Chapter 4
Stem Cells and Asymmetric Cell Division

Frank Hirth

Abstract Asymmetric stem cell division is a fundamental process used to generate cellular diversity and to provide a source of new cells in developing and adult organisms. Asymmetric stem cell division leads to another stem cell via self-renewal, and a second cell type which can be either a differentiating progenitor or a postmitotic cell. Experimental studies in model organisms including the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster* and the laboratory mouse, *Mus musculus*, have identified interrelated mechanisms that regulate asymmetric stem cell division from polarity formation and mitotic spindle orientation to asymmetric segregation of cell fate determinants and growth control. These mechanisms are mediated by evolutionary conserved molecules including Aurora-A, aPKC, Mud/NuMa, Lgl, Numb and Brat/TRIM-NHL, which in turn regulate a binary switch between stem cell self-renewal and differentiation. The mechanistic insights into asymmetric cell division have enhanced our understanding of stem cell biology and are of major therapeutic interest for regenerative medicine as asymmetrically dividing stem cells provide a powerful source for targeted cell replacement and tissue regeneration.

Abbreviations

Ago1	Argonaute protein 1
AurA	Aurora-A
Baz	Bazooka
Brat	Brain tumor

F. Hirth, B.Sc., M.Sc., Ph.D., PD (✉)
Department of Neuroscience, MRC Centre for Neurodegeneration Research,
Institute of Psychiatry, King's College London, PO Box 37, De Crespigny Park,
Denmark Hill, London, SE5 8AF, UK
e-mail: Frank.Hirth@kcl.ac.uk

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Cdc2	Cell division cycle 2
Cdc42	Cell division cycle 42
Cdc25	Cell division cycle 25
Cdk	cyclin dependent kinase
Cnn	centrosomin
CNS	Central Nervous System
c-Myc	cellular myelocytomatosis oncogene
DaPKC	Drosophila atypical protein kinase C
Dctn1	dynactin
Dlg	Discs large
DmPar6	Drosophila melanogaster Partitioning defective 6
ESC	embryonic stem cell
ECT-2	epithelial cell transforming gene 2
Galphai	G-protein alpha, subunit i
GMC	Ganglion Mother Cell
GoLoco	G-protein 0, Locomotion defects domain
GDPase	guanosine diphosphatase
GTPase	guanosine triphosphatase
Insc	Inscuteable
Khc-73	Kinesin heavy chain 73
Lgl	Lethal (2) giant larvae
Mira	Miranda
Mud	Mushroom body defect
NB	Neuroblast
NHL	NCL-1, HT2A, and LIN-41 domain
NuMa	Nuclear Mitotic apparatus
PAR	partitioning defective
Par-3	partitioning defective 3
Par-6	partitioning defective 6
PDZ	Post synaptic density 95, Discs large, and Zonula occludens-1 domain
Pins	Partner of Inscuteable
Pon	Partner of Numb
Pros	Prospero
RNA	Ribonucleic Acid
Sqh	Spaghetti squash
TRIM 3	tripartite motif protein 3
TRIM 32	tripartite motif protein 32
VNC	Ventral Nerve Cord

4.1 Introduction

Stem cells are characterised by their potential to self-renew and to differentiate into every cell type of the organism. Stem cells are found in developing and adult tissue, starting with the totipotent zygote which subsequently leads to

pluripotent stem cells of the early embryo. Later during germ layer formation and organogenesis, stem cells become increasingly restricted in their lineage potential and give rise to progeny that contribute to mature tissue (Eckfeldt et al. 2005; Slack 2008; Murry and Keller 2008; Metallo et al. 2008; Mitalipov and Wolf 2009). Because of their origin and pluripotency, stem cells are of major therapeutic interest in regenerative medicine as they provide a powerful source for cell replacement and tissue regeneration. This is evident in cases of damage-, disease and age-related cellular degeneration, such as spinal cord injury (Nandoe Tewarie et al. 2009; Kim and de Vellis 2009) or age-related neurodegeneration seen for example in Alzheimer's and Parkinson's disease (Daniela et al. 2007; Li et al. 2008), where stem cell therapy may become one way of replacing lost cells (Rosser et al. 2007; Ormerod et al. 2008).

