhibits both proliferation and myogenic differentiation program and could be a part of the pathway responsible for maintenance of reserve cell pool during differentiation. Global analyses of gene expression during the regeneration of injured mouse muscles documented that p130 expression changes concurrently with those of E2F1 and E2F2, i.e., factors responsible for the expression of the crucial cell cycle regulators, and is induced during the initial stages of regeneration (Yan et al. 2003). However, E2F1 but not E2F2 gene was shown to be necessary for correct regeneration of mice skeletal muscles. Moreover, concomitantly with the progress of skeletal muscle regeneration, the expression of E2F3, E2F4, and E2F5 increases. Expression of pRb, however, correlates with the cell cycle withdrawal and myoblast differentiation, which once again underlines the importance of pRb in the regulation of muscle differentiation (Yan et al. 2003).

Analysis of E2F1 expression in C2C12 cells showed that myoblasts differentiation is accompanied by its downregulation. On the other hand, overexpression of this transcription factor results in the decrease of myogenin levels and induction of

cyclin D1 expression, thus preventing myogenic differentiation (Wang et al. 1995). Another E2F family member – E2F3a and E2F3b, was previously shown to play opposite roles in the cell cycle regulation. E2F3a was expressed in proliferating cells, predominantly in S-phase, while E2F3b is active throughout the cell cycle and also in the G0 arrested cells (Leone et al. 2000). E2F3a was widely accepted to be a transcriptional activator, while in differentiated or quiescent cells E2F3b acts as transcriptional repressor (Kong et al. 2007). These proteins were also shown to play opposite role in myoblasts (Asp et al. 2009). E2F3a as well as E2F1 was shown to be downregulated during myogenic differentiation of C2C12 cells, but the level of E2F2, E2F4, and also E2F3b did not change (Asp et al. 2009). Asp et al. showed that in proliferating myoblasts E2F3a binds to the promoters activating certain subset of cell cycle genes, while in myotubes E2F3b interacts with the same regions repressing them. Moreover, these factors regulate subsets of specific genes. Among the E2F3a targets are genes involved in the regulation of the cell cycle proliferation-associated genes, while E2F3b is predominantly recruited to genes involved in differentiation (Asp et al. 2009).

Satellite cells maintain quiescent state balancing between cell cycle progression and differentiation. While analyzing MPCs, one has to remember that quiescence in contrast to terminal differentiation, apoptosis, and senescence is reversible (Coller et al. 2006). Conclusions drawn from the initial studies devoted to myoblast proliferation stated that only proliferating myoblast can be induced to differentiate (Nameroff and Holtzer 1969; Dienstman and Holtzer 1977; Yeoh and Holtzer 1977). If this holds true only the cycling, but not quiescent cells, would respond to the factors inducing myogenesis. Indeed, expression of MyoD in cycling human fibroblasts induced their myogenic differentiation (Coller et al. 2006). However, also the quiescent fibroblasts, which were cultured under serum-free conditions or subjected to contact inhibition, differentiated as a result of MyoD overexpression. Importantly, concomitant expression of MyoD and $p21^{CIP1}$ also led to myogenic differentiation, supporting the view that such cells do not need to transiently reenter cell cycle to differentiate (Coller et al. 2006).

20.4.4 Regulating Proliferation of MPCs

Satellite cells can be induced to reenter the cell cycle either by the muscle injury or after their isolation and in vitro culture. In regenerating muscles, the first cycling myoblasts were detectable as soon as 30 h, and intensive proliferation approximately 5 days after muscle injury (McGeachie and Grounds 1987). Increased levels of MyoD and myogenin expression occurs in mononuclear cells, i.e., activated satellite cells, 6 h after injury, and the numbers of MyoD-positive cells were augmented markedly by 24 h. MyoD expression declined to preinjury levels at approximately 8th day of regeneration (Grounds et al. 1992; Yan et al. 2003). However, as it was documented in analyses of injured skeletal muscles (Grounds et al. 1992; Yan et al. 2003), in vitro cultured murine MPCs (Yablonka-Reuveni

et al. 1999), or isolated mouse muscle myofibers (Zammit et al. 2004), MyoD upregulation is correlated with satellite cell activation and induction of differentiation. As long as the myoblasts proliferate, they synthesize high levels of cyclin D1 (Rao et al. 1994; Skapek et al. 1995), the expression of which is controlled by c-jun and c-fos transcription factors (Fig. 20.3). It should be mentioned that one of these factors – c-jun also binds to bHLH domain of MyoD. This interaction prevents the formation of MyoD–protein E complexes and in turn inhibits MyoD transcriptional activity (Bengal et al. 1992; Li et al. 1992a). In proliferating MPCs, increase in the cyclin D1 level promotes translocation of CDK4 to nucleus and interaction with MyoD (Fig. 20.3). MyoD bound through CDK4 to cyclin D1–CDK4 complex is unable to bind DNA and, therefore, cannot induce transcription (Li et al. 1992a; Zhang et al. 1999a). As a result of cyclin D1 degradation that accompanies cell cycle withdrawal, D1–CDK4 complexes are disassembled liberating MyoD (Walsh and Perlman 1997). In consequence, myoblasts cease to proliferate and start to differentiate (Fig. 20.4).

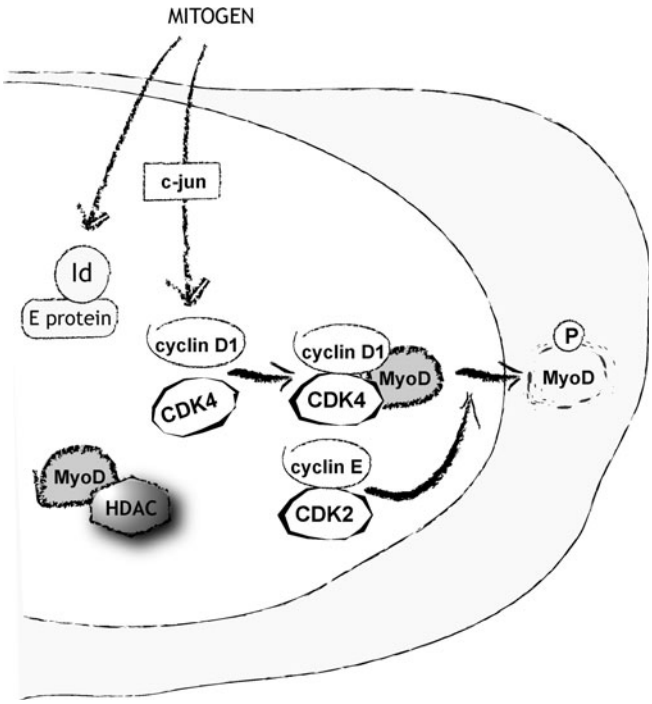

Fig. 20.3 Inhibition of MyoD in proliferating myogenic cells. In proliferating cells c-jun, cellular protooncogene activated in response to mitogens, directly binds MyoD preventing its heterodimerization with E protein and interfering with MyoD-dependent transcription. E protein is itself inhibited by Id factor. Elevated level of cyclin D1 induces CDK4 translocation to nucleus, where it forms complexes with MyoD. MyoD is also directed for degradation after phosphorylation mediated by cyclin E–CDK2 complexes. Finally, deacetylation of MyoD by HDAC prevents its binding to DNA

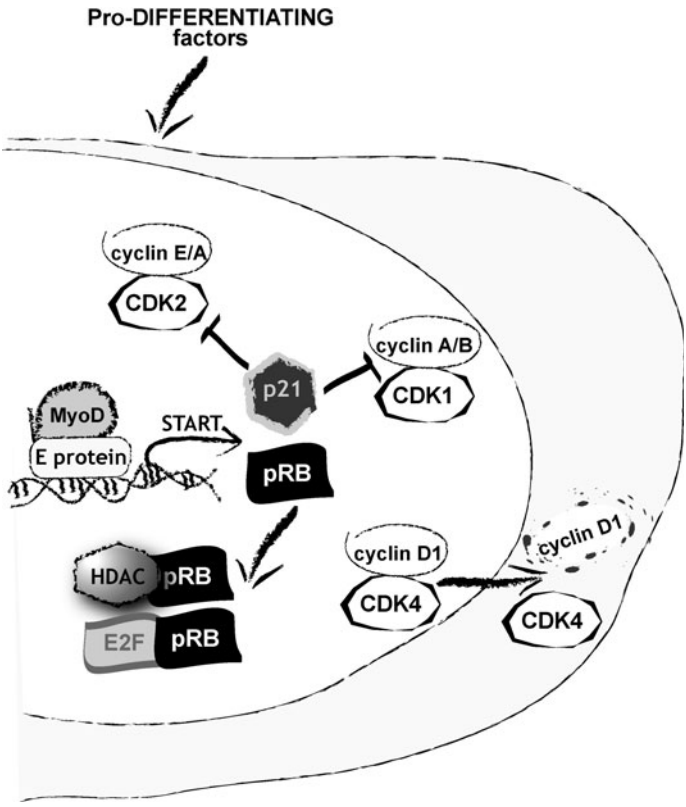

Fig. 20.4 Differentiation-associated changes in cellular machinery of myogenic cells. As a result of cyclin D1 degradation, CDK4 is released into the cytoplasm, and MyoD becomes liberated. MyoD induces expression of $p21^{CIP1}$ and pRb. $p21^{CIP1}$ inhibits CDK2 and CDK1 and thus promotes cell cycle withdrawal. pRb protein binds and inactivates E2F transcription factors preventing the transcription of cell cycle progression genes. pRb also displaces HDACs from MyoD and translocates it to the regions encoding cell cycle genes leading to their silencing. Liberated MyoD together with E protein promotes expression of muscle-specific genes and thus promotes the myogenic differentiation

Cyclin D3 was the other cyclin postulated to be involved in the regulation of myoblast proliferation and differentiation (Bartkova et al. 1998, see Sect. 20.3.2). Significantly, overexpression of cyclin D3 increases expression of negative cell cycle regulators, e.g., $p21^{CIP1}$, and muscle-specific genes such as myogenin or MyHCs (Fig. 20.5). On the other hand lack of cyclin D3 leads to the decrease in $p21^{CIP1}$, MyHCs, and α-actin (De Santa et al. 2007). Ectopic expression of cyclin D3 was shown to correct the defects in myogenic differentiation associated with myotonic dystrophy type 1 (Salisbury et al. 2008). As far as other cyclins are concerned, their roles are proproliferative, since increasing the CDK activity by overexpression of cyclins A, D, or E prevents myogenic differentiation by means of

inhibiting MyoD (Skapek et al. 1995; Guo and Walsh 1997). Moreover, cyclin E–CDK2 complexes phosphorylate MyoD at Ser^{200} directing it to proteolytic degradation, since hyperphosphorylated MyoD becomes ubiquitinated (Song et al. 1998; Kitzmann et al. 1999; Fig. 20.3). Besides MyoD, other MRF family members are also regulated by changes in their phosphorylation status. For example, phosphorylation of myogenin catalyzed by protein kinase C inhibits its interaction with DNA (Li et al. 1992b), and protein kinase A represses activity of both Myf-5 and MyoD (Winter et al. 1993).

Id proteins were shown to be other important factors regulating both proliferation and differentiation acting via the cell cycle regulators and MRFs (see Sect. 20.3.2, Fig. 20.3). Id1 was detected in activated satellite cells and then in rapidly proliferating myoblasts (Ono et al. 2009), in which it prevents expression of $p21^{CIP1}$, and thus allows cell cycle progression (Prabhu et al. 1997). Besides Id1, Id2 and Id4 are also constitutively synthesized in proliferating myoblasts. Dramatic increase in Id2 was also noticed at the initial stages of skeletal muscle regeneration, i.e., during the period of satellite cell activation and myoblasts proliferation (Zhao and Hoffman 2004). Interestingly, Id3 was postulated to be controlled by MyoD transcription factor, suggesting that the members of Id family perform independent function during myoblasts differentiation (Wyzykowski et al. 2002). Several other lines of evidence strongly supported the notion that Id3, similarly to Id1 or Id2, does not play any unique role, but also interferes with MyoD function (Loveys et al. 1996; Melnikova et al. 1999; Wu and Lim 2005). In differentiating myoblasts, i.e., exiting the cell cycle, Id gene promoters were shown to be repressed (Biggs et al. 1995), and resulting loss of these proteins correlated with the formation of MyoD–E protein complexes, induction of myogenin expression, and terminal differentiation (Jen et al. 1992; Ono et al. 2009). Id2 was also shown to be involved in the regulation of apoptosis-associated atrophy of skeletal muscles (Alway et al. 2003). Its ablation in proliferating myoblasts results in the decrease of proliferation and increase of apoptosis (Butler et al. 2009).

20.4.5 Molecular Signature of Satellite Cells and MPCs

Molecular and cytological in situ studies of quiescent satellite cells, activated or differentiated MPCs, and also in vitro experiments were supplemented with the elegant molecular analyses involving microarray and CHIP-on-CHIP techniques (Blais et al. 2005; Fukada et al. 2007; Pallafacchina et al. 2010). Obtained data confirmed previous observations documenting that negative regulators of cell cycle are highly upregulated in quiescent satellite cells. $p57^{KIP2}$ was expressed at a very high level in quiescent cells and downregulated in proliferating MPCs. However, $p21^{CIP1}$, $p27^{KIP1}$, and also p130, which were previously described as factors involved in the maintenance of quiescence, were not significantly upregulated in quiescent cells. Quiescence was also accompanied with the high expression of specific

myogenic inhibitors, e.g., Bmp4, Bmp6, Musculin/MyoR, and also some of the positive myogenic regulators, e.g., Gli2, Pax3, and Pax7 regulator Meox2 (Fukada et al. 2007). Genes encoding cell–cell adhesion molecules, the key elements in the regulation of quiescent celli niche, were also highly expressed. Among them were VE-cadherin, VCAM1, ICAM1, and Pcdhb9 (protocadherin beta 9). Interestingly, Esam, which is characteristic for HSC and mammary gland SPs, and claudin 5, which along with Esam is expressed in blood vessels, were also upregulated in quiescent satellite cells (Fukada et al. 2007; Pallafacchina et al. 2010). Satellite cell activation and cell cycle reentry were accompanied by the upregulation of other gene sets, including gene encoding factors involved in cell cycle progression, DNA, RNA, and protein synthesis, e.g., Cyclin E, CDK1, and many others (Fukada et al. 2007; Pallafacchina et al. 2010). Surprisingly, CHIP-on-CHIP assays revealed relative lack of canonical cell cycle genes that would be controlled by MRFs; however, they documented that E2F4 transcription factor, similar to E2F3, is involved in cell cycle gene expression in MPCs (Blais et al. 2005; Asp et al. 2009).

20.5 Last But Not Least – The Impact of Extracellular Factors on the MPCs Cell Cycles

Extracellular signals such as mitogens, cell–cell, and cell–ECM (extracellular matrix) interactions are crucial factors regulating cell cycle reentry of quiescent cells and their proliferation and differentiation (Charge and Rudnicki 2004). The levels of mitogens stimulating proliferation of MPCs increase drastically when the skeletal muscle becomes injured and inflammatory cells begin to infiltrate damaged tissue. These mitogens, e.g., growth factors, are mainly secreted by neutrophils, macrophages, and to a lesser extent by T-cells and platelets. Vasculature, motor neurons, and MPCs themselves are also responsible for the production and secretion of several growth factors (Cannon and St Pierre 1998). Importantly, some of the factors impacting on satellite cells and MPCs persist within ECM bound by proteoglycans (Taipale and Keski-Oja 1997) and can be easily released as a result of ECM remodeling by metalloproteases occurring after muscle injury (Levi et al. 1996; Kurisaki et al. 2003).

The first described growth factor that was shown to trigger the activation of quiescent MPCs was HGF/SF (hepatocyte growth factor/scatter factor; Nakamura et al. 1986). It binds the c-met receptor expressed by MPCs and activates multiple intracellular signaling pathways, such as MAPK pathway, eventually leading to MPCs divisions (Leshem et al. 2002). The function of other growth factors, such as insulin-like growth factors I and II (IGF-I, IGF-II), platelet-derived growth factor (PDGF), fibroblast growth factors 1, 2, 4, 6, and 9 (FGF-1, -2, -4, -6, and -9), is to promote the proliferation of already activated MPCs and also to stimulate their differentiation (Johnson and Allen 1995; Sheehan and Allen 1999; Czifra et al. 2006). Among other growth factors influencing the cell cycle of myogenic cells are

EGF and PDGF-BB (platelet growth factor BB), which in combination with other factors, such as IGF-I or FGF-2, were shown to stimulate myoblasts proliferation (Doumit et al. 1993). Other factors that impact on MPCs are cytokines, such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and leukemia inhibitory factor (LIF) (Li 2003; Cantini et al. 1995; Spangenburg and Booth 2002). Besides the induction of the cell cycle reentry and progression, extracellular factors also regulate cell cycle withdrawal and myoblast terminal differentiation. Some of them, e.g., IGFs and HGF, play a dual role, i.e., stimulate proliferation and also are able to induce differentiation. As a result, growth factors can either up- or downregulate the expression of cell cycle associated or myogenic genes (Hawke and Garry 2001). Therefore, the sequence of growth factors expression and their crosstalk is of vital importance for the proper progression of skeletal muscle regeneration and myoblasts differentiation (Fig. 20.5).