However, there are several obstacles that need to be resolved before stem cell based therapies can be translated clinically. These obstacles include the unlimited proliferation potential of stem cells as well as our incomplete knowledge about the molecular machinery underlying cellular differentiation programs. Thus, a major challenge is the identification of stem cell-derived molecular determinants inherited by differentiating progenitor cells that are required for the specification of the variety of different cell types in the adult organism. Successful cell replacement and tissue regeneration is only achieved once the new cells differentiate into the desired cell type and integrate into existing cell clusters, tissues and organs. This is particularly evident for the nervous system, where the majority of cells are post-mitotic and integrated into elaborate neural circuits underlying complex behaviour. For example, a major challenge will be to induce effective functional integration of stem cell-derived neurons into existing neural circuits with the ultimate goal to restore behavioural deficits caused by progressive neurodegeneration (Lindvall and Kokaia 2006; Ormerod et al. 2008).

Equally important is the need to understand how growth and proliferation of stem cells is regulated at the molecular level in order to regenerate tissue without unwanted over-proliferation that may lead to cancer formation, but also to control undesired growth that may jeopardize final tissue and organ size. Here, regenerative medicine faces two challenges at the same time. First, stem cell proliferation needs to be restricted to a certain number of mitotic divisions until a defined and limited amount of differentiating progeny is generated. Second, and at the same time, the size of each individual differentiating cell needs to be regulated in the context of its neighbours so that a cell cluster, tissue or organ reaches an appropriate final and functional size. It is obvious from these obstacles that a solid and comprehensive understanding of the molecular mechanisms underlying stem cell proliferation and differentiation are fundamental prerequisites for the successful application of stem cells in regenerative medicine.

The majority of our current understanding comes from studies investigating asymmetric stem cell division in model organisms such as the nematode *Caenorhabditis elegans* (*C. elegans*), the insect *Drosophila melanogaster* and the laboratory mouse, *Mus musculus*. These animals are seemingly very different to humans and the ancestors of worms and flies already separated from the vertebrate lineage more than 600 Ma ago during the course of evolution (Adoutte et al. 2000;

Peterson et al. 2004). However, worms, flies and mice share several key features relevant to human stem cell biology and tissue regeneration. Whole genome sequencing revealed striking similarities in the structural composition of individual genes of *Homo sapiens*, mouse, *Drosophila* and *C. elegans*. For example, the nucleotide sequence of the gene encoding actin is almost similar in all four species, providing compelling evidence for structural conservation due to common origin (homology). Moreover, the molecules and mechanisms underlying core modules of cell biology are conserved as well: homologous genes mediate homologous mechanisms such as cyclin/cdk modules regulating the eukaryotic cell cycle (Edgar and Lehner 1996; Bähler 2005; Sánchez and Dynlacht 2005), or insulin signalling regulating metazoan cell growth (Stocker and Hafen 2000; Hietakangas and Cohen 2009). These data provide compelling evidence for a deep homology underlying cell biological mechanisms. This notion is further supported by experiments demonstrating that *Drosophila* and human genes can substitute each other in species-specific but evolutionary conserved mechanisms underlying embryonic brain development in insects and mammals (Leuzinger et al. 1998; Nagao et al. 1998; Hanks et al. 1998).

These principles of homology seem to apply to stem cell biology as well. There is mounting evidence that the mechanisms underlying asymmetric stem cell division are conserved across species. Therefore, knowledge gained in model organisms is invaluable to enhance our understanding of stem cell biology for its successful application in regenerative medicine. Experimental studies in *C. elegans*, *Drosophila* and mice have identified molecules involved in cell-intrinsic and cell-extrinsic mechanisms underlying asymmetric stem cell division which are outlined in this article.

4.2 Classifications and Definitions

Stem cells are classified by the range of commitment options and thus their lineage potential available to them (Smith 2006). *Totipotent* stem cells are sufficient to form an entire organism, whereas *pluripotent* stem cells are able to form all the body's cell lineages, including germ cells; a typical example for the latter is an embryonic stem cell (ESC). *Multipotent* stem cells can form multiple lineages that constitute an entire tissue or tissues, such as hematopoietic stem cells, whereas *unipotent* and *oligopotent* stem cells are able to form one (uni-), two or more (oligo-) lineages within a tissue.

Stem cells can continuously produce daughter cells that are either similar resulting from *symmetric* stem cell division, or they generate different daughter cells by *asymmetric* stem cell division (Fig. 4.1a). Asymmetric division leads to two distinct daughter cells from a single mitosis, usually a self-renewing stem cell, and a progenitor cell that has the capacity to differentiate. *Self-renewal* is a defining property of stem cells and the term *commitment* characterises their exit from self-renewal leading to differentiation. Self-renewal and asymmetry can be established and maintained by intrinsic and extrinsic signals.

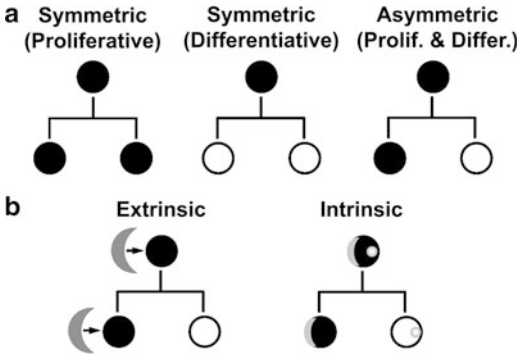

Fig. 4.1 Regulation of stem cell division. (**a**) Different modes of stem cell division. Stem cells (*filled circles*) show different modes of divisions, which can be either symmetric or asymmetric thereby regulating the number of stem cells and differentiating progeny (*open circles*) in developing and adult organisms. Symmetric, proliferative stem cell division expands the stem cell pool, whereas symmetric, differentiative stem cell division depletes the stem cell pool by generating differentiating progenitor and/or postmitotic cells. Asymmetric stem cell division can be regarded as a mixture of both proliferative and differentiative stem cell division, as it results in a self-renewing stem cell and a differentiating progenitor and/or postmitotic cells. Thus, asymmetric stem cell division maintains the stem cell pool while at the same time generates differentiating progeny. (**b**) Asymmetric stem cell division can be regulated by extrinsic or intrinsic mechanisms. Extrinsic regulation relies on asymmetric contact of the stem cell (*filled circles*) with a "niche" (*grey crescent* adjacent to stem cell) that provides support and stimuli necessary for self-renewal and to prevent differentiation. Following cell division, the cell adjacent to the niche remains a self-renewing stem cell, whereas the second daughter cell lacking contact to the niche is committed to differentiate. Intrinsic mechanisms regulate the exclusive segregation of cell fate determinants into daughter cells, with apical polarity cues (*grey crescent* within the stem cell) required for stem cell maintenance, and basal cell fate determinants (*grey circle*) required for terminal differentiation (Modified after Caussinus and Hirth 2007)

Extrinsic mechanisms are usually summarised by the term "*niche*" which characterises a cellular micro-environment that provides stimuli and support necessary to maintain self-renewal to the stem cell located adjacent to the niche (Fig. 4.1b). At the same time, the niche can generate asymmetry provided that the plane of cell division is parallel to the signals of the niche, resulting in only one daughter cell (the self-renewing stem cell) that retains contact to the niche (Fig. 4.1b). The sibling daughter cell does no longer receive niche signals, and hence can no longer maintain a self-renewing mode of division and is forced into cell cycle exit and differentiation (for review see, Li and Xie 2005; Roeder and Lorenz 2006; Martinez-Agosto et al. 2007; Mitsiadis et al. 2007; Morrison and Spradling 2008; Kuang et al. 2008; Losick et al. 2011).

Intrinsic signals refer to mechanisms and molecules acting within a dividing stem cell; they regulate the mode of division and hence the fate and commitment of its daughter cells (Fig. 4.1b). The majority of our knowledge about stem cells and asymmetric cell division come from insights into cell-intrinsic mechanisms which are outlined below.