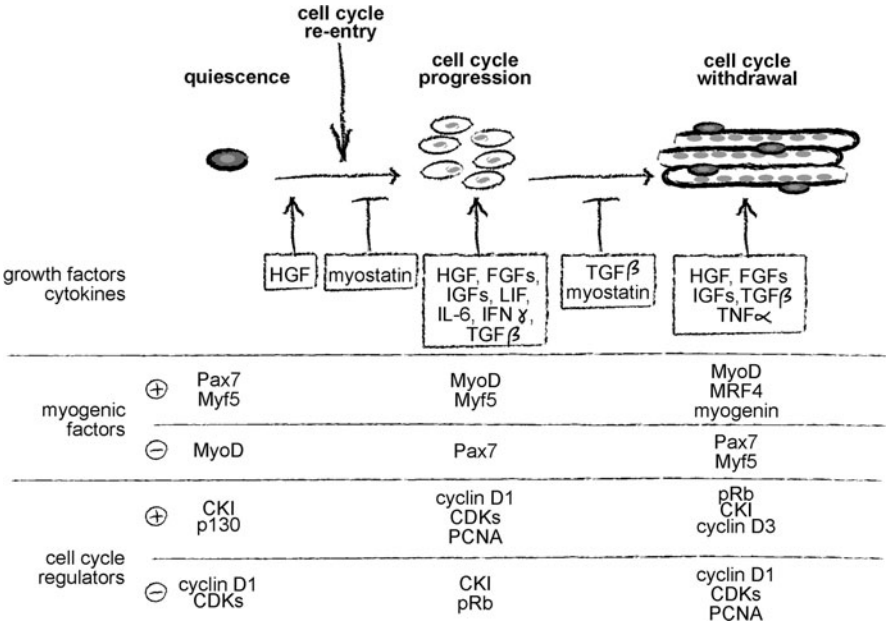

Fig. 20.5 General overview of the extracellular factors acting at the myogenic cells, and changes in the expression of crucial cell cycle regulators and myogenic factors. Cell cycle reentry of satellite cells, proliferation of MPCs, and cell cycle withdrawal associated with either terminal differentiation or quiescence remain under control of various growth factors and cytokines, and result in changes of the expression/activity of myogenic factors and cell cycle regulators. Among the myogenic factors are Pax proteins and MRFs. Cell cycle regulators include such factors as pocket proteins, cyclins, CDKs, CDK inhibitors (CKI), CIP/KIP, and PCNA. The scheme illustrates only gross changes; it does not include the subtle ones. Upregulation of the expression or increase in the activity is marked by plus; downregulation or decrease is marked by minus. *Arrows* indicate cell cycle reentry, stimulating impact of the mitogens at myogenic cells. *Blocking marks* indicate the inhibitory actions of mitogens

20.5.1 Signaling Pathways Activated in Myogenic Cells

Among the pathways activated by growth factors that regulate satellite cells or myoblasts behavior are MAP kinases, i.e., ERK1/ERK2, ERK5 or p38, phosphatidylinositol 3-kinase (PI3K), and protein kinase B (PKB or Akt). Activation of ERK1/ERK2 MAPK results in the cell cycle reentry, while other MAP kinases, PKB, and PI3K influence both proliferation and differentiation of myoblasts (Coolican et al. 1997; Gredinger et al. 1998; Jones et al. 2001, 2005; Perdiguero et al. 2007). Among the receptors that are common for various growth factors or cytokines are receptor tyrosine kinases (RTKs). RTKs interaction with the ligand leads to their dimerization and autophosphorylation enabling binding of effector proteins containing SH2 (Src homology 2) domains. Next steps include interaction with the adaptor proteins, such as Gab-1, which is the adaptor of Met kinase, and Grb2 – the adaptor of Sos-GEF (Guanine nucleotide Exchange Factor), and then downstream factors such as monomeric GTPase Ras. Induction of Ras leads to activation of Raf kinase that phosphorylates and activates MAP kinase kinase – MEK1, which in turn phosphorylates and activates MAPK ERK1/ERK2. Subsequently, active ERK1/ERK2 enters the cell nucleus and mediates the activation of factors involved in chromatin remodeling, such as histone H3 or high mobility group proteins (HMGs), or transcription factors (Sassone-Corsi et al. 1999). Among the first genes that are activated via MAPK pathway are genes encoding crucial cell cycle regulators such as cyclin D1, which allows the cell cycle reentry (Albanese et al. 1995; Lavoie et al. 1996). Interestingly, besides RTKs, MAPK pathway and other pathways, such as PI3K and Akt, can also be activated by ligand binding to G-protein-coupled receptors (Luttrell et al. 1995; Murga et al. 1998). Other growth factors, such as TGFβ, bind to serine/threonine receptors and activate the effector proteins such as Smads, which also translocate to the nucleus and induce expression of their target genes. Activation of c-Jun N-terminal kinase (JNK/SAPK1, Stress Activated Protein Kinase 1), which is also a member of MAPK family, is induced by TNF-α, a cytokine that was shown to impact on cell cycle of murine C2 myoblasts (Alter et al. 2008). Other pathways that were shown to participate in the regulation of proliferation and differentiation of MPCs are, for example, those that involve nuclear factor kappa-light-chain-enhancer (NFκB), also impacting on the expression of cell cycle regulators, such as cyclin D1 (Guttridge et al. 1999).

20.5.2 Growth Factors Impacting on MPCs

Up-to-date multiple extracellular factors were shown to affect the proliferation and differentiation of myogenic cells in vitro and in vivo. The impact of those factors on satellite cells or MPCs depends on their concentration, presence of their receptors, and functionality of signaling pathways that are activated. Presence of the other growth factors within the satellite cells or myoblasts environment is also crucial for

the final cellular reaction. As far as satellite cells and myoblasts are concerned, the role of HGF, IGFs, FGFs, TGFβs, and cytokines, such as LIF and IL-1, was extensively studied.

20.5.2.1 Hepatocyte Growth Factor

HGF/SF was first purified from rette hepatocytes (Nakamura et al. 1986). It is known, however, that its synthesis occurs also in other cells including MPCs. Moreover, it is one of the very few mitogens that was confirmed to activate quiescent satellite cells (Ten Broek et al. 2010). Thus, its action on satellite cells or myoblasts can be achieved not only by endocrine but also by paracrine/autocrine mechanisms (Anastasi et al. 1997). Importantly, HGF can be stored within ECM as a complex with heparan sulfate proteoglycans (Suzuki et al. 2002). During embryonic development, HGF is involved in the migration of muscle precursor cells, but not in their proliferation, differentiation, or survival (Dietrich et al. 1999). As far as satellite cells are concerned, HGF was shown to be able to activate them via its binding to c-met receptor, and trigger cell cycle reentry, both in vitro and in vivo. Depending on the pathway activated, e.g., MAPK and PI3K, c-met binding may sustain myoblast proliferation, or result in their differentiation (Allen et al. 1995; Anastasi et al. 1997; Tatsumi et al. 1998; Leshem et al. 2000; Miller et al. 2000; Halevy and Cantley 2004). Upregulation of HGF expression accompanies the phase of intensive MPCs proliferation and, thus, improves the regeneration of injured muscle (Tatsumi et al. 1998; Miller et al. 2000). As it was shown in murine C2C12 cells, HGF induces cyclin D1 expression and CDK4 translocation into the nucleus, pRb hyperphosphorylation, and release of E2F transcription factors (Ponzetto et al. 2000; Leshem and Halevy 2002). Simultaneously, expression of $p27^{KIP1}$ decreases, and via this mechanisms G1/S transition is secured. Yamada et al. revealed that the function of HGF in MPCs might be more complex and dependable on the concentration of this growth factor. High levels of HGF, accompanied by the expression of myostatin and $p21^{CIP1}$, was shown to inhibit proliferation of rette MPCs and induce their quiescence. On the other hand, low concentrations of HGF allowed cell proliferation (Yamada et al. 2010). Moreover, ectopic expression of HGF in chicken MPCs increases the expression of myogenic inhibitory bHLH factor Twist and decreases the expression of cell cycle inhibitor $p27^{KIP1}$ (Leshem et al. 2000, 2002). Activation of PI3K pathway by HGF is accompanied by the upregulation of myogenin and inhibition of the ERK1/ERK2 MAPK pathway. As a consequence, MPCs exit the cell cycle and differentiate (Leshem et al. 2002). Moreover, PI3K phosphorylates and activates Akt, one of the major regulators of apoptosis, indicating that, apart from sustaining quiescence of MPCs, HGF may be involved in myogenic cell survival (Halevy and Cantley 2004). Interestingly, activity of PI3K was also shown to be involved in ERK1/ERK2 MAPK activation, opposing the thesis that PI3K signaling participated only in the control of myogenic cell differentiation (Halevy and Cantley 2004).

20.5.2.2 IGF-I and IGF-II

Extensive studies devoted to the role of insulin-like growth factors in myogenesis clarified that, similarly to HGF, IGF-I and IGF-II are also able to promote both proliferation and differentiation of MPCs. IGFs are able to induce both effects in a biphasic manner. First, they stimulate proliferation and expression of genes, such as cyclin D1, next they promote differentiation and activation of myogenic genes (Coolican et al. 1997). Moreover, these factors induce both processes by binding the same receptors, but the final result depends on the signaling pathway utilized (Coolican et al. 1997). IGF-I stimulates the proliferation of MPCs via binding to RTKs, and stimulating various signaling pathways, such as ERK1/ERK2 MAPK (Coolican et al. 1997), calcineurin/NFAT (Musaro et al. 1999), p70 S6K1 (Haddad and Adams 2004), and PI3K (Chakravarthy et al. 2000; Machida and Booth 2004). Muscle injury induces increase in IGF-I levels which stimulates MPCs' proliferation resulting in muscle fiber hypertrophy (Hayashi et al. 2004). Moreover, infusion of IGF-I into rette tibialis anterior muscle leads to the increased expression of cyclin D1, higher proportion of cells in S-phase, and enhanced MPCs proliferation (Haddad and Adams 2004). However, this effect apparently did not depend on ERK1/ERK2 MAPK pathway, since simultaneous infusion of skeletal muscle with IGF-I and MEK1 inhibitor PD-098059 did not result in the significant decrease in the proliferation of analyzed MPCs (Haddad and Adams 2004). Thus, it was suggested that MAPK pathway is not essential for the IGF-I induced proliferation of MPCs. On the other hand, ERK1/ERK2 MAPK pathway was shown to transduce IGF-I-mediated mitogenic signal in L6A1 myoblasts, whereas the PI3K/p70 S6K was involved in differentiation of those cells (Coolican et al. 1997). Other studies documented that in MPCs IGF-I might "utilize" other than ERK1/ERK2 MAPK signaling pathway. Czifra et al. postulated that IGF-I action on human MPCs is exclusively mediated by PKCδ, but in C2C12 myoblasts also by ERK1/ERK2 MAPK (Czifra et al. 2006). However, other lines of evidence argued against IGF-I involvement in the regulation of human MPCs proliferation, suggesting that it causes muscle hypertrophy by increasing the cell recruitment rather then by stimulating cellular divisions (Jacquemin et al. 2004).

Besides ERK1/ERK2 MAPK, PI3K/Akt pathway is also considered as the one crucial for the regulation of MPCs proliferation mediated by IGFs. MPCs isolated from transgenic mice overexpressing IGF-I were characterized by the presence of activated PI3K and Akt, and increased proliferation potential (Chakravarthy et al. 2000). Activation of PI3K/Akt is associated with the increase in CDK2 activity, hyperphosphorylation of pRb and decrease in the level of $p27^{KIP1}$, securing the proper S-phase progression. Interestingly, ectopic expression of $p27^{KIP1}$ in murine MPCs inhibited proliferation of those cells, even if they were stimulated with IGF-I (Chakravarthy et al. 2000). IGF-I-mediated activation of PI3K/Akt pathway regulates the activity of forkhead transcription factor FOXO1 which controls cell proliferation and survival. Activation of Akt leads the phosphorylation of FOXO1 on Ser^{256} which inhibits its translocation to the nucleus. This in turn leads to decrease of the $p27^{KIP1}$ promoter activity which is regulated by FOXO1, and

downregulation of $p27^{KIP1}$ expression (Machida et al. 2003). Thus, in MPCs FOXO1 has been postulated to "mediate" between phosphorylation of Akt and regulation of activity of $p27^{KIP1}$ promoter (Machida and Booth 2004).

Another mode of IGF-I action includes the increase of MPCs survival. Results obtained by Barton et al. revealed that IGF-I ameliorates the dystrophic muscle phenotype of mdx mice, i.e., reduces necrosis and prevents apoptosis of MPCs (Barton et al. 2002). Generation of the transgenic mdx mice, characterized by IGF-I synthesis directed by myosin light chain promoter (mdx:$mIGF^{+/+}$ mice), allowed to achieve muscle-specific overexpression of this growth factor. Such mice were characterized by muscle hypertrophy and improved regeneration of their dystrophic muscles resulting from increased MPCs survival (Barton et al. 2002). The phenotype of mdx:$mIGF^{+/+}$ mice was explained by the higher expression and persistent activation of Akt kinase which is crucial for survival of MPCs. In addition, muscles of mdx:$mIGF^{+/+}$ mice were characterized with reduced fibrosis and increased strength in comparison to mdx dystrophic muscles (Barton et al. 2002).

Interestingly, IGFs regulate not only the proliferation, but can also impact on the MPCs differentiation. Their mode of action greatly depends on the duration of the stimulation. Brief exposition (1–8 h) of L6E9 myoblasts to IGF-I stimulates their proliferation by inducing cyclin D1 expression. However, 30 and 48 h long treatment leads to myogenin and MRF4 expression, and promotes the differentiation of those cells (Engert et al. 1996). Both IGFs were shown to promote terminal differentiation of C2 and C2C12 myoblasts via activation of PI3K that leads, to the induction of MyHC expression (Shanely et al. 2009). As it was described terminal differentiation of murine MPCs is correlated with the expression of the cell cycle inhibitors $p21^{CIP1}$ or $p57^{KIP2}$ and cell cycle exit (Parker et al. 1995; Zhang et al. 1999b). Similar mechanisms operate also in human myotubes that upon IGF-I treatment upregulate $p57^{KIP2}$, and increase the synthesis of myogenin and MyoD (Jacquemin et al. 2007). Interestingly, IGF-I is not able to induce differentiation when p38 MAPK is blocked with a specific inhibitor, implicating that this kinase mediates IGF-I "differentiating" action (Wu et al. 2000). Besides IGF-I, IGF-II is also able to induce the cell cycle withdrawal and myoblasts differentiation acting via ERK5 signaling pathway. Activated ERK5, similarly to ERK1/ERK2 MAPK translocates into the cell nucleus and increases MyHCs and MEF2 expression (Carter et al. 2009). Introduction of antisense IGF-II constructs into C2 myoblasts reduces both ERK5 activity and MyHC expression (Carter et al. 2009). Thus, the role of IGF-I and IGF-II in myoblast proliferation depends on the origin of analyzed myogenic cells, and their concentration present in the cellular environment.

20.5.2.3 Fibroblast Growth Factors

Among known FGFs, FGF-1 (also termed as acidic FGF, or aFGF), FGF-2 (also termed as basic FGF, or bFGF), FGF-4, -6, and -9, were shown to have a significant impact on the myogenic cell proliferation (Doumit et al. 1993; Johnson and Allen 1995; Sheehan and Allen 1999). The notion that FGFs play a crucial

function as regulators of myogenesis is also supported by the observation that their receptors, such as FGFR1, are involved in the regulation of growth and differentiation, not only of embryonic, but also of adult myoblasts (Itoh et al. 1996; Flanagan-Steet et al. 2000). Thus, it is not surprising that FGFs up-regulation is also observed during skeletal muscle regeneration.

In regenerating rette plantaris muscle expression of FGF-1, FGF-5, and -7 is associated with the expression of "early" MRF - MyoD and of PCNA protein that is involved in DNA replication and serves as a marker of actively dividing cells (Tanaka et al. 2008). Inhibition of FGF2 by specific antibodies impairs not only the number, but also the diameter of newly formed myofibers (Lefaucheur and Sebille 1995). Moreover, injection of FGF2 into tibialis anterior of mdx mice improves proliferation capacity of MPCs, and leads to the increase in number of newly regenerated myofibers (Lefaucheur and Sebille 1995). The impact of FGFs on muscle regeneration was confirmed by the analyses of the phenotype of mice lacking FGF-6. Their skeletal muscles were characterized by impaired ability to regenerate, reduced number of myoblasts expressing MyoD and myogenin, and decreased proliferative capacity of MPCs (Floss et al. 1997). However, these results were questioned in other study documenting that during skeletal muscle regeneration of FGF6-deficient mice formation of new fibers is accelerated, due to stimulation of differentiation, accompanied by increase in $p16^{INK4a}$ and $p21^{CIP1}$ (Armand et al. 2005). Importantly, Armand et al. revealed that during muscle regeneration the influence of FGF-6 is dose-dependent. Injection of high amounts of human recombinant FGF-6 into muscles of FGF-6-deficient mice results in accumulation of cyclin D1 transcripts, and stimulation of proliferation. Lower doses of FGF-6 stimulate their differentiation (Armand et al. 2005). FGF-2, -4, -6 and -9 were shown to act synergistically with HGF increasing its mitogenic effect on MPCs (Sheehan and Allen 1999). However, the detailed mechanisms of FGFs impact on the cell cycle machinery are still not well characterized. Moreover, the interpretation of experiments in that the levels of certain FGF were manipulated is difficult due to the possible functional redundancy between other existing isoforms.

The effect of FGFs on myogenic cell proliferation depends on signaling pathway activated. This was confirmed by the overexpression of FGFR1 in L6 myoblasts, which resulted in significantly higher activation of Raf-1 kinase and ERK1/ERK2 MAPK, than in the case of the overexpression of FGFR4 (Vainikka et al. 1994). Moreover, overexpression of a truncated form of FGFR1 resulted in decreased proliferation and enhanced differentiation of murine Sol 8 myoblasts (Scata et al. 1999). It was also shown that in embryonic and adult turkey MPCs FGF-2 activates ERK1/ERK2 MAPK and in murine MPCs also p38 MAPK (McFarland and Pesall 2008; Shi et al. 2010). Involvement of ERK1/ERK2 MAPK pathway in repression of MPCs differentiation was questioned by the results of experiments in that inhibition of this pathway did not enhance the myogenic differentiation of MM14 myoblasts (Jones et al. 2001). In conclusion, the influence of FGFs on MPCs' cell cycle may be regulated by acting through different receptors and activation of distinct signaling pathways. As it was shown for other growth factors their action depends on their concentration, receptor specificity, and activated pathways.

20.5.2.4 TGFβ Family

TGFβ is a family of growth factors that include TGFβs, myostatin and bone morphogenic proteins (BMPs). Depending on the cell type and conditions these factors can either promote or suppress cellular proliferation. In general, members of this family acting on their target cells bind to TGFβR-I and -II serine/threonine kinase receptors, trigger their phosphorylation, and activate the cascade of kinases leading to the phosphorylation of Smad transcription factors (Whitman 1998). Among the signaling pathways which were shown to be involved in TGFβs action are PI3K, ERK1/ERK2 MAPK and JNK pathways.

In 1980s, TGFβ was determined as a factor that negatively regulates myoblasts differentiation (Massague et al. 1986). In vitro experiments underscored the proproliferative function of TGFβ1, TGF2, and TGF3, which stimulated C2C12 murine myoblasts proliferation via inhibition of the expression of the $p21^{CIP1}$ and induction of PCNA translocation to the cell nucleus (Schabort et al. 2009). Interestingly, TGFβ1, TGFβ2, and TGFβ3 isoforms delayed myogenic commitment by increasing MyoD degradation and by decreasing myogenin and MyHCs expression (Martin et al. 1992; Schabort et al. 2009). Increase in the expression of TGFβ accompanies skeletal muscle regeneration (Sakuma et al. 2000; Zimowska et al. 2009). However, it also results in the development of fibrosis and inhibition of TGFβ with specific antibodies leads to the improved muscle repair (Zimowska et al. 2009).

A member of TGFβ family, myostatin (Mstn), was documented to be specifically expressed in the developing myotome and in the different types of adult muscles. Contrary to TGFβ1, TGFβ2, and TGFβ3, Mstn arrests muscle growth by decreasing myoblast proliferation and inhibiting G1/S progression. This is achieved via activation of GSK-3β that phosphorylates and induces degradation of cyclin D1 and leads to the drop in CDK4 activity (Yang et al. 2007). In addition, $p21^{CIP1}$ expression becomes upregulated leading to CDK2 inhibition and as a result pRb becomes dephosphorylated, i.e., able to bind and inactivate E2F (Thomas et al. 2000; Rios et al. 2001; McCroskery et al. 2003). Overexpression of Mstn in C2C12 cells was shown to prevent myoblast differentiation via reversible repression of MyoD and myogenin expression (Rios et al. 2001). Analysis of mutant mice lacking Mstn documented that the absence of Mstn leads to enhanced proliferation and delayed differentiation, resulting in the increased muscle mass and higher number of larger myofibers (McPherron et al. 1997; McCroskery et al. 2003). Such phenotype was confirmed in the analyses of other mouse and bovine models, such as Belgian Blue or Piedmontese (Grobet et al. 1997; Kambadur et al. 1997; McPherron and Lee 1997; Szabo et al. 1998).

The other members of TGFβ family, i.e., BMPs also impact on the myogenesis (Wang et al. 2010). During somite development BMP2, together with the FGF4 and Shh, inhibits the expansion of cells within the limb buds and induces chondro- and osteogenesis (Wall and Hogan 1995). Modulating the BMP-dependent signaling pathways was shown to have a significant impact on the proliferation of mesodermal precursor cells. In mice null mutation of BMP receptor fully inhibits the mesoderm development and causes early embryonic lethality by 9.5 dpc

(Mishina et al. 1995). Activation of BMP signaling pathway induces Smad phosphorylation and MPCs proliferation (Wang et al. 2010). The presence of the phosphorylated form of Smad was also observed in dividing satellite cells localized at the isolated muscle fibers, indicating that BMP signaling is active in those cells. The inhibition of BMP signaling by the Noggin overexpression represses Smad phosphorylation and leads to the decrease in the number of muscle precursor cells (Wang et al. 2010).

20.5.2.5 Cytokines Impacting on MPCs

Cytokines, such as TNF-α or IFN-γ (interferon γ), are produced by inflammatory cells, such as macrophages, natural killer cells, and T-cells migrating to the site of muscle injury, and also by endothelial cells and MPCs. TNF-α binds to TNF-α receptors p55 and p75, and IFN-γ binds and induces phosphorylation of the IFN-γ receptor, which results in the activation of such signaling pathways as p38 MAPK pathway. In injured skeletal muscle, their action concerns MPCs recruitment and their stimulation to reentry or to withdraw the cell cycle and also to differentiate (Lafreniere et al. 2006). To make the story even more complicated, the expression of cytokines and chemokines and their receptors is regulated by other cytokines and growth factors, such as IGF-I (e.g., Reem and Yeh 1984; Sironi et al. 1989). The significance of these complicated relations for proper myogenic differentiation during development or muscle regeneration is not fully understood yet. However, it is well known that various cytokines, such as TNF-α, IFN-γ, LIF, and interleukins, such as IL-1β, IL-4, or IL-13, participate in the recruitment of MPCs and their differentiation (Horsley et al. 2003; Broussard et al. 2004; Charge and Rudnicki 2004; Li et al. 2005; Jacquemin et al. 2007; Cheng et al. 2008). Moreover, LIF, IL-6, and IFN-γ were shown to have an impact on cell divisions of MPCs and also various cell lines such as C2C12 myoblasts (Charge and Rudnicki 2004; Cheng et al. 2008).

The two best studied cytokines in terms of their involvement in muscle regeneration and also in vitro differentiation of myoblasts are IFN-γ and TNF-α. IFN-γ improves murine muscle regeneration, and blocking of its action reduces proliferation of MPCs and decreases the number of MyoD-positive cells, which in turn leads to the drop in the number of reconstructed myofibers (Cheng et al. 2008). Interfering with IFN-γ receptor with specific antibody represses proliferation and fusion of in vitro cultured C2C12 myoblasts (Cheng et al. 2008). On the other hand, the other proinflammatory cytokine TNF-α was proved to regulate differentiation of C2C12 myoblasts and murine MPCs, acting via p38 MAPK pathway (Chen et al. 2005, 2007). Importantly TNF-α, both in vitro and in vivo, stimulates cell cycle withdrawal, by inducing the expression of $p21^{CIP1}$, and myogenic differentiation via expression of MEF2C and myogenin (Chen et al. 2005, 2007). Moreover, regeneration of Soleus muscle in mice lacking TNF-α receptors, i.e., p55 and p75, was accompanied by increased expression of cyclin D1 (Chen et al. 2005), proving that TNF-α is involved in cell proliferation.

Among other cytokines that regulate cycling of MPCs are LIF and IL-6 (Barnard et al. 1994; De Rossi et al. 2000). Both factors utilize the same receptor subunit gp130 and thus probably are able to activate similar signaling pathways, such as JAK/STAT pathway (Lord et al. 1991; Hibi et al. 1996; Heinrich et al. 1998). Results obtained by Cantini and Cararro showed that IL-6 stimulates proliferation of in vitro cultured human MPCs (Cantini and Carraro 1995). However, Kami and coworkers suggested that this interleukin promotes rather degeneration of damaged fibers than proliferation of myogenic cells (Kami and Senba 1998). Moreover, it was shown that muscle regeneration of LIF-deficient mice is repressed, and infusion of LIF can rescue this phenotype (Kurek et al. 1996). LIF administration into muscles of mdx mice ameliorates their regeneration, probably by stimulating myoblast proliferation (Kurek et al. 1996; Austin et al. 2000). LIF was also shown to activate JAK2/STAT3 pathway and increase proliferation of in vitro cultured C2C12 myoblasts (Spangenburg and Booth 2002). Although it was showed that IL-1 activates the p38 MAPK, and NFκB (Li et al. 2009), the detailed mode of action of interleukins, i.e., signaling pathways that are activated in myoblasts, is not well described.

Cell cycle of MPCs is orchestrated by various extracellular factors that not only stimulate their entry but also withdrawal from the cell cycle both in vivo and in vitro. Although action of growth factors and cytokines depends on experimental models that are used, it is clear that appropriate spatiotemporal pattern of their expression secures effective MPCs differentiation and also muscle regeneration.

20.6 Concluding Remarks

Development and regeneration of skeletal muscle may serve as excellent examples of processes during which cell cycle progression and withdrawal must be perfectly controlled. Spatiotemporally regulated transitions of cells from proliferating state to differentiating and quiescent ones and conversely are crucial for proper function of both growing and regenerating muscles. The number of identified factors controlling both proliferation and differentiation of myogenic cells still increases; however, the exact mechanism of their action remains, in many cases, still uncovered. The reciprocal interactions of these factors, for example, cell cycle regulators, MRFs, other muscle-specific genes, and epigenetic machinery, make this picture even more complicated. However, sooner or later due to development of precise molecular methods such as microarrays or CHIP technology combined with "traditional" ones, and detailed analyses of knockout animals, these puzzles will be put together.

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Chapter 21
Drosophila Neural Stem Cells: Cell Cycle Control of Self-Renewal, Differentiation, and Termination in Brain Development

Heinrich Reichert

Abstract The wealth of neurons that make up the brain are generated through the proliferative activity of neural stem cells during development. This neurogenesis activity involves complex cell cycle control of proliferative self-renewal, differentiation, and termination processes in these cells. Considerable progress has been made in understanding these processes in the neural stem cell-like neuroblasts which generate the brain in the genetic model system *Drosophila*. Neuroblasts in the developing fly brain generate neurons through repeated series of asymmetrical cell divisions, which balance self-renewal of the neuroblast with generation of differentiated progeny through the segregation of cell fate determinants such as Numb, Prospero, and Brat to the neural progeny. A number of classical cell cycle regulators such as cdc2/CDK1, Polo, Aurora A, and cyclin E are implicated in the control of asymmetric divisions in neuroblasts linking the cell cycle to the asymmetrical division machinery. The cellular and molecular identity of the postmitotic neurons produced by proliferating neuroblasts is influenced by the timing of their exit from the cell cycle through the action of a temporal expression series of transcription factors, which include Hunchback, Kruppel, Pdm, and Castor. This temporal series is also implicated in the control of termination of neuroblast proliferation which is effected by two different cell cycle exit strategies, terminal differentiative division or programmed cell death of the neuroblast. Defects in the asymmetric division machinery which interfere with the termination of proliferation can result in uncontrolled tumorigenic overgrowth. These findings in *Drosophila* brain development are likely to have general relevance in neural stem cell biology and may apply to cell cycle control in mammalian brain development as well.

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21.1 Introduction

A defining feature of all stem cells is their ability to simultaneously generate copies of themselves while giving rise to more differentiated progeny. This involves both proliferation and differentiation processes and, in cell cycle terms, implies highly controlled reentering as well as exiting of the cell cycle (Ohnuma and Harris 2003; Alvarez-Buylla et al. 2001). In consequence, stem cells are faced with a number of crucial tasks. First, they must self-renew at each division while avoiding cell cycle exit and differentiation. Second, they must be able to generate numerous daughter cells that are committed to lineage-specific differentiation. Third, during their self-renewal process they must prevent uncontrolled proliferation which can lead to tumor formation. Indeed, there is now increasing evidence that stem cells might be the cells of origin of certain cancers ("tumor stem cells") suggesting that errors in stem cell division rate or in the fine balance between self-renewal and differentiation could result in tumorigenesis (Morrison and Kimble 2006; Read et al. 2006; Dirks 2008). Finally, once the appropriate number of progeny is generated, stem cells must terminate their proliferative activity.

In brain development, the spatially and temporally regulated proliferation of neural stem cells generates the enormous number and remarkable diversity of neuronal cells that characterize the central nervous system. How do neural stem cells regulate self-renewal and differentiation during neurogenesis in the brain, what determines the cellular identity of the generated postmitotic neurons, and how is this complex process of neurogenesis shut down at the appropriate time? Here, we review some of the recent progress made in addressing these issues and highlight the numerous ways in which the cell cycle is linked to neural development in the brain. We focus primarily on the developing brain of *Drosophila* where neural stem cell-like progenitors, called neuroblasts, have been utilized as a highly successful model to unravel the processes of stem cell-based self-renewal, generation of differentiated neuronal progeny, and controlled termination of proliferation.

21.2 *Drosophila* Neuroblasts are Neural Stem Cell-Like Progenitors

Based on their position in the developing brain of *Drosophila*, neuroblasts are subdivided into central brain neuroblasts and optic lobe neuroblasts. The neuroblasts of the central brain, like those of the ventral nerve cord, derive from the ventral neuroectoderm and are specified by a process involving proneural genes and Notch signaling (reviewed in Artavanis-Tsakonas and Simpson 1991; Skeath and Carroll 1992, 1994; Skeath and Thor 2003; Egger et al. 2008; Technau et al. 2009). During embryogenesis, they undergo a limited first phase of proliferation which gives rise to the neurons of the larval CNS (Younossi-Hartenstein et al. 1996; Campos-Ortega and Hartenstein 1997; Urbach and Technau 2003a, b; Urbach et al. 2003).

Towards the end of embryogenesis, most of the neuroblasts enter a reversible G1 arrest known as quiescence, which separates embryonic from postembryonic neurogenesis (Truman 1990). Subsequently during larval development, most of the neuroblasts reenter the cell cycle and resume proliferation in a second, more substantial neurogenesis phase that gives rise to the majority of the neural cells in the adult brain (Truman and Bate 1988; Hofbauer and Campos-Ortega 1990; Prokop and Technau 1991; Ito and Hotta 1992; Truman et al. 1993; Hartenstein et al. 2008). Reactivation of neuroblast divisions following quiescence involves enlargement of the neuroblasts and entry into the S-phase of the cell cycle, and is regulated by intrinsic and extrinsic influences including nutrition, a glial cell niche, and several mitogenic signals (Ebens et al. 1993; Datta 1995; Britton and Edgar 1998; Voigt et al. 2002; Bello et al. 2003; Maurange and Gould 2005; Tsuji et al. 2008).

In contrast to the neuroblasts of the central brain, the neuroblasts of the optic lobes are all generated during larval development. They derive from two neuroepithelial placodes known as the inner proliferation center and the outer proliferation center, and, at least in the case of the outer proliferation center, this process involves a wave of proneural gene expression which spreads through the placode and triggers the transition of epithelial cells into neuroblasts (Hofbauer and Campos-Ortega 1990; Green et al. 1993; Ceron et al. 2001; Egger et al. 2007; Yasugi et al. 2008). The neuroepithelial-to-neuroblast conversion continues for a remarkably long time (by *Drosophila* standards) lasting approximately 4 days until the optic lobe neuroepithelia are depleted. The generation of optic lobe neuroblasts exemplifies the transition from symmetric ("proliferative") divisions of neuroepithelial progenitors, which serve to increase the neural stem cell pool, to asymmetric ("differentiative") divisions of neuroblasts, which lead to the generation of differentiating cells. This two-step process in the developing optic lobe, which results in a dramatic increase in progenitor numbers and, hence, in neural proliferation, may have close parallels to the mode of neurogenesis that operates in parts of the vertebrate brain (Noctor et al. 2004; Gotz and Huttner 2005).

All neuroblasts in the brain generate sets of lineally related neural progeny by dividing asymmetrically into a larger daughter cell, which retains neuroblast features, and a smaller daughter cell, which is committed to the differentiation pathway. In most cases (type I neuroblasts), the smaller daughter cell, referred to as a ganglion mother cell (GMC), divides only once more and both of its daughters exit the cell cycle to give rise to two differentiating neural cells (Doe 1992). By undergoing repeated rounds of these stem cell-like divisions, each type I neuroblast typically generates a stereotyped lineage of approximately 100 neural progeny during its proliferative lifetime (Fig. 21.1a). In contrast, a small set of dorsomedially located central brain neuroblasts has a more complex proliferation pattern (Bello et al. 2008, Boone and Doe 2008; Bowman et al. 2008). These type II neuroblasts also undergo multiple rounds of asymmetric cell divisions, and each of these gives rise to a self-renewed neuroblast and a smaller daughter cell referred to as an intermediate progenitor. The intermediate progenitor, a transit amplifying cell, reenters the cell cycle a limited number of times and in this process self renews and generates a sublineage of GMCs, each of which undergoes a terminal division

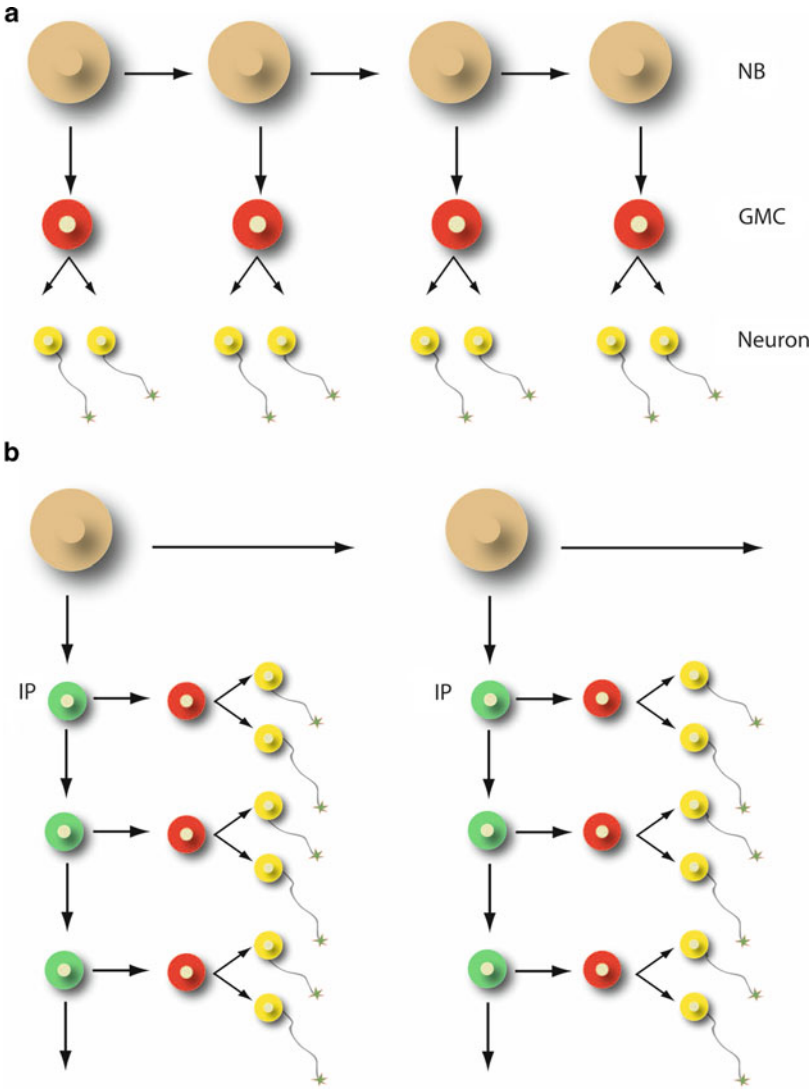

Fig. 21.1 Two types of neuroblasts in the central brain. (**a**) Type I neuroblasts (NB) repeatedly self renew and generate a smaller ganglion mother cell (GMC) which divides only once to produce two daughter cells that exit the cell cycle and differentiate into neural cells. (**b**) Type II neuroblasts repeatedly self renew and generate an intermediate progenitor which itself reenters the cell cycle a limited number of times to self renew and generate a sublineage of GMCs; each of these GMCs divides only once to produce two postmitotic neural cells

into two differentiating neural cells. As each type II neuroblast generates numerous intermediate progenitors and each intermediate progenitor again generates numerous GMCs, a considerable amplification of neural proliferation results (Fig. 21.1b). Due to this amplification, lineages of up to 500 neurons can be generated postembryonically

by a single type II neuroblast (Bello et al. 2008). These exceptionally large lineages make a substantial contribution to an extensive midline neuropile structure, the central complex of the *Drosophila* brain (Izergina et al. 2009). The restricted proliferative potential of intermediate progenitors as compared to neuroblasts is maintained by the dFezf/Earmuff transcription factor which limits mitotic activity of intermediate progenitors and prevents their dedifferentiation into neuroblasts (Weng et al. 2010). It is noteworthy that comparable intermediate progenitors have been found in the developing brains of mammals (Gotz and Huttner 2005; Merkle and Alvarez-Buylla 2006; Kriegstein and Alvarez-Bullya 2009). This may be one indication that the molecular mechanisms that control neurogenesis in insects and vertebrates are evolutionarily conserved.