4.3 Principles and Mechanisms

In general, four interrelated mechanisms underlie the molecular machinery regulating intrinsic asymmetric cell division: symmetry break, polarity formation, mitotic spindle orientation and segregation of cell fate determinants (see Fig. 4.2). Asymmetric stem cell division usually results in two cells that differ in fate but sometimes also in size: a self-renewing stem cell and a differentiating daughter cell.

4.3.1 Generating Asymmetry

Initially, stem cell symmetry is broken by signals from the niche or by an overlaying polarity inherited from the tissue of origin from where the stem cell derives, as in the case of epithelial cells (Fig. 4.2a). For example, in the *Drosophila* embryonic nervous system, a symmetry break already occurs in the polarised neuroectodermal epithelium from which neural stem cells, termed neuroblasts delaminate. In the *C. elegans* zygote, symmetry is broken by an actomyosin network present on the cell cortex of newly fertilized embryos. This actomysoin network drives surface contractions around the circumference which requires activity of the small GTPase Rho and its activator, the Rho guanine nucleotide exchange factor ECT-2 (for review, see Gönzy 2008). At the end of the first cell cycle the contractile cortex covers the anterior half of the embryo and the non-contractile cortex covers the posterior half, resulting in broken symmetry and initial polarity formation.

The activity of Rho GTPases is highly conserved and plays a key role in the initial steps of polarity formation in various tissues and cell types, including T-cells and epithelial cells of the lung, gut and skin (for review, see Iden and Collard 2008). Rho GTPases function as molecular switches and cycle between an active, GTP-bound state predominantly associated with membranes, and an inactive, GDP-bound state that is present in the cytoplasm. In all cases, Rho GTPases regulate and coordinate cytoskeleton remodelling, thereby providing a scaffold for symmetry break.

4.3.2 Polarity Formation

As soon as symmetry is broken, the emerging cell polarity becomes stabilized by evolutionary conserved *partitioning defective* (PAR) proteins and associated components (Fig. 4.2b). This is the case in *C. elegans* and *Drosophila*, but also for stem cells in other organisms including mammals (for review, see Schneider and Bowerman 2003; Cowan and Hyman 2004; Wodarz 2005; Suzuki and Ohno 2006; Goldstein and Macara 2007; Johnson 2009; Knoblich 2010).

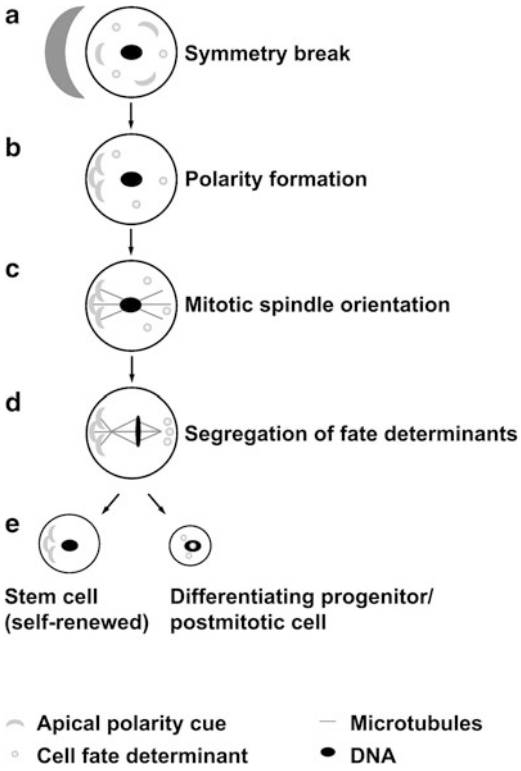

Fig. 4.2 Intrinsic asymmetric cell division. Four consecutive steps underlie intrinsic asymmetric cell division. (**a**) Symmetry break occurs in the parental stem cell either by external signals coming from the "niche" (*grey crescent* adjacent to stem cell) or from signals that have been inherited from the place of stem cell origin, such as epithelia. (**b**) Broken symmetry in turn is used to establish polarity formation, which usually involves reorganization of the actomyosin network and segregation of polarity cues along the new symmetry axis. (**c**) Subsequently, mitotic spindles are aligned perpendicular to the axis of polarity and the future cleavage plane. (**d**) Cell fate determinants are segregated along the axis of polarity and determine the fate of the future daughter cells. (**e**) Asymmetric stem cell division takes place along the axis of polarity, resulting in the unequal distribution of cell fate determinants; these in turn implement the fate of the two resulting daughter cells: a self-renewing stem cell and a differentiating progenitor/postmitotic cell (Modified after Gönzy 2008)