21.3 Asymmetric Cell Divisions Balance Self-Renewal and Differentiation

The asymmetric cell divisions manifested by proliferating *Drosophila* neuroblasts exemplify a fundamental process whereby the unequal distribution of cell fate determinants leads to the generation of daughter cells with two different fates. From the perspective of the cell cycle, the fate of the larger of these daughter cells is to reenter the cell cycle as a proliferating neuroblast, while the fate of the other smaller cell, the GMC, is to exit the cell cycle after one final division which generates two postmitotic cells. Many of the molecular elements that control asymmetric neuroblast divisions in *Drosophila* have now been identified (for recent reviews see Doe 2008; Knoblich 2008, Wu et al. 2008; Zhong and Chia 2008; Neumuller and Knoblich 2009).

The asymmetry in cell fate is based on the maintained inheritance of apical protein complexes by the self-renewing neuroblast and the segregation of basal protein complexes into the GMC daughter (Fig. 21.2). The apical protein complexes comprise the proteins Par-3, Par-6, and atypical PKC ("Par complex"), as well as Inscuteable, Pins, Gαi, and Mud, all of which accumulate on the apical side of the cell cortex before mitosis (Kuchinke et al. 1998; Schober et al. 1999; Wodarz et al. 1999; Parmentier et al. 2000; Petronczki and Knoblich 2001; Izumi et al. 2004, 2006; Bowman et al. 2006; Siller et al. 2006). The apical protein complexes establish an axis of polarity and, during mitosis, are preferentially inherited by the apical daughter cell, which remains a neuroblast (Yu et al. 2000, 2003, 2005; Schaefer et al. 2001). However, these protein complexes do not appear to influence cell fate directly. Rather, they are involved in orientation as well as inherent asymmetry of the mitotic spindle and direct the asymmetric localization of specific cell fate determinants to the opposite, basal side of the dividing cell, probably through cell cycle-dependent phosphorylation of the determinants or their adaptor molecules (reviewed by Wodarz and Huttner 2003; Neumuller and Knoblich 2009). During neuroblast mitosis, the basal cell fate determinants then segregate exclusively

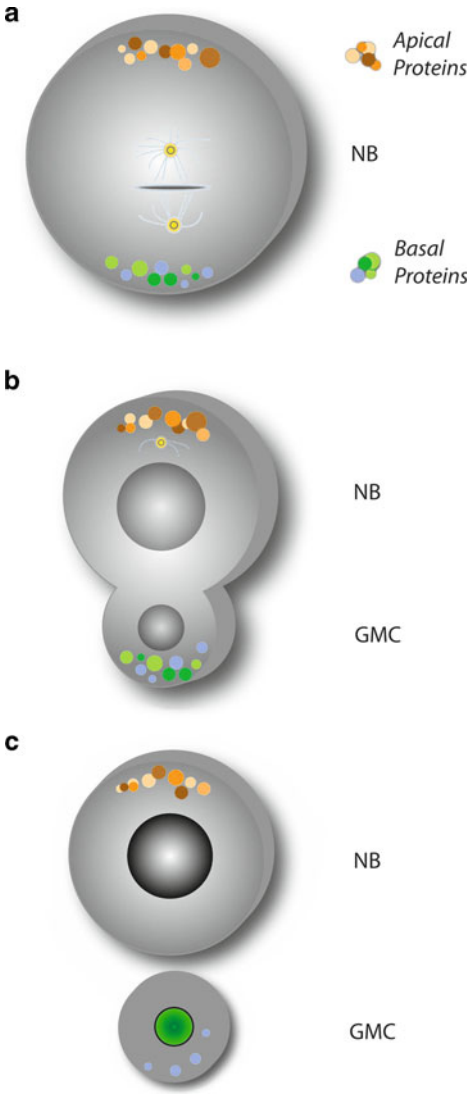

Fig. 21.2 Asymmetric cell division of neuroblasts results in the production of two cells with different fates. The asymmetry of cell fates is based on the inheritance of apical protein complexes by the apical daughter which remains a self-renewing neuroblast and on the segregation of basal protein complexes into the smaller basal daughter where they act in the specification of GMC fate and in neural differentiation of the GMC's progeny. Different stages in asymmetric neuroblast division are schematically represented (**a**, metaphase; **b**, telophase; **c**, postmitosis)

into the GMC at telophase where they subsequently act in the specification of GMC fate.

Three important asymmetrically segregated cell fate determinants have been analysed in some detail in brain neuroblasts. The first is Numb, a tissue-specific repressor of the Notch pathway (Uemura et al. 1989; Rhyu et al. 1994; Knoblich et al. 1995). The second is Pros (Prospero), a homeodomain transcription factor that can act as transcriptional activator and repressor (Doe et al. 1991; Vaessin et al. 1991; Matsuzaki et al. 1992; Hirata et al. 1995; Knoblich et al. 1995; Spana and Doe 1995).

The third is Brat (Brain tumor), a member of the NHL domain family involved in translational regulation and cell growth inhibition (Kurzik-Dumke et al. 1992; Arama et al. 2000; Sonoda and Wharton 2001; Frank et al. 2002). The asymmetric segregation of Numb, Pros, and Brat is mediated by two adaptor proteins, Mira (Miranda) and Pon (Partner-of-Numb). Mira prevents Pros from regulating transcription in the neuroblast by tethering it to the basal cortex during mitosis; however, once segregated into the daughter GMC, Mira is degraded and Pros enters the nucleus (Ikeshima-Kataoka et al. 1997; Shen et al. 1997; Matsuzaki et al. 1998). Like Pros, Brat also binds to Mira and hence is cosegregated into the GMC during neuroblast division. Pon assists in the asymmetric localization of Numb but is not essential for its segregation into the GMC during late stages of mitosis.

Once in the GMC, Numb, Pros, and Brat are all thought to inhibit self-renewal and promote cell cycle exit and differentiation. Numb probably does this by promoting endocytosis of the Notch receptor, making levels of Notch signaling lower than in the neuroblast (Almeida and Bray 2005; Lee et al. 2006a; Wang et al. 2006). Pros represses expression of cell cycle genes and activates genes that specify cell fate and are required for terminal differentiation (Choksi et al. 2006; Li and Vaessin 2000; Liu et al. 2002). For example, Pros is required to negatively regulate the expression of the cell cycle genes cyclin A, cyclin E, and String (the fly Cdc25 homolog), and to positively regulate the cyclin-dependent kinase inhibitor Dacapo. Brat is thought to be a posttranscriptional inhibitor of the transcription factor dMyc and may act to prevent cell growth (Bello et al. 2006; Betschinger et al. 2006; Choksi et al. 2006; Lee et al. 2006b). Consistent with the functions of these genes in repressing growth and self-renewal, loss of *pros*, *brat* or *numb* in the larva results in neuroblast lineages that escape differentiation (see below). This causes overgrowth characterized by the overproduction of neuroblast-like cells at the expense of differentiated neurons (reviewed in Januschke and Gonzalez 2008).

It is noteworthy that all three cell fate determinants in *Drosophila* have comparable mammalian homologs. Numb is required for mouse neurogenesis (Zhong et al. 1996, 2000; Petersen et al. 2002; Li et al. 2003; Bultje et al. 2009). Mouse Pros controls proliferation in the retina but has not yet been described to segregate asymmetrically (Dyer et al. 2003). One of the murine Brat homologs, TRIM3, is expressed in cortical neural stem and progenitor cells where it is asymmetrically localized and has a conserved inhibitory effect on the regulation of stem cell proliferation (Schwamborn et al. 2009).

21.4 Cell Cycle Regulators Can Affect Asymmetric Neuroblast Divisions

There is increasing evidence that cell cycle regulators can impinge on the molecular machinery that controls asymmetric divisions in neuroblasts. Mutations in several genes encoding key regulators of cell cycle events can affect asymmetric localization of cell fate determinants, specification of distinct daughter cell fates, and the decision

to self-renew or differentiate. These cell cycle regulators include protein kinases, Cdc2/CDK1, Aurora A, and Polo, as well as cyclin E and anaphase-promoting complex/cyclosome (APC/C) core components (for recent reviews see Chia et al. 2008; Budirahardja and Gonczy 2009).

Cdc2 contributes to the kinase activity (CDK1) required to drive cells from the G2 phase into mitosis and cells defective in CDK1 activity arrest in the G2 phase. Mutant analysis of the role of Cdc2/CDK1 in neuroblast divisions indicates that high levels of CDK1 activity during mitosis are necessary to maintain asymmetric localization of both apical and basal components of the asymmetry machinery (Tio et al. 2001). If these CDK1 levels are not attained, asymmetric division of the neuroblasts is converted into a symmetric division. This implies that Cdc2/CDK1 levels play a role in determining whether a neuroblast's division is symmetric or asymmetric.

Aurora A and Polo, two other highly conserved kinases, also affect asymmetric divisions (Lee et al. 2006a; Wang et al. 2006, 2007). Both kinases are involved in several cell cycle-associated processes such as centrosome maturation, metaphase arrest, and cytokinesis. In the absence of either Aurora A or Polo in developing neuroblasts, the asymmetric localization of Numb and its adaptor Pon is defective, such that cell division can occur but asymmetric localization of determinants is disrupted. Accordingly Pon, which facilitates the asymmetric localization of Numb, has been shown to be an important downstream target of Polo in the control of neuroblast asymmetric division, thus providing a direct biochemical link between a cell cycle regulator and a component of the asymmetry machinery (Wang et al. 2007). Aurora A and Polo also affect other elements involved in controlling the self-renewal versus differentiation decision in neuroblasts. Thus, in mutants of Aurora A or Polo, the asymmetrical localization of aPKC fails and the orientation of the mitotic spindle is perturbed. Moreover both kinases act as tumor suppressors and prevent excess self renewal of neuroblasts (Lee et al. 2006a; Wang et al. 2006, 2007).

Cyclin E has a general role in the cell cycle in regulating the G1- to S-phase transition. Additionally, at least in identified neuroblasts of the ventral nerve cord, cyclin E can act downstream of Hox genes to convert a symmetric neuroblast division into an asymmetric division (Berger et al. 2005). This role of Cyclin E in mediating asymmetric cell division appears to be independent of its role in the cell cycle. At the molecular level Cyclin E is thought to inhibit the function of Pros and facilitate its cortical localization in the neuroblast, which is critical for its self-renewal during asymmetric division (Berger et al. 2010). Cyclin E is also thought to confer self-renewing asymmetric division potential to GMCs, and upregulation of Cyclin E has been observed in brain tumors (Bhat and Apsel 2004; Betschinger et al. 2006).

APC/C, in transient association with the activating subunits Cdc20 and Cdh1, promotes cell cycle transitions through several key processes including regulation of DNA replication, centrosome duplication and mitotic spindle assembly as well as the destruction of mitotic cyclins and chromosome separation inhibitors. In neuroblasts, APC/C core function is additionally required for asymmetric localization of Mira and its cargo proteins Pros and Brat (Slack et al. 2007). Mutations in any one of several APC/C core component proteins cause Mira to mislocalize to a pericentrosomal region. In molecular terms, APC/C is thought to facilitate the ubiquination

of Mira, which in turn is required for the asymmetrical cortical localization of this adaptor molecule and its cargo proteins.

21.5 Neural Differentiation is Influenced by the Timing of Cell Cycle Exit

Most neuron types in the *Drosophila* central brain are specified according to their lineage of origin and depend on the identity of their parent neuroblast, which is thought to be determined by positional information encoded in the neurogenic region by anteroposterior and dorsoventral patterning genes (reviewed in Hartenstein et al. 2008; Lichtneckert and Reichert 2008; Urbach and Technau 2008). Neural specification in the developing brain also depends, in part, on whether Notch signaling is active or suppressed by asymmetrically segregated Numb in newly generated neurons, since the two postmitotic daughter cells of a given GMC apparently undergo an opposing, Notch-dependent binary cell fate decision once they exit the cell cycle (Kumar et al. 2009; Lin et al. 2010; Das et al. 2010). Importantly, however, the specification of neural identity in the developing brain, as in other parts of the nervous system, is also critically dependent on the time point at which the neural cells are generated in their neuroblast lineage of origin.

The time at which a given daughter cell exits the cell cycle is referred to as its birth date. In *Drosophila* brain development, there is a marked correlation between birth date and cell fate for the neural progeny that are generated by a given neuroblast. This is most clearly evident during embryonic neurogenesis when neuroblasts generate neural cells with different fates in an invariant temporal sequence. Recent work has identified a neural stem cell division-dependent timing mechanism which underlies this phenomenon (Fig. 21.3). This cell cycle-dependent timing mechanism is based on a series of transcription factors that are expressed within each neuroblast in a characteristic developmental sequence, known as the temporal series (for recent reviews see Jacob et al. 2008; Kao and Lee 2009; Sousa-Nunes et al. 2010).

The temporal expression series, which consists of the nonoverlapping expression of the transcription factors Hunchback, Kruppel, Pdm, and Castor during sequential neuroblast division cycles, ensures that each neuroblast generates a relatively invariant temporal sequence of specified GMCs. Each of these GMCs then gives rise to daughters with defined, and often individually identifiable, neural cell fates (Kambadur et al. 1998; Brody and Odenwald 2000; Isshiki et al. 2001, Grosskortenhaus et al. 2005; Tran and Doe 2008). This linkage between birth order and neural fate during early neurogenesis is based on the fact that GMCs and their neural progeny maintain the expression of the cell fate-determining transcription factor that is present in the neuroblast at the time of the GMC's birth date (Fig. 21.3). Loss of function of the temporal factors or their persistent expression can block progression of the temporal series of differentially specified GMCs (Isshiki et al. 2001; Pearson and Doe 2003; Cleary and Doe 2006; Grosskortenhaus et al. 2006). Interestingly, mutations in general cell cycle control elements such as String also lead to

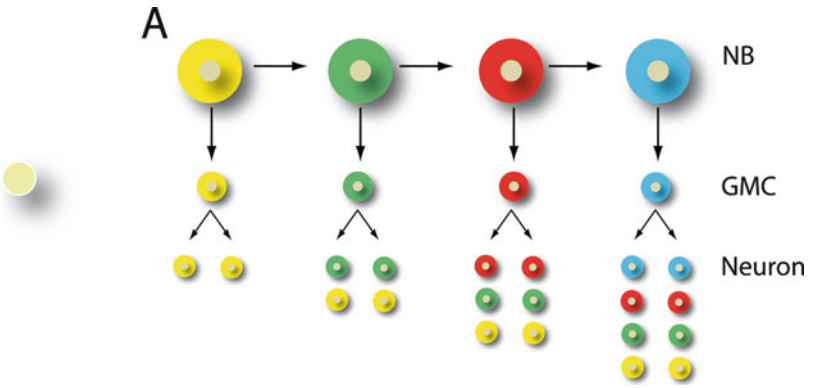

Fig. 21.3 A neuroblast division-dependent temporal transcription factor series acts in generating neural cells with different cell fates. This cell timing mechanism causes the GMC and its progeny to maintain the expression of the cell fate determining transcription factor present in the neuroblast at the time of the GMC's generation. Different transcription factors in the temporal series are represented by different colors

a block in the sequential expression of the temporal series transcription factors indicating that progression of the cell cycle is required to progress a molecular clock that generates neurons of different fate.

In addition to the canonical temporal series of four transcription factors, most of which act primarily during embryonic neurogenesis, neuroblasts express a fifth transcription factor, Seven up, both during embryogenesis and during larval development (Kanai et al. 2005; Mettler et al. 2006; Maurange et al. 2008). The larval expression of Seven up, together with a larval expression pulse of Castor, is required for neuroblasts to switch from the generation of neurons with early temporal identities to the generation of neurons with late temporal identities (Neurons with early postembryonic temporal identities are relatively large and express the BTB zinc finger protein Chinmo; neurons with late postembryonic temporal identities are smaller and express the BTB zinc finger protein Broad Complex). Although the molecular basis of transcription factor switching in the temporal sequence is not yet clear, multiple crossregulatory interactions have been observed between the temporal transcription factors in the proliferating neuroblasts and these may contribute to the sequential progression of the observed expression patterns (Jacob et al. 2008).

21.6 Programmed Cell Death Contributes to the Termination of Proliferation

In development, the time at which neuroblasts irreversibly exit the cell cycle and stop proliferation is important for attaining the appropriate number of neurons in different parts of the brain, for achieving the correct balance of early versus late neural cell fates, and for the prevention of uncontrolled overgrowth in the brain.

The neuroblast termination process occurs at different times in different regions but is largely complete by the end of metamorphosis so that there are no identifiable neuroblasts in the adult brain of *Drosophila* (Ito and Hotta 1992). Some type I neuroblasts terminate early in development and contribute only a small set of neurons to the adult CNS. This has been especially well studied in the ventral nerve cord where specific neuroblasts terminate proliferation by undergoing Hox gene-mediated programmed cell death (White et al. 1994; Prokop et al. 1998; Bello et al. 2003). In the ventral nerve cord, neuroblast apoptosis occurs towards the end of embryogenesis and is heavily biased towards neuroblasts in the abdominal neuromeres; this round of early programmed cell death makes a major contribution to the neuromere-specific sculpting of the adult nervous system (reviewed in Blaschke et al. 1998; Maurange and Gould 2005).

Most type I neuroblasts in the central brain terminate proliferation postembryonically shortly after the onset of pupation (Truman and Bate 1988). One group of these neuroblasts also terminates proliferation via Hox gene-mediated apoptosis (Bello et al. 2003; Cenci and Gould 2005). This is thought to involve activation of the proapoptotic proteins Reaper, Hid, and Grim by a transient expression pulse of the Hox gene *abdominal A*. Inappropriate termination of type I neuroblast proliferative activity through apoptosis induced by aberrant or ectopic Hox gene expression is prevented by genes of the Polycomb group which are part of a general epigenetic cellular memory system that maintains the correct inactive states of Hox gene expression in *Drosophila* (Bello et al. 2007). A second group of neuroblasts terminates proliferation by cell cycle withdrawal. These neuroblasts undergo a specific series of modifications in early pupal stages, which include lengthening of the cell cycle, reduction in cell size, expression of nuclear Pros and finally cell cycle exit through a symmetric differentiative division that produces two postmitotic daughters (Maurange et al. 2008).

In contrast to the other type I neuroblasts, the four brain neuroblasts that give rise to the mushroom bodies do not terminate proliferation until the end of pupation. Since they also do not undergo a quiescent phase at the end of embryogenesis, these mushroom body neuroblasts proliferate throughout most of brain development from embryonic to late pupal stages, thus, producing approximately 500 neurons in 8 days (Ito and Hotta 1992; Ito et al. 1997; Lee et al. 1999). The mushroom body neuroblasts do not appear to undergo Hox gene-mediated apoptosis nor do they require Pros for termination of proliferation suggesting that they might utilize a third different type of caspase-dependent termination mechanism (Kurusu et al. 2009; Siegrist et al. 2010). The termination processes that occur in type II neuroblasts or in neuroblasts of the optic lobe are currently unknown.

Remarkably, the timing of neuroblast termination in the central brain appears to be regulated by the temporal transcription factors and their target genes, regardless of whether the termination of proliferation involves Hox gene-induced programmed cell death or Pros-induced cell cycle exit (Maurange et al. 2008). Indeed, despite different cell cycle exit strategies (apoptosis vs. terminal division) all neuroblasts apparently use a similar molecular timer, namely the temporal series, to shut down proliferation and, hence, prevent uncontrolled overgrowth in the brain. At least for

the type I neuroblasts studied so far, the speed of their cell cycle, the identity of their progeny, as well as the time of their termination is controlled, in part, by the same set of temporal transcription factor genes.

Termination of proliferation, be it through apoptosis or terminal differentiative division, does not occur normally in neuroblasts that are mutant for asymmetric cell fate determinants such as Pros, Numb, or Brat. These mutant neuroblasts undergo excess self-renewal, continue to divide into adult stages, and produce excess neuroblast-like cells at the expense of differentiated progeny (Choksi et al. 2006; Bello et al. 2006; Betschinger et al. 2006; Lee et al. 2006b). Moreover, transplantation of the corresponding mutant brain tissue into normal hosts results in immortalized transformed cells and lethal malignant neoplastic overgrowth, and the resulting tumors can be successively reimplanted into new hosts for years (Caussinus and Gonzalez 2005). Unlike wild type transplants, these mutant-derived transplants fail to respond to signals which normally terminate division and appear to be immortal, and they exhibit genome instability as indicated by high frequencies of cytologically abnormal karyotypes and defects in centrosome morphology and number (Caussinus and Gonzalez 2005; Castellanos et al. 2008). In terms of growth rate, cell types and metastatic activity, the transplant-induced tumors are essentially indistinguishable from one another regardless of the mutant from which they derive suggesting a common underlying etiology (Gonzalez 2007).

21.7 Conclusions

Recent investigations show that the cell cycle-dependent control of proliferation, differentiation and termination in *Drosophila* neuroblasts is much more diverse than previously thought. There are multiple types of neuroblasts in different regions of the developing brain and their developmental origins, from ventral neuroectoderm or cephalic placodes, as well as their modes of proliferation, through GMCs or intermediate progenitors, differ substantially. Some neuroblasts transiently exit the cell cycle and enter quiescence while others continue to proliferate throughout development. Termination of neuroblast proliferation can occur early through apoptosis or late through apoptosis or cell cycle exit and terminal division.

Remarkably, however, some of the most fundamental molecular control mechanisms that underlie these processes appear to be general. The asymmetric cell division machinery that balances self-renewal with differentiation is present in all neuroblasts studied so far. The molecular components of this machinery such as the apical protein complex and basal protein complex are universal throughout all the neural stem cells in *Drosophila*. Moreover, some of the classical cell cycle regulators known to act in eukaryotic mitosis, appear to be involved in the control of asymmetric cell division in the neuroblasts. Similarly, the members of the same temporal transcription factor sequence, which are probably expressed in all neuroblasts, are involved in specifying different temporal identities of differentiating neuroblast daughter cells as well as in timing and executing the termination of proliferation in the neuroblasts themselves.

In view of the universality of these molecular control mechanisms in *Drosophila*, it is perhaps not surprising that some of these regulatory principles are conserved in mammalian brain development. Although there are significant differences in these processes as well between insects and vertebrates, the striking conservation of the basic molecular machinery for asymmetric cell divisions in both groups does imply that analysis of brain development in *Drosophila* will provide important insights into normal and abnormal function of neural stem cells that apply to mammalian and human neural stem cells as well.

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Chapter 22
Control of Neuronal Ploidy During Vertebrate Development

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Abstract Somatic tetraploid neurons are present in different structures of the vertebrate nervous system, including cortex and retina. In this chapter, we provide evidence that these neurons can be widely detected in the chick nervous system. We also discuss mechanisms creating neuronal tetraploidy in vertebrates, concluding that the neurotrophin receptor p75 could be responsible for the generation of these neurons in most neural tissues, as previously observed in the retina. Somatic tetraploidy in the chick retina correlates with increased neurons' soma size and dendritic arborization, giving rise to neurons known to innervate a specific layer of the optic tectum. Tetraploidy could therefore account for neuronal diversity in the normal nervous system. De novo generation of tetraploid neurons has been shown to occur in Alzheimer's disease. This suggests that the morphological changes expected to occur in the affected neurons could lead to altered neuronal function, thus providing a basis for neurodegeneration.

22.1 Introduction

Vertebrate neurons have classically been assumed to be postmitotic cells with a 2C amount of DNA in their nuclei (Swift 1953), but this view was challenged when Lapham (1968) suggested that most Purkinje cells are tetraploid.[1] Following this initial observation, an ample debate took place about whether tetraploid neurons are actually present in vertebrates. Although several authors claimed that other large vertebrate neurons are tetraploid (Herman and Lapham 1968, 1969; Museridze et al. 1975), many other studies questioned this concept (Swartz and Bhatnagar 1981), and the absence of reliable procedures for DNA quantification made it impossible to reach a clear conclusion at that time (Swartz and Bhatnagar 1981).

[1]Note that somatic tetraploidy as used in this chapter refers only to the amount of DNA in the cell nucleus without implying a doubling of chromosomes rather than chromatids.

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Up-to-date techniques such as flow cytometry, fluorescent in situ hybridization (FISH), slide-based cytometry (SBC), and quantification of genomic DNA from isolated nuclei can all reliably estimate the amount of nuclear DNA in neurons (Mosch et al. 2007; Morillo et al. 2010). The use of these techniques has shown, for instance, that neurons with DNA content higher than 2C can readily be observed in the human cortex (Mosch et al. 2007). Using SBC, flow cytometry, and FISH, we have recently demonstrated that tetraploid neurons also exist in the normal vertebrate retina (Morillo et al. 2010). This latter observation can be extrapolated to other areas of the central nervous system (CNS), including the optic lobes, dorsal root ganglia (DRG), cerebellum, and spinal cord of the posthatching day 2 (P2) chick (Fig. 22.1). The presence of nuclei with a 4C DNA content in these tissues was analyzed by flow cytometry in dissociated cells immunostained for the neuronal marker βIII tubulin. This analysis indicated that 10.79 ± 2.49% (mean ± SEM; $n = 2$) of optic lobe neurons, 10.86 ± 0.06% (mean ± SEM; $n = 2$) of DRG neurons, 4.80 ± 0.54% (mean ± SEM; $n = 7$) of spinal cord neurons, and 3.67 ± 0.44% (mean ± SEM; $n = 7$) of cerebellar neurons contain double the normal amount of DNA in their nuclei (Fig. 22.1, right panels). Furthermore, as previously observed in the retina (Morillo et al. 2010), most βIII tubulin-negative cells were observed to contain nuclei with a 2C DNA content in all analyzed structures (Fig. 22.1, left panels). These results indicate that subpopulations of tetraploid neurons, representing a variable amount depending on the specific area that is analyzed, can readily be detected in the normal nervous system.

22.2 Mechanisms Creating Somatic Tetraploidy in Neurons

Vertebrate neurons derive from neuroepithelial cells constituting the walls of the neural tube (see Fig. 22.2). These cells are highly packed, forming a pseudostratified neuroepithelium characterized by the to-and-fro migration of the nuclei as the cell cycle proceeds (a nuclear behavior referred to as interkinetic nuclear movement; Sauer 1935; Frade 2002). Nuclei migrate to the basal side during G1 and remain there during S-phase (labeled as S-phase1 in Fig. 22.2). Then, they migrate back to the apical side during G2, before undergoing mitosis at the apical surface. At this place, neural precursors acquire capacity to express neurogenic and proneural genes (Latasa et al. 2009), and then they divide. Some of the daughter cells maintain the expression of the proneural genes and become neurons as they migrate basally toward the differentiated region of the CNS (gray cell in Fig. 22.2). In this critical period, neuronal progenitors are supposed to come out from the cell cycle giving rise to postmitotic neurons (Farah et al. 2000; Ochocinska and Hitchcock 2009).

The observation that tetraploid neurons exist in the vertebrate CNS raises the question as to how and where these neurons are generated. One possibility is that tetraploid neurons could derive from cell fusion of previously differentiated neurons, generating heterokaryons. Indeed, stable reprogrammed heterokaryons have been shown to be formed spontaneously in Purkinje neurons after bone marrow

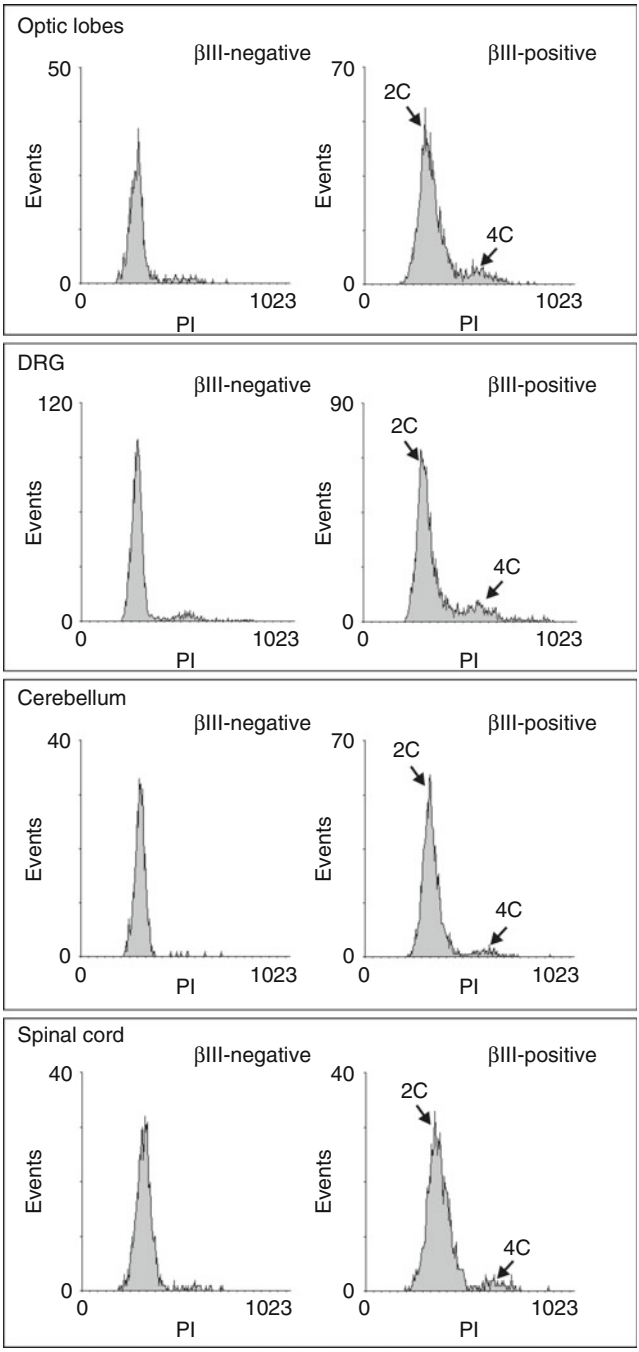

Fig. 22.1 Somatic tetraploid neurons in the P2 chick. Optic lobes, cerebella, dorsal root ganglia (DRG), and spinal cords were dissected out from P2 chicks, dissociated, and processed for flow

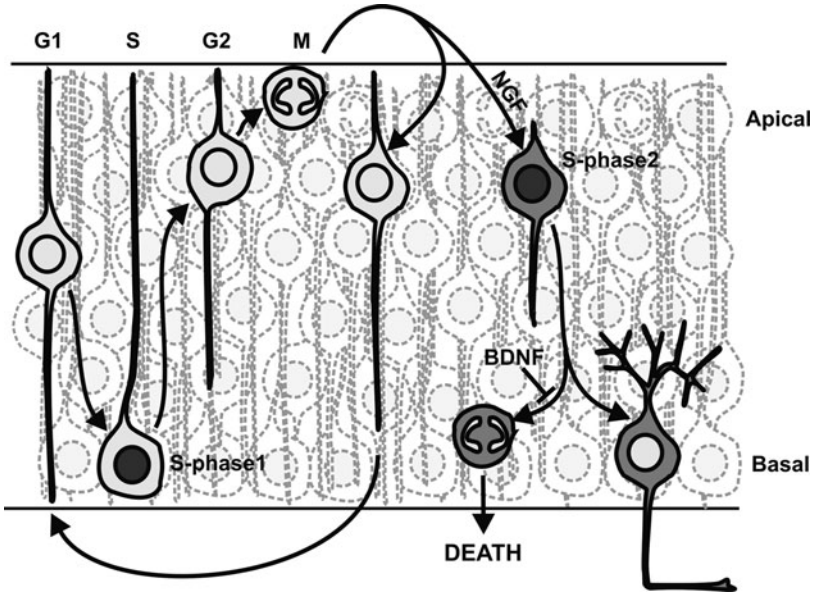

Fig. 22.2 Scheme showing the dynamics of neuroepithelial cells during a neurogenic cycle and the possible fate of the differentiating neurons. Precursors incorporate BrdU (*dark gray nucleus*) at basal positions where S-phase1 takes place, and they divide at the apical neuroepithelium. This division gives rise to either progenitor cells that displace their nuclei to the basal neuroepithelium to undergo a new round of S-phase1 or differentiating neurons (*dark gray cytoplasm*). Some of these differentiating neurons are susceptible to undergo a new round of DNA synthesis (S-phase2, *dark gray nucleus*) at the apical half of the neuroepithelium. In the presence of BDNF, these cells remain in a G2-like status (thus acquiring somatic tetraploidy), while in the absence of BDNF they undergo mitosis and subsequently they die

transplant (Weimann et al. 2003). In vivo, rare events of neuron–neuron or neuron–glia fusion can readily be observed in the rat prefrontal cortex in response to stroke (Paltsyn et al. 2008). Double-nucleated neurons have also been observed in Alzheimer's disease, an observation that has been interpreted in terms of nuclear division without cytokinesis (Zhu et al. 2008). Although neuronal fusion can also be observed in the control brain (Paltsyn et al. 2008), the frequency at which it occurs is too low to explain the proportion of tetraploid neurons observed in vivo. Furthermore, this process seems to be of stochastic nature. An alternative mechanism generating somatic tetraploidy and increase of cell size in proliferating cells is endomitosis (i.e., mitotic cycle in the absence of anaphase/cytokinesis thus

Fig. 22.1 (Continued) cytometry as previously described (Morillo et al. 2010). Flow cytometric analysis of propidium iodide (PI)-labeled cells demonstrates that some βIII tubulin-positive cells (i.e., neurons) are tetraploid (4C) in all tissues that were studied (see text for a quantification), whereas most βIII tubulin-negative cells show a 2C DNA content. At P2, these tissues are fully differentiated (Fujita 1964; Rohrer et al. 1985; Feirabend 1990; Mey and Thanos 2000), thus ruling out the possibility that the βIII tubulin-positive cells with 4C DNA content are actually proliferating neuronal precursors

resulting in genomic DNA duplication). In vertebrates, this mechanism for polyploidization has been shown to take place during differentiation of megakaryocytes (Italiano and Shivdasani 2003), and it could theoretically account for neuronal tetraploidization. Finally, several eukaryotic cells are also known to undergo endoreduplicative cycles (or endocycles). Endoreduplication is a modified version of the cell cycle characterized by S-phase without mitosis, which leads to somatic polyploidy in specific tissues (Edgar and Orr-Weaver 2001; Ullah et al. 2009).