In *Drosophila* (see Fig. 4.3), delaminating neuroblasts inherit the PDZ domain proteins Par-3 (Bazooka) (Wodarz et al. 1999; Schober et al. 1999), Par-6 (DmPar6) (Petronczki and Knoblich 2001) and the *Drosophila* atypical protein kinase C (DaPKC) (Betschinger et al. 2003; Rolls et al. 2003; Izumi et al. 2004). Once the neuroblast has delaminated from the neuroectoderm, mitotic spindle align perpendicular to the epithelial plane (Kaltschmidt et al. 2000) and the adaptor protein Inscuteable (Insc) (Kraut et al. 1996) binds to the apical protein complex through Bazooka. Inscuteable, in turn, recruits another adaptor protein, Partner of Inscuteable

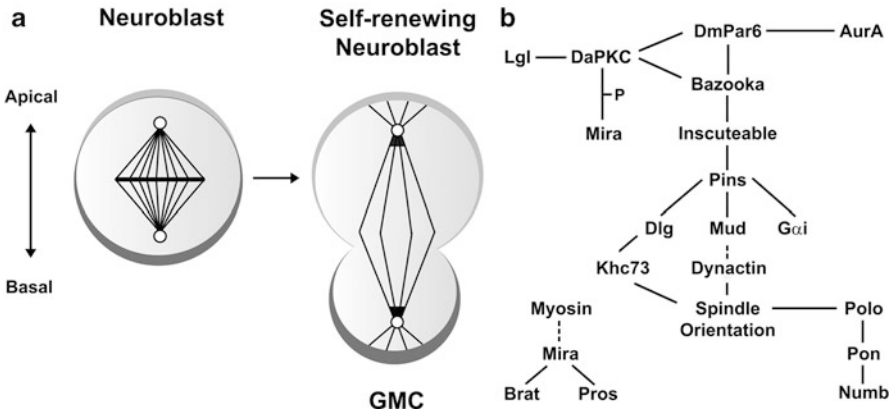

Fig. 4.3 Asymmetric stem cell division in the nervous system of *Drosophila*. (**a**) Asymmetric neuroblast division in *Drosophila*. In the developing nervous system of *Drosophila*, neural stem cells called neuroblasts divide asymmetrically along their apical-basal axis to give rise to another, self-renewing neuroblast and an intermediate progenitor cell, called ganglion mother cell (GMC). The GMC in turn exits the cell cycle and differentiates into neurons (or glial cells) by one terminal division. (**b**) Molecular machinery underlying asymmetric neuroblast division in *Drosophila*. Dividing neuroblasts are polarized along the apical-basal axis: Apical polarity cues include Lgl, DaPKC, Baz, Par6, Insc, Pins, Dlg, Mud, Gαi and AurA; spindle orientation is directed by Mud, dynactin and Khc73. Basal cell fate determinants and their adaptor proteins include Mira, Pros, Brat, Polo, Pon and Numb. During asymmetric neuroblast division, apical complex formation and basal targeting simultaneously ensure stem cell self-renewal (apical) and the formation of a differentiating GMC (basal). See text for details (Modified after Kim and Hirth 2009)

(Pins) which together bind the heterotrimeric G-protein α-subunit Gαi into the complex to form an apical crescent at late interphase/early prophase (Yu et al. 2005; Nipper et al. 2007).