Current evidence indicates that neuronal tetraploidy in vertebrates is caused by endoreduplication taking place in newborn neurons. In the vertebrate retina, retinal ganglion cells (RGCs) are the first neurons to come out from the cell cycle. These cells express early neuronal markers such as RA4 already 15 min after the last mitosis (Waid and McLoon 1995), and they acquire a differentiated morphology as they displace to the basally located ganglion cell layer (GCL) (Prada et al. 1981). Short pulses of 5-bromo-2-deoxyuridine (BrdU) result in the labeling of a population of retinal cells in S-phase within the apical half of the neuroepithelium, most of them expressing both βIII tubulin and the RGC marker RA4 (Morillo et al. 2010). These cells would be differentiating neurons that undergo S-phase (referred to as S-phase2 in Fig. 22.2). BrdU pulse-chase experiments have also demonstrated that most migrating BrdU-positive RGCs remained in the GCL with a 4C DNA content (Morillo et al. 2010). It can therefore be concluded that postmitotic RGC progenitors replicate their DNA in vivo as they migrate to the GCL and remain in a G2-like state, giving rise to tetraploid RGCs.

We have studied whether cell cycle reentry in differentiating βIII tubulin-positive neurons can be extrapolated to other developing neural tissues. In this regard, we have focused in the early chick embryo (stage HH19–20; Hamburger and Hamilton 1951) since the first chick neurons are known to be born at this stage (McConnell and Sechrist 1980). βIII tubulin immunostaining was used in these experiments since this marker is known to be expressed by neuronal progenitors soon after their last mitosis (Menezes and Luskin 1994). After a 1-h pulse, BrdU was readily observed in βIII tubulin-positive cells from different CNS structures, including spinal cord, rhombencephalon, and diencephalon (see examples of BrdU incorporation in the spinal cord in Fig. 22.3a; for quantification see Table 22.1). These results are consistent with the widespread presence of tetraploid neurons in the vertebrate nervous system (see Sect. 22.1), and they suggest that endoreduplication in differentiating neurons is a general mechanism creating neuronal tetraploidy throughout the whole vertebrate nervous system.

22.3 Regulation of Endoreduplication and Somatic Tetraploidy in Neuronal Progenitors

We have provided evidence that the neurotrophins play a critical role in the control of endoreduplication and somatic tetraploidization in RGCs (Morillo et al. 2010). The neurotrophin family in mammals is constituted by four members, namely nerve

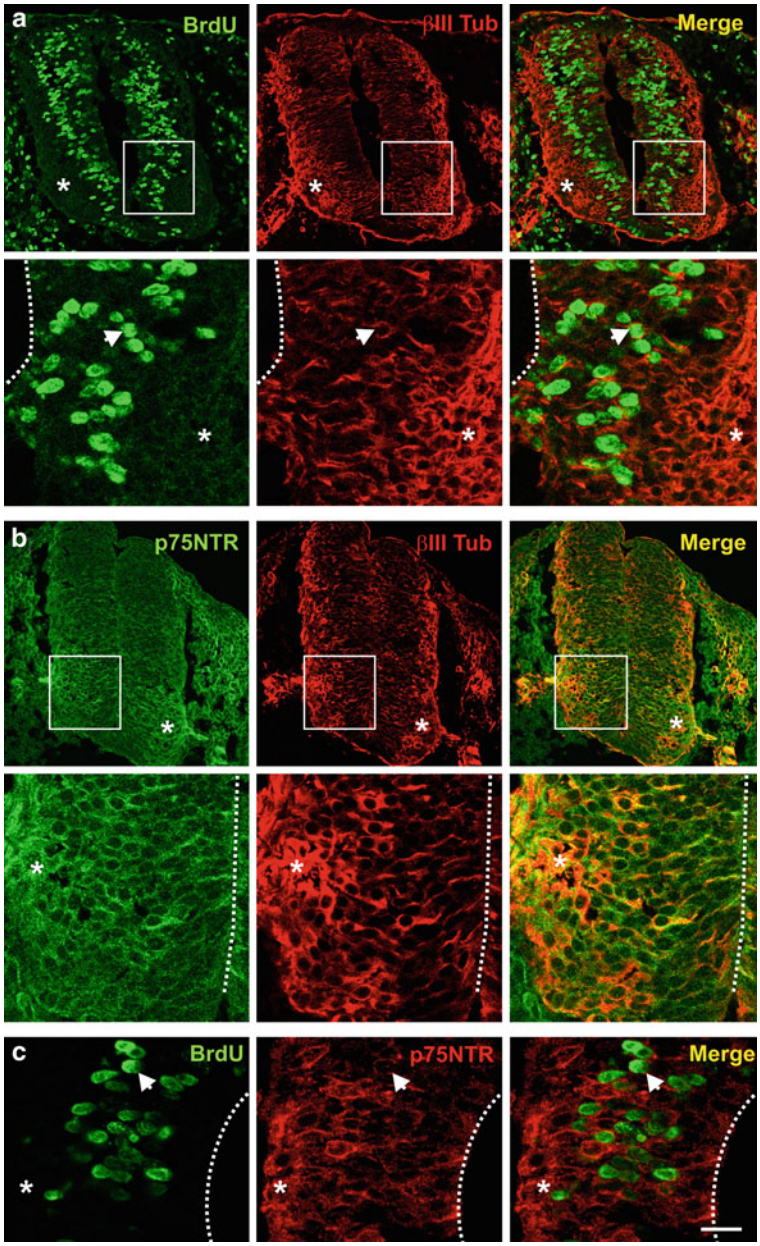

Fig. 22.3 Coexpression of the neuronal marker βIII tubulin and $p75^{NTR}$ at early developmental stages of the spinal cord and incorporation of BrdU in differentiating $p75^{NTR}$-positive neurons. HH19–20 chick embryos were treated with BrdU in ovo for 1 h, fixed, processed for cryosectioning (10 μm), and then subjected to immunohistochemistry as previously described (Morillo et al. 2010). Double immunostaining with an anti-βIII tubulin monoclonal antibody (mAb) (clone 5G8, Millipore) and an anti-BrdU mAb (ICR1, Serotec) is shown in panel (**a**). Double immunolabeling with the anti-$p75^{NTR}$ polyclonal antibody (pAb) [9992] (kindly provided by Moses V. Chao,

Table 22.1 Average number (±SEM) of cells positive for βIII tubulin (i.e., neurons) per confocal section (1.5 μm) showing BrdU incorporation after a 1-h pulse

	BrdU-βIII tubulin double-labeled cells/section	n^a
Spinal cord (ventral horn)	1.42 ± 0.30	3
Rhombencephalon	1.00 ± 0.82	3
Diencephalon	3.67 ± 0.47	3

Ten sections per embryo (HH19–20 stage) were analyzed.
[a]Number of embryos analyzed.
See Fig. 22.3 legend for further details.

growth factor (NGF) (Levi-Montalcini and Hamburger 1951), brain-derived neurotrophic factor (BDNF) (Barde et al. 1982), neurotrophin (NT)3 (Hohn et al. 1990; Maisonpierre et al. 1990; Rosenthal et al. 1990; Jones and Reichardt 1990), and NT4/5 (Hallböök et al. 1991; Berkemeier et al. 1991). These molecules were initially described as trophic factors inhibiting cell death in specific neuronal subsets of the vertebrate peripheral nervous system (Lewin and Barde 1996). Nevertheless, neurotrophins have also been involved in many other functions, including cell death induction in particular neuron types, axonal outgrowth, neuronal differentiation, induction of cell movements, regulation of synaptic plasticity, and control of cell cycle progression (López-Sánchez and Frade 2002; Reichardt 2006). Neurotrophins exert its effects through two main types of receptors, the Trk tyrosine kinase receptor family and the neurotrophin receptor p75 ($p75^{NTR}$), a known member of the tumor necrosis factor family.

$p75^{NTR}$ participates in different signaling platforms crucial for the development and maintenance of the vertebrate nervous system (Barker 2004), and it has been shown to modulate the cell cycle in several systems (López-Sánchez and Frade 2002). In this regard, $p75^{NTR}$ can interact with a number of proteins known to regulate the cell cycle, including the neurotrophin receptor interacting factors (NRIF)1 and NRIF2 (Casademunt et al. 1999; Benzel et al. 2001), SC-1 (Chittka and Chao 1999; Chittka et al. 2004), Bex1 (Vilar et al. 2006), Sall2 (Pincheira et al. 2009), and different members of the melanoma antigen (MAGE) protein family

Fig. 22.3 (Continued) New York University) and the anti-βIII tubulin mAb indicated above is shown in panel (**b**). Double immunostaining with the anti-$p75^{NTR}$ pAb [9650] (kindly provided by Moses V. Chao, New York University) and the anti-BrdU mAb mentioned above was performed in panel (**c**). BrdU in panel (**c**) was revealed using a protocol modified from Gonchoroff et al. (1986). Briefly, cryosections were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, rinsed twice with PBS, and incubated for 1 h at 37°C in 100 mM Tris–HCl buffer (pH 8.5) containing 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 μg/ml DNAse I (Roche). Then, cryosections were rinsed twice with PBS and subjected to immunohistochemistry as described by Morillo et al. (2010). (**a**) Some nuclei (*arrow*) positive for BrdU (*green*) are present in βIII tubulin cells (*red*). *Bottom panels* represent the area included in the *squares* indicated in the *top panels*. (**b**) Immunoreactivity for $p75^{NTR}$ (*green*) largely coincides with βIII tubulin immunoreactivity (*red*). *Bottom panels* represent the area included in the *squares* indicated in the *top panels*. (**c**) Double immunostaining demonstrates that some nuclei (*arrow*) positive for BrdU (*green*) are present in $p75^{NTR}$-positive cells (*red*). *Dashed line:* ventricle; *asterisk:* ventral horn differentiated region. *Bar*: 15 μm

including neurotrophin receptor-interacting MAGE homolog (NRAGE)/MAGE-D1 (Salehi et al. 2000), MAGE-H1 (Tcherpakov et al. 2002), necdin (Kuwako et al. 2004), and chicken MAGE (CMAGE) (López-Sánchez et al. 2007).

In the chick retina, migrating RGC neuroblasts express $p75^{NTR}$ in the absence of the NGF-specific receptor TrkA (Frade et al. 1996). Our work has shown that endoreduplication in differentiating RGCs is triggered by an endogenous source of NGF acting through $p75^{NTR}$ (Morillo et al. 2010), an observation consistent with the capacity of NGF to induce $p75^{NTR}$-dependent cell cycle reentry in these neurons (Frade 2000). Interestingly, $p75^{NTR}$ colocalizes with the early neuronal marker βIII tubulin in many other neural tissues from the HH18–19 chick embryo, including spinal cord, rhombencephalon, and mesencephalon (see, for instance, a double immunostaining for $p75^{NTR}$ and βIII tubulin in the spinal cord in Fig. 22.3b). These results, along with the observation that βIII tubulin-positive neurons can incorporate BrdU during early retinal development (Fig. 22.3a), suggest that $p75^{NTR}$ is actually a general inducer of endoreduplication and neuronal tetraploidy in the vertebrate nervous system. This hypothesis is further supported by the observation that BrdU-specific immunoreactivity can be detected in $p75^{NTR}$-positive cells from the spinal cord of HH19–20 embryos treated for 1 h with this nucleotide analog (Fig. 22.3c).

We have shown that cell cycle reentry takes place in differentiating RGCs expressing the transcription factor E2F1, but lacking retinoblastoma (Rb) protein expression (Morillo et al. 2010). E2F1 is crucial for G1/S-phase progression, whereas Rb is known to prevent E2F1 activity during G1, being the expression of these two proteins tightly controlled during neurogenesis (Kusek et al. 2001). Activation of $p75^{NTR}$ in differentiating retinal neurons induces E2F1 activity in vitro, a result consistent with the observed decrease of BrdU incorporation in the developing retina of $p75^{NTR}$ knockout mice (Harada et al. 2006). Interestingly, different MAGE proteins have been shown to mimic the E2F1 blocking effect of Rb (Kuwako et al. 2004; López-Sánchez et al. 2007), thus giving a direct link between $p75^{NTR}$ and the cell cycle. CMAGE is known to colocalize with $p75^{NTR}$ in the developing RGCs (López-Sánchez et al. 2007), thus representing a candidate protein for the regulation of endoreduplication in these neurons. One possibility could be that the intracellular domain of $p75^{NTR}$, known to be released in a secretase manner in response to neurotrophin binding (Frade 2005) and able to inhibit CMAGE (López-Sánchez et al. 2007), could favor E2F1 activity in RGC neuroblasts as previously observed in differentiated neuroblastoma cells (López-Sánchez et al. 2007).

22.3.1 Maintenance of the G2-Like Status in the Tetraploid Neurons and Early Cell Death During Nervous System Development

In the chick retina, a peak of cell death can be detected during the period of RGC production and prevention of this cell death with BDNF results in higher production

of RGCs (Frade et al. 1997; Mayordomo et al. 2003). This early peak of cell death has been shown to be induced by endogenous NGF through $p75^{NTR}$ (Frade et al. 1996), and it requires cell cycle reentry (Frade 2000). A connection between somatic tetraploidization and early apoptosis in RGC neuroblasts should therefore exist. Our work has provided evidence that cell death associated with the genesis of RGCs occurs in a small portion of tetraploid RGCs that lose their G2-like state and undergo G2/M transition as they come close to the GCL (Frade 2000; Morillo et al. 2010). BrdU pulse-chase experiments performed in vivo have clearly demonstrated that ectopic mitosis in these neurons is rapidly followed by apoptosis (Morillo et al. 2010). In some instances, cell death affecting differentiating RGCs is rapid enough to be visualized even during late anaphase. For instance, Fig. 22.4 illustrates an example of an anaphase occurring in a partially pyknotic $p75^{NTR}$-positive neuron.

We have provided evidence that BDNF prevents cyclin B1 expression and mitosis in differentiating retinal neurons (Frade 2000), suggesting that the anti-apoptotic effect of this neurotrophin in the retina (Frade et al. 1997) relies on its effects on the cell cycle (see Fig. 22.2). In the developing mouse retina, the BDNF receptor TrkB is expressed by layered RGCs and basally located neuron progenitor cells, but not by other neuroepithelial cells (de Melo et al. 2008), an observation consistent with the effect of BDNF in late differentiating chick RGCs as they approach to the GCL. Blocking endogenous BDNF in chick retinal explants with TrkB receptor bodies resulted in an increased proportion of differentiating neurons showing mitotic figures (Morillo et al. 2010). Therefore, endogenous BDNF seems to prevent G2/M transition followed by apoptosis in tetraploid neurons, thus maintaining the proportion of these latter cells in the developing retina. These observations are consistent with our current understanding about how cell cycle machinery

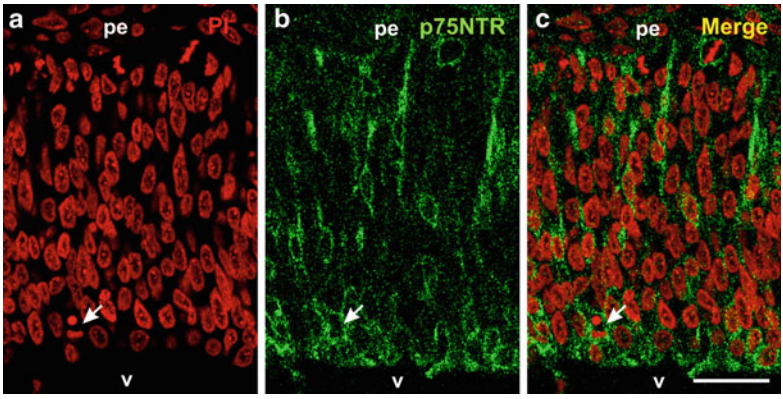

Fig. 22.4 Cell death takes place soon after mitosis in tetraploid RGCs. E5 chick embryos were fixed, processed for immunohistochemistry with the $p75^{NTR}$-specific antibody [9992] (kindly provided by Moses V. Chao, New York University), and their DNA was labeled with propidium iodide (PI), as previously described (Morillo et al. 2010). An example of a $p75^{NTR}$-positive cell in anaphase containing a partial pyknosis is shown (*arrow*). *Red*: propidium iodide; *green*: $p75^{NTR}$-specific labeling; *right panel*: merged image. *Bar*: 30 μm

is modified to convert a mitotic cycle to an endocycle (Sigrist and Lehner 1997; Shcherbata et al. 2004). In this regard, endoreduplication is contingent on the downregulation of the levels (and/or the activity) of the mitotic cyclin-dependent kinase (CDK)1. In *Drosophila*, removing the mitotic cyclins or the mitotic kinase CDK1 induces cells programmed to be in the mitotic cycle to undergo a self-sustaining endocycle (Hayashi 1996; Weigmann et al. 1997). In plants, inhibition of the mitotic CDK1/cyclin complex is also required for endoreduplication (Grafi and Larkins 1995). While evidence exists that BDNF prevents G2/M transition in tetraploid RGCs, the mechanisms involved in this regulation remain so far unknown.

22.3.2 Avoidance of Multiple S-Phase Reentries in Tetraploid Neurons

In several instances, endoreduplication results in multiple rounds of DNA synthesis, giving rise to cells with high levels of ploidy (Edgar and Orr-Weaver 2001; Ullah et al. 2009). Maybe the most dramatic example so far documented in animals is the mollusk *Aplysia californica*, whose giant neurons have been shown to contain DNA amounts ranging from 2,000C to 200,000C (Lasek and Dower 1971). By contrast, tetraploidization of RGCs can be considered as a particular case of endoreduplication in which these neurons undergo a single round of DNA synthesis, thus remaining in a G2-like state with a 4C DNA content. Therefore, a mechanism impeding further rounds of DNA replication must exist in tetraploid RGCs, thus allowing these neurons to be maintained with a 4C DNA content.

Before thinking of possible mechanisms preventing hyperploidization of vertebrate tetraploid neurons, it is important to first analyze what is known about regulation of S-phase entry in normal eukaryotic cells (see Fisher 2011). DNA replication in eukaryotes is tightly controlled by posttranslational mechanisms to assure that only a single round of DNA replication is allowed in each mitotic cycle. In this way, S-phase entry requires a previous drop of CDK1 activity during early G1, which leads to the assembly onto DNA replication origins of prereplication complexes (preRCs) containing several proteins, including origin replication complex (ORC) and minichromosome maintenance (MCM) proteins, Cdc6, and Cdt1/Dup1 (Donaldson and Blow 1999). These preRCs remain DNA-bound during G1, but once that CDK2 becomes activated by Cyclin E at the G1/S transition, it fires preRCs by phosphorylation of yet unidentified components therein (Duronio et al. 1998). This then leads to removal of Cdc6 and the MCM proteins from their interaction with the DNA, followed by DNA replication. It is only after levels of CDK1 activity drop during M/G1 transition that assembly of preRCs takes place again, thus assuring only one round of DNA replication per cycle takes place.

DNA replication in endocycling cells also requires the completion of the previous S-phase, and a mitosis-independent drop of CDK1 activity prior to a new round

of DNA synthesis takes place (Follette et al. 1998; Weiss et al. 1998; Edgar and Orr-Weaver 2001; Lilly and Duronio 2005). The machinery involved in reassembly of preRCs onto DNA replication origins might therefore be inhibited in tetraploid RGCs, thus allowing these cells to remain as tetraploid neurons. Fluctuations in cyclin E levels are also known to be necessary for multiple rounds of endocycle S-phase in *Drosophila* (Follette et al. 1998), suggesting that periodic CDK2 activation is also crucial for hyperploidization. E2F1 has been shown to be crucial for cyclin E transcription and endoreduplication (Royzman et al. 1997). Therefore, absence of E2F-1 activity in layered RGCs might also underlie the mechanism preventing several rounds of DNA synthesis in tetraploid RGCs.

22.4 Somatic Tetraploidy: A Physiological View

Endoreduplication is normally associated with cellular hypertrophy (Edgar and Orr-Weaver 2001; Ullah et al. 2009). In the nervous system, endoreduplication-associated hypertrophy has been elegantly demonstrated in studies comparing neurons from tetraploid strains of *Xenopus laevis* with analogous neurons in comparably sized diploid frogs. These studies concluded that both somal diameters and total length of the dendritic arbors increased significantly in tetraploid neurons when compared with the diploid counterparts (Szaro and Tompkins 1987). Accordingly, we have observed that somatic tetraploidy correlates with the presence of large somas and extensive dendritic trees in RGCs (Morillo et al. 2010), suggesting that somatic tetraploidization is associated with morphological and functional diversity among retinal projection neurons. This concept has been demonstrated in the chick retina. In this tissue, tetraploid neurons represent a subpopulation of RGCs that express the β2 nicotinic acetyl choline receptor subunit (β2AChR), a known marker of RGCs innervating lamina F in the stratum griseum et fibrosum superficiale of the tectal cortex (Yamagata and Sanes 1995). Tetraploid RGCs in the chick show features of primate parasol cells; a population of RGCs equivalent to α-Y cells in the cat (Crook et al. 2008) with large somas and wide receptive fields, which are involved in motion processing. Parasol cells make up about 10% of the RGCs, they establish contacts with cholinergic amacrine cells, and they project to specific layers of the lateral geniculate nucleus, the major retinorecipient tissue in mammals (Callaway 2005; Jacoby et al. 1996). These large neurons are involved in motion processing and show a specific distribution pattern along the GCL. Therefore, adjusting the numbers of the large versus small RGCs may be crucial to assure the proper functioning of the retina. In this regard, the density of large RGCs is known to be increased in the peripheral retina of the chick (Naito and Chen 2004), while early apoptosis is known to be high in the center of this tissue during chick development (Frade et al. 1997). Early cell death in the central retina may therefore be required to reduce the number of tetraploid RGCs and increase the ratio between small and large RGCs in this region. Variable levels of BDNF expression along the retinal tissue may regulate the degree of apoptosis in large RGCs. TGFβ might also

participate in the removal of tetraploid RGCs as it has been shown to cooperate with NGF in triggering retinal apoptosis (Dünker et al. 2001), mainly acting on large RGCs (Beier et al. 2006). Adjustment of the ratio between tetraploid and diploid neurons could also explain early cell death in other regions of the developing nervous system, a phenomenon known for decades but, so far, of unclear physiological significance (Yeo and Gautier 2004).

22.5 Somatic Tetraploidy in Neurons and Neurodegeneration: A Pathophysiological View

Neurodegeneration, including Alzheimer's disease (AD), is often associated with neuronal cell cycle reentry (see Moh et al. 2011). Nevertheless, mitotic figures are hardly observed in the affected neurons (Bowser and Smith 2002). This suggests that, like the tetraploid RGCs generated at early developmental stages, most neurons reentering the cell cycle under pathological situations could remain for long time as tetraploid cells in the affected brain, a view consistent with the chronic course of neurodegenerative diseases. In the AD brain, the presence of tetraploid neurons has been described by several laboratories. Karl Herrup in 2001 provided evidence that a significant fraction of the hippocampal pyramidal and basal forebrain neurons in AD have fully replicated four separate genetic loci on three different chromosomes (Yang et al. 2001), an observation recently confirmed in the frontal cortex of both control and AD-affected brains (Iourov et al. 2009). Thomas Arendt has also provided compelling evidence for the presence of tetraploid neurons in both normal and AD cortex (Mosch et al. 2007). Using three independent methods for DNA quantification, this laboratory has demonstrated that the cortex of AD brain contains a population of cyclin B1-positive neurons showing tetraploid nuclei, representing approximately 2% of all neurons (Mosch et al. 2007). Importantly, these authors have recently shown that neurons increasing their DNA content are those that selectively die at late stages of AD (Arendt et al. 2010). As previously discussed (see Sect. 22.4), neuronal tetraploidization correlates with increased cell size. This suggests that neurodegeneration-associated tetraploidization likely results in neuronal hypertrophy. Functional changes in the affected neurons, derived from the morphological alterations, may therefore participate in the course of disease (for details, see Frade and López-Sánchez 2010).

$p75^{NTR}$ has several links with AD and other neurodegenerative diseases (Dechant and Barde 2002; Diarra et al. 2009; Coulson et al. 2009), and an endogenous source of NGF (proNGF) has been shown to be accumulated in the cortex and hippocampus of AD brain (Fahnestock et al. 2001). Therefore, AD-associated cell cycle reentry and neuronal tetraploidization may result from the activation of $p75^{NTR}$ by proNGF in the affected brain, as occurs during development. The known decline of BDNF/TrkB expression in AD (Ferrer et al. 1999; Hock et al. 2000) could facilitate G2/M transition in tetraploid neurons, thus favoring their

death by apoptosis and further degeneration of the affected brain (for further details, see Frade and López-Sánchez 2010).

22.6 Concluding Remarks

Somatic tetraploidy in neurons seems to be a general phenomenon affecting several regions of the vertebrate nervous system. Evidence indicates that somatic tetraploidy arises by endoreduplication taking place in differentiating neurons soon after they come out from the cell cycle, as they migrate from the ventricular surface of the neuroepithelium to their final destination in the adult nervous system. These cells undergo one round of DNA replication and then they remain in a G2-like status with a 4C DNA content in their nuclei. Activation of $p75^{NTR}$ by NGF is responsible for endoreduplication in the developing chick retina, but its widespread expression as an early marker of neurogenesis and the presence of BrdU incorporation in $p75^{NTR}$-positive neurons suggest that this neurotrophin receptor represents a general inducer of neuronal tetraploidy in the vertebrate nervous system. The interaction of $p75^{NTR}$ with NGF is able to activate the transcription factor E2F1 in retinal neuroblasts lacking Rb expression, thus explaining the induction of DNA transcription in these cells. In addition, other mechanisms must exist to prevent both G2/M transition and further rounds of S-phase in neurons that undergo somatic tetraploidization. In this regard, evidence indicates that BDNF is necessary for the avoidance of tetraploid neurons to undergo G2/M transition, but the molecular mechanism regulating this process remains unknown. In the absence of BDNF, tetraploid neurons undergo apoptosis soon after trespassing the G2/M check point. Therefore, neurogenesis-associated cell death seems to be linked to the endoreduplicative process. This process may be important for the adjustment of neuronal phenotypes in the nervous system. Indeed, somatic tetraploidy seems to participate in the creation of phenotypic diversity during nervous system development. This is clearly the case in the chick retina since tetraploid neurons represent a subpopulation of large RGCs that innervate a specific layer in the target tissue. Therefore, these neurons seem to be not only morphologically but also functionally different from diploid RGCs. Further research will be important to determine whether somatic tetraploidization may account for the creation of large neurons innervating specific areas and having specific functions in other regions of the nervous system. Somatic tetraploidy in neurons may also participate in pathology. Indeed, neurodegeneration is clearly associated with cell cycle reentry, and neuronal tetraploidy has been observed in the AD brain. It will be crucial to test whether somatic tetraploidy in neurons is important for the occurrence of these pathologies.

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Chapter 23
Cell Cycle Deregulation in the Neurons of Alzheimer's Disease

Calvin Moh, Jacek Z. Kubiak, Vladan P. Bajic, Xiongwei Zhu, Mark A. Smith, and Hyoung-gon Lee

Abstract The cell cycle consists of four main phases: G_1, S, G_2, and M. Most cells undergo these cycles up to 40–60 times in their life. However, neurons remain in a nondividing, nonreplicating phase, G_0. Neurons initiate but do not complete cell division, eventually entering apoptosis. Research has suggested that like cancer, Alzheimer's disease (AD) involves dysfunction in neuronal cell cycle reentry, leading to the development of the two-hit hypothesis of AD. The first hit is abnormal cell cycle reentry, which typically results in neuronal apoptosis and prevention of AD. However, with the second hit of chronic oxidative damage preventing apoptosis, neurons gain "immortality" analogous to tumor cells. Once both of these hits are activated, AD can develop and produce senile plaques and neurofibrillary tangles throughout brain tissue. In this review, we propose a mechanism for neuronal cell cycle reentry and the development of AD.

23.1 Introduction

The cell cycle consists of four main phases that are necessary for division and replication: G_1, S, G_2, and M. Most cells undergo these cycles up to 40–60 times in their life. Neuronal precursors in developing brain and in the whole nervous system proliferate and undergo regular cell cycles and divisions (Kubiak and Smith 2010). Thereafter, in adults, this process ceases and the vast majority of neurons, save

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neuronal progenitor cells, never replicate. As such, most neurons are terminally differentiated, meaning that they remain in a nondividing, nonreplicating phase, G_0, for most of their lives. If such neurons enter the cell cycle, recent evidence has shown that these neurons can initiate, but cannot complete, cell division and in consequence eventually enter an apoptotic-type neurodegeneration (Raina et al. 2001; Lee et al. 2009). Because of these properties, neurons are vulnerable to destructive neuropathies, such as Alzheimer's disease (AD).

AD is a condition characterized by the destruction of neurons with two hallmarks: the senile plaque and the neurofibrillary tangle. Senile plaques are aggregations of amyloid-β (Aβ) protein that localize extracellularly (reviewed in Castellani et al. 2010). Senile plaques originate when the amyloid-β protein precursor (AβPP) is cleaved by α- and γ-secretases, resulting in Aβ aggregation and deposition in the brain (Korenberg et al. 1989). Currently, there is a debate about the role of amyloid aggregation and deposition. Some researchers suggest that Aβ is a toxic protein aggregate that causes the destruction of neurons (Robakis 2010), whereas others argue that Aβ has a protective effect, shielding neurons from oxidative damage (Moreira et al. 2008; Castellani et al. 2009). There may be some truth in both of these arguments such that while Aβ has known antioxidative properties (Hayashi et al. 2007; Nakamura et al. 2007), the large aggregates observed in AD would hinder such properties and lead to neuronal dysfunction and death (Zhu et al. 2007).

Neurofibrillary tangles, the other hallmarks of AD, are collections of protein found within neurons. They consist of hyperphosphorylated tau protein, which is typically associated with microtubules. Tau protein stabilizes microtubules and, at least in vitro, disassociates from microtubules when phosphorylated (Conde and Caceres 2009). As with Aβ, there is considerable debate over the role of tau phosphorylation in disease pathogenesis with some arguing that hyperphosphorylation of tau protein leads to microtubule destabilization and neuronal dysfunction (Iqbal et al. 1984; Grundke-Iqbal et al. 1986), whereas other investigators posit tau phosphorylation as the protective adaptation of neurons during stress (Smith et al. 2002; Lee et al. 2005).

In AD, senile plaques and neurofibrillary tangles are widespread throughout brain tissue and mirror other pathological changes. For example, in the past decade, research has shown that neuronal cell cycle reentry plays a fundamental role in the pathogenesis of AD. As such, AD can be considered as a disease of deregulation of cell cycle in neurons. Such an idea provided novel insights for the treatment of AD. However, before effective interventions can be implemented, a better understanding of cell cycle reentry involvement in AD must be achieved.

23.2 The Cell Cycle

To progress through the cell cycle, cells use proteins called cyclins and cyclin-dependent kinases (Cdks). In each cell stage, one set of cyclins are expressed while others are downregulated through controlled proteolysis (Udvardy 1996). To advance to the next stage, the current stage cyclins are downregulated, so that the

next stage cyclins can be upregulated. Cyclin metabolism is largely dependent on the ubiquitin–proteasome pathway responsible for precisely regulated proteolysis. Anaphase-promoting complex/cyclosome is the major ubiquitin ligase involved in cell cycle regulation via cyclins recognition and targeting for destruction.

23.3 Alzheimer's Disease and the Cell Cycle Reentrant Neuron

Through this inducible progression, neurons will occasionally reenter the cell cycle from G_0 to G_1. Although this transition is regulated by the same cyclins/Cdks as normal mitosis, there are some key differences. In AD neurons, there are significantly elevated levels of cyclin D, Cdk4, and Ki67 (McShea et al. 1997; Zhu et al. 2007). The abundance of these markers signifies progression through the G_1 phase and exit from G_0. Interestingly, these markers are found in the cytoplasm of AD neurons rather than in the nucleus, their typical site of action (Vincent et al. 1997). Also, M-phase markers are found in AD neurons: increased MPM2 phosphoepitopes, Cdc25 A and B phosphatases, and binucleation, which may result of abortive mitotic karyokinesis (Vincent et al. 1998, 2001; Ding et al. 2000; Spremo-Potparevic et al. 2008; Zhu et al. 2008; Bajic et al. 2009). The ubiquitination system is also altered in AD (including ubiquitin-1 mutations; Tan et al. 2007; Tank and True 2009), which may influence both neuronal cell cycle regulation (Kubiak and Smith 2010) and, protein aggregation and accumulation (Haapasalo et al. 2010). Moreover, as some ubiquitin ligases, e.g., BRCA1, are overexpressed in AD neurons, the ubiquitination substrates, and thus, ubiquitination-dependent signaling, are most likely highly altered in AD (Evans et al. 2007).

Hernandez-Ortega et al. (2007) found that a hippocampal excitotoxic lesion would upregulate cell cycle markers in a progressive fashion in the entorhinal complex and dentate gyrus. They measured the levels of cyclin D1 and Cdk6 (G_0/G_1 transition), PCNA (late G_1/early S transition), Cdk2 (G_2/S transition), and cyclin B (G_2 phase) and found that these markers elevate and decline in a sequential fashion in response to an AD-like stimulus. Levels of cyclin D1 and Cdk6 increased 1 day after kainic acid injection and remained elevated until day 15. PCNA rose for the first 7 days and then diminished in correlation with the rise of cyclin B. Cdk2 was detected over the first 15 days and then declined until day 30 (Hernandez-Ortega et al. 2007). Taking the progressive increase of cell cycle markers into account, these changes are representative of intentional reentry into the cell cycle rather than incidental increases in marker levels. The neurons seem to reenter the cell cycle with the intent of apoptosing or replicating (Fig. 23.1).

Further evidence for reentry into the cell cycle in AD neurons was demonstrated by Lopes et al. (2009). This study found that the pathway of Cdk5, a serine-threonine kinase involved in axonal guidance, cortical layering, and synaptic structure/plasticity, was overactivated and relocalized in AD and prion-induced

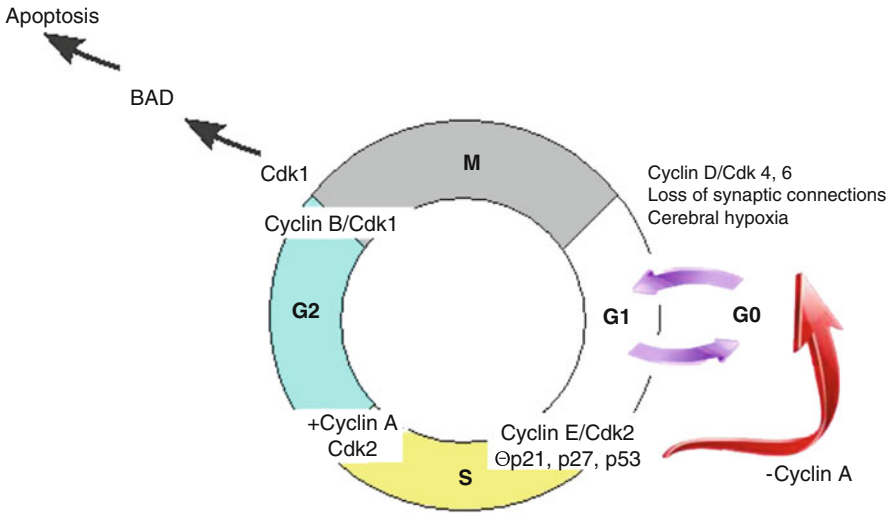

Fig. 23.1 Neurons subject to loss of connections or other stressors exit G_0 and reenter into the cell cycle that is abortive and leads to cell death

pathologies. In cultured cortical neurons, levels of Cdk4, a downstream effector of Cdk5 and cell cycle marker of the G_0/G_1 transition, increased 13% by Aβ injection. Although the Cdk4 coactivator, cyclin D1, was not upregulated, it condensed into a nuclear/perinuclear pattern in response to Aβ. Levels of PCNA, a marker of S phase, increased 24% in response to Aβ. The number of apoptotic cells also increased by threefold in this study in response to Aβ. In contrast to findings in AD (Ogawa et al. 2003), levels of phosphorylated histone H3 (phH3), a marker of M phase, did not change, suggesting that these neurons did not undergo the G_2/M transition. To prove that these changes in cell cycle markers were related, Lopes et al. (2009) treated the cells with roscovitine, a Cdk5 blocker. The increases in each case were inhibited with this treatment, suggesting that they were all mediated through the Cdk5 pathway. This study demonstrated that in response to Aβ, neurons will reenter and transit through the cell cycle up until M phase, and that this process is mediated by Cdk5 and its downstream cell cycle effectors.

Another interesting characteristic that separates the neuron from normal mitotic cells is the highly polarized state of its cytoskeleton (Nguyen et al. 2002). Because the neuron is constantly creating new synapses, neuronal microtubules are often in a state of flux, resulting in high levels of tau phosphorylation. This increased tau phosphorylation could cause problems for the neuron when it reenters the cell cycle, since mitosis requires microtubule remodeling for spindle assembly and transformation of its cytoskeleton (Conde and Caceres 2009). Under the circumstances of cell cycle reentry of this highly specialized neuron, mitotic-like hyperphosphorylation of tau could occur, producing neurofibrillary tangles and a disorganized mass of microtubule subunits (Bonda et al. 2010).

As a consequence, hyperphosphorylation of tau could undermine proper microtubule reorganization of cell replication and result in premature centromere division (PCD). PCD is a phenomenon where the centromeres prematurely divide in the G_2 phase of the cell cycle, immediately after DNA replication in the S phase. Spremo-Potparevic et al. (2008) found that there was a three times higher incidence of PCD in the frontal lobe cortex of AD specimens than that of controls. This study suggests that since neurons underwent PCD, they must have reentered the cell cycle. Induction signals for PCD in these cases included loss of synaptic connections, cerebral hypoxia, Aβ, hormonal factors (estrogen), and mutations in presenilin 1 (Spremo-Potparevic et al. 2008). Upon review, these induction factors are associated with chromosomal damage and abortive mitogenesis, suggesting that premature division is the first step in neuronal apoptosis or dedifferentiation.

While there are differences between neuronal and normal mitotic cell cycle reentry, neuronal cell cycle reentry in control cases is in no way identical to neuronal reentry in AD. In 2001, Raina et al. discovered that AD does not activate the full set of caspases required for neuronal apoptosis. Instead, upstream caspases (caspase 8 and 9) were upregulated in AD, whereas downstream caspases (caspase 3, 6, and 7) stayed at control levels (Raina et al. 2001). This study suggests that AD neurons lacked effective apoptotic signal propagation to downstream caspase effectors, resulting in abortosis, a phenomenon consisting of apoptotic avoidance and neuronal survival (Raina et al. 2000, 2001).

To appreciate this unique process in AD, it is important to consider the differences between diseased and healthy neurons. For one, oxidative damage is highly associated with AD neurons and not with healthy neurons (Smith 2006). In 1998, Hampton et al. found that chronic oxidative stress inhibits the downstream propagation of caspase-mediated apoptotic signals (Hampton et al. 1998). If chronic oxidative stress induced apoptotic avoidance, or abortosis, in AD neurons, then this unique process would explain neuronal survival in the disease. Furthermore, recent evidence has shown Aβ to have antioxidant properties (Hayashi et al. 2007; Nakamura et al. 2007; Moreira et al. 2008). In response to the accumulation of oxidative damage in these resilient AD neurons, α- and γ-secretases could be induced to produce more Aβ for neutralization of future free radicals (Tamagno et al. 2002, 2005; Kim and Shen 2008).

23.4 Cell Cycle-Related Pathology of Alzheimer's Disease

From the activation of cell cycle reentry induced by Aβ to the disruption of the microtubule network due to hyperphosphorylated tau protein, the cell cycle is intertwined with the pathology of AD. An important protein that initiates the pathology of AD is Aβ, which has numerous reported effects in the cell, ranging from the induction of apoptosis, promotion, or attenuation of cell survival, the activation of mitogen-activated protein kinase (MAPK), the promotion of tau phosphorylation, increases in oxidative stress and synapse loss, and the production of more Aβ.

One of the main pathways that mediate the effects of Aβ is the activation of the nerve growth factor (NGF) receptor, specifically the p75NTR isoform (Sakono and Zako 2010). Once activated, the NGF receptor can result in either a cell survival cascade or phosphorylation of JNK, a MAPK that is responsive to the accumulation of oxidative stress in the cell. Phosphorylated JNK negatively inhibits Bcl2, an antiapoptotic protein, resulting in upregulation of caspase 3 and apoptosis (Zhu et al. 2004b). What determines the path that the NGF receptor chooses must still be elucidated. Perhaps its choice for cell survival rather than apoptosis is a reflection of the circumstances that lead to AD. Other stimuli for the activation of JNK include macrophages and reactive oxygen species (ROS), which are both products of inflammation and another Aβ pathway (Zhu et al. 2001, 2003; Thakur et al. 2007).

Aβ can also bind to the *N*-methyl D-aspartate (NMDA) receptor. Activation of this receptor results in abnormal Ca^{2+} homeostasis, perhaps mediated through dysregulation of Ca^{2+} ion channels and upregulated Ca^{2+} influx (Sakono and Zako 2010). Increased levels of Ca^{2+} can be disastrous for a cell, leading to increased oxidative stress, synapse loss, and activation of lipases and proteases that will destroy the cell and lead to apoptosis.

Another pathway of Aβ is the Frizzled (Fz) receptor. When Aβ is bound to Fz, Wnt signaling is inhibited. Wnt inhibits GSK-3β, which inhibits β-catenin. The net result is an upregulation of β-catenin and an increase in tau phosphorylation (Sakono and Zako 2010). The effects of Aβ are mediated through several pathways, which can determine the pathology of AD and the fate of the cell. To better understand this relationship, we postulate a mechanism for neuronal cell cycle reentry in neurons and how this might lead to AD.

In the normal neuron, a mitotic stimulus (Aβ, estrogen, FGF, BMP, TGF-β, etc.) induces the cell to undergo cell cycle reentry. Because of its highly polarized cytoskeleton and the high activity of tau phosphorylation due to synapse creation, cell cycle reentry hyperphosphorylates tau and creates neurofibrillary tangles. These tangles aggregate with one another, disrupting the microtubule network and scattering microtubule-associated proteins across the cell. Despite this chaotic process, the neuron tends to reorganize its microtubule network in preparation for mitosis, but instead ends up in a cell crisis state. As a consequence of the disrupted microtubules, PCD is initiated, which activates the G_2/M checkpoint. Activation of this checkpoint prevents the cell from progressing into mitosis and prolongs Cdk1 activity. Cdk1 is an interesting protein because it can act as a proapoptotic factor by phosphorylating Bcl2, an antiapoptotic protein, in addition to its role as a prerequisite of mitosis (Spremo-Potparevic et al. 2008). Thus, apoptosis is initiated, cellular proteins are degraded in a regulated fashion, and the cell dies before mitosis occurs (Fig. 23.2). This mechanism corresponds with findings showing that Cdk1 is expressed at higher levels in AD and localizes to the glia and neurofibrillary tangles (Vincent et al. 1997). Cdk1 promotes mitosis when localized in the nucleus and induces apoptosis in the cytoplasm.

In the AD neuron, a mitotic stimulus (Aβ, estrogen, FGF, BMP, TGF-β, etc.) induces the cell to undergo reentry. Again, the high activity of tau phosphorylation in the neuron combines with the reorganization of the microtubule network to

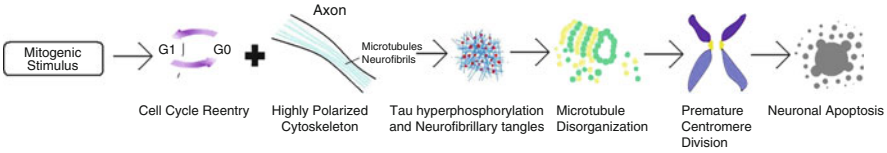

Fig. 23.2 Cell cycle reentry in a normal neuron leads only to death

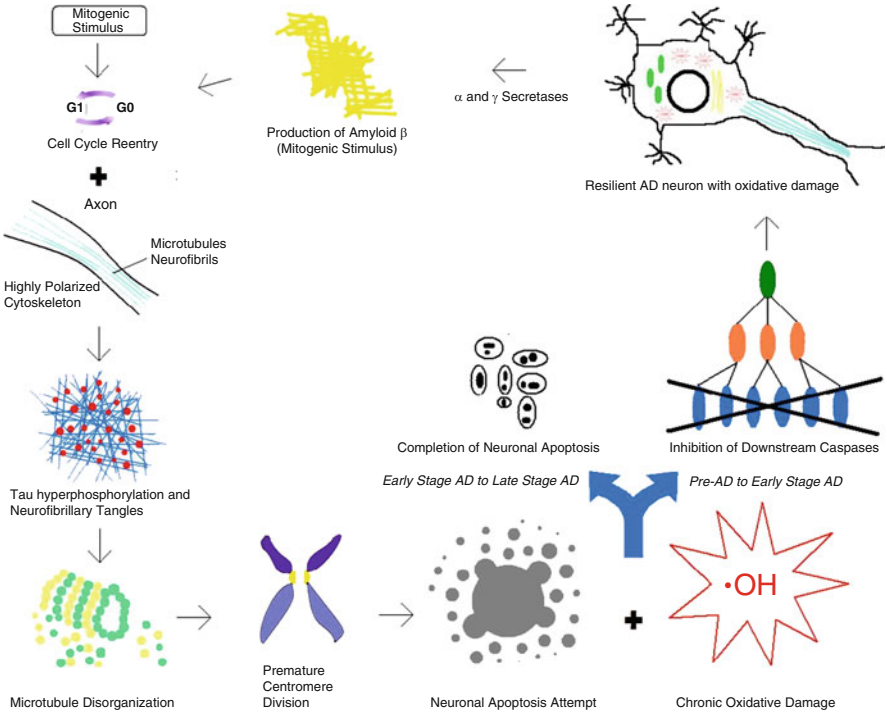

Fig. 23.3 Cell cycle reentry in AD leads to a host of downstream sequelae including oxidative stress and death

hyperphosphorylate tau protein. These proteins then aggregate with one another to create neurofibrillary tangles, disrupting the microtubule network. This disruption then initiates PCD and attempts to undergo apoptosis. However, the AD neuron contains significant amounts of oxidative damage-inducing activities, preventing the downstream caspases of apoptosis from immediately destroying the cell. Because of the neuronal accumulated oxidative damage, Aβ is upregulated to prevent future free radicals from further damaging the cell. Aβ can then begin a new cycle of reentry in other AD neurons. Eventually, after a few cycles of this process, the AD neuron is successful in its attempt to apoptose and dies (Fig. 23.3).

23.5 Cell Cycle Dysregulation Commonalities for Alzheimer's Disease and Cancer

Despite having different pathological results, cancer and AD do share similar etiologies. In cancer, abnormal cell cycle reentry instigates the uncontrolled proliferation and apoptotic avoidance resulting in tumor development and malignancy. In AD, abnormal reentry into the cell cycle initiates the pathway resulting in neurofibrillary tangles, apoptotic avoidance, and Aβ production. In 2000, Raina et al. described how even cell cycle control elements (Cdk4, p16, and p21) behave as oncoproteins in AD neurons (Raina et al. 2000).

In addition to cell cycle reentry, AD and cancer both require apoptotic avoidance to progress to a disease state. In AD, apoptotic avoidance allows the neuron to arrest in G_2, accumulating oxidative damage and Aβ production. Oxidative damage may accumulate from the excessive amounts of mitochondria replicated in S phase (Sousa et al. 1997). In cancer, apoptotic avoidance is clearly necessary for the oncogenic cells to persist and proliferate indefinitely.

Since cancer and AD share similar etiologies, it is important to consider what result in their different conditions. On the most superficial level, AD is mediated through changes in the level of certain proteins, such as cell cycle regulators, Aβ, and regulators of tau phosphorylation, whereas cancer is mainly mediated through genetic mutations. However, this distinction becomes confusing because cell cycle reentry is involved in both processes. In AD, dysfunctional cell cycle reentry results in apoptosis/abortosis and delayed neuronal apoptosis. In cancer, dysfunctional cell cycle reentry leads to cell survival and the development of an immortal cell population.

Kim et al. suggests that these differences can be explained through the pathways of MAPK associated with each disease (Kim and Choi 2010). MAPKs are signaling cascades that involve a MAPK1, MAPK2, and MAPK3. MAPK3 phosphorylates MAPK2, which phosphorylates MAPK1, which then phosphorylates downstream effectors. AD is mostly associated with the MAPK1s, p38, and JNK. Oxidative stress activates the MAPK3, ASK1, which can then phosphorylate either MKK4/7 or MKK6. MKK4/7 phosphorylates JNK, which then activates caspase 3 and initiates apoptosis. MKK6 activates p38, which then leads to tau hyperphosphorylation.

Cancer is more associated with the MAPK pathway involving ERK 1/2. This pathway is mediated through activation of a GTPase, ras, which then goes downstream to activate K-ras, MEK, and then ERK. MEK 1/2 and phosphorylated ERK upregulate matrix metalloproteinases (MMP) and protect cancer cells. MMPs are critical for cancer progression because they degrade the extracellular matrix to allow for cancer cell migration. ERK 1/2 also downregulates proapoptotic BIM and upregulates antiapoptotic MCL-1 by phosphorylating FOXO3a and MCL-1. Phosphorylation of FOXO3a degrades the transcription factor, which is necessary for the production of BIM. Phosphorylation of MCL-1 stabilizes the protein (Kim and Choi 2010). The net effect of ERK is to inhibit apoptosis and promote cancer cell survival. Because AD and cancer use different MAPK pathways, the final results of

their pathologies are different. In addition, ERK 1/2 has a negative feedback on β-secretase, an enzyme that cleaves AβPP to produce Aβ, whereas JNK and p38 have a positive feedback on the enzyme (Tamagno et al. 2005, 2008). This difference in MAPK regulation explains the excessive production of Aβ in AD and its absence in cancer.

These events in cancer have led to the establishment of the two-hit hypothesis (Knudson 1971). This theory suggests that there are two requirements for a cell to become cancerous. The first requirement is that the cell must have an upregulating mutation in an oncogene or a gene that promotes proliferation of the cell. An activated oncogene would result in abnormal cell cycle reentry and unlimited replication. The second requirement is that the cell must have an inactivating mutation in a tumor suppressor gene or a gene that inhibits cell proliferation. A tumor suppressor gene could produce a protein that promotes cell cycle arrest or induces apoptosis.

From the two-hit hypothesis of cancer, Zhu et al. (2004a, 2007) proposed the two-hit hypothesis of AD. This theory states that oxidative damage and cell cycle reentry are both necessary for a healthy neuron to become an AD neuron. The first hit in this theory also originates from abnormal cell cycle reentry. The process of AD is initiated when a mitogenic stimulus pushes the neuron to reenter the cell cycle. Typically, neurons that experience this "hit" undergo apoptosis and never progress to a disease state. However, when a neuron accumulates the second hit of chronic oxidative damage, apoptosis can be avoided, resulting in an "unlimited proliferation" of mitochondrial free radical production and Aβ deposition.

23.6 Conclusion

The dysregulation of cell cycle control is an integral part of both AD and cancer. Abnormal cell cycle reentry in a normal neuron leads to apoptosis. In aged subjects with AD, on the other hand, abnormal reentry triggers a cycle of oxidative damage and mitogen production with neurofibrillary tangles and Aβ deposition as a result of the condition. Cell cycle reentry is also a requirement of carcinogenesis, during which initiated cells must undergo dysregulation of the cell cycle to proliferate indefinitely and create tumors.

The similarities in these disease processes have led to the development of the two-hit hypothesis of AD. AD neurons must undergo the first hit of abnormal cell cycle reentry to develop the condition. With this event, neurons typically die, preventing the development of AD. However, with the second hit of chronic oxidative damage preventing apoptosis of the cell, neurons gain "immortality" analogous to tumor cells. Once both of these hits are activated, AD can develop and produce the pathophysiology commonly seen in this condition. Most cancers, as well as AD, are age-related diseases reflecting problems arising at the end of the human developmental process. Thus, the cell cycle control seems to escape fine

regulation at those final steps, resulting in pathologies abbreviating our lives. It remains unclear how far this slippage is imprinted to our developmental program.

Abnormal cell cycle reentry raises the possibility of a new target for therapeutic intervention. Cell cycle inhibitors could be a possible solution to the progression of AD. In combination with current drug therapies for AD, millions of people could improve their AD and delay progression for a substantial number of years.

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