Binding of Gαi to Insc enables Pins to recruit an additional protein called Mushroom body defect (Mud) (Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006) which is the *Drosophila* homolog of the microtubule and dynein binding protein NuMA (Zheng 2000; Sun and Schatten 2006). Mud is thought to interact with the astral microtubules to 'fix' one of the spindle poles on the apical cortex of the neuroblast, thus contributing to the orientation of the mitotic spindle. Pins also binds to a membrane associated guanylyl kinase protein called Discs large (Dlg), that is known to interact with Kinesin heavy chain 73 (Khc-73), localised at the plus ends of astral microtubules. These interactions polarise the complex of proteins localised at the apical cortex of *Drosophila* neuroblasts in the direction of the mitotic spindle, which aligns perpendicular to the overlying epithelial plane (Yu et al. 2005; Wang et al. 2006a; Siegrist and Doe 2007). Thus, molecules of the apical complex direct apical-basal spindle orientation in dividing neuroblasts, and thereby establish an axis of polarity along which cytokinesis takes place.

Comparable mechanisms have been found in *C. elegans* and mammals (Gönzy 2008; Siller and Doe 2009; Knoblich 2010), suggesting that interactions between

the PAR complex, heterotrimeric G-proteins and mitotic spindle orientation represent a highly conserved mechanism underlying polarity formation. As a result of polarity formation, cell fate determinants are distributed unequally along the axis of polarity, which is usually the apical-basal axis and the future axis of division (Fig. 4.2c). Importantly, as the name states, these cell fate determinants predefine the destiny of the resulting daughter cells and their unequal segregation implements proper asymmetric cell division.

4.3.3 Apical Polarity and Mitotic Spindle Orientation

As soon as an axis of polarity is established, asymmetric cell division secures the segregation cell fate determinants into only one of the resulting daughter cells, thereby regulating a binary switch between stem cell self-renewal and differentiation. This is achieved by asymmetric localisation and subsequent unequal segregation of fate determinants that promote either stem cell identity or intermediate progenitor cell identity (Fig. 4.2d). In dividing *Drosophila* neuroblasts this results in apically localised proteins being maintained in self-renewing neuroblasts, whereas basally localised proteins are segregated into differentiating progenitor cells, termed ganglion mother cells (GMCs) (for review, see Kim and Hirth 2009).

In *Drosophila* neuroblasts (Fig. 4.3a), asymmetric segregation of cell fate determinants requires key substrates including the cortically localised tumour suppressor proteins Discs large (Dlg) and Lethal (2) giant larvae (Lgl) (Ohshiro et al. 2000; Peng et al. 2000; Albertson and Doe 2003; Betschinger et al. 2003). DaPKC and Lgl are key players in the establishment and maintenance of apical polarity, thereby providing neuroblasts with the capacity to self-renew. Lgl is a cytoskeletal protein known to specify the basolateral domain and to restrict DaPKC, Bazooka, and DmPar6 to the apical cortex (Wirtz-Peitz and Knoblich 2006). Lgl does not directly influence spindle orientation and apical localisation of the PAR complex. However, phosphorylation of Lgl by DaPKC leads to Lgl inactivation, or exclusion of Lgl from the apical cortex (Betschinger et al. 2003), thereby restricting cortical recruitment of basal cell fate determinants (Fig. 4.3b).

These observations have been substantiated by mutant studies, showing that neural lineages mutant for Lgl lead to supernumerary postembryonic neuroblasts due to occasional ectopic self-renewal (Rolls et al. 2003; Lee et al. 2006a). Furthermore, overexpression of a membrane-targeted DaPKC, but not a kinase-dead mutant isoform leads to a similar phenotype, whereas a decrease in DaPKC expression reduces neuroblast numbers. Genetic interaction experiments showed that Lgl, DaPKC double mutants have normal numbers of neuroblasts and that DaPKC is fully epistatic to Lgl, suggesting that DaPKC directly promotes neuroblast self-renewal (Lee et al. 2006a).

How are DaPKC and Lgl directed to the apical cortex? A partial answer to that comes from recent data suggesting that the mitotic kinase Aurora-A (AurA) is required for the asymmetric localisation of DaPKC (Lee et al. 2006b; Wang et al.

2006b; Wirtz-Peitz et al. 2008). AurA phosphorylates DmPar6, a member of the PAR complex, which in turn prevents an interaction between DmPar6 and DaPKC (Fig. 4.3b). Subsequently, phosphorylated DaPKC can act independently of DmPar6 and is able to phosphorylate Lgl, leading to Lgl inactivation/exclusion of Lgl from the apical cortex (Betschinger et al. 2003; Lee et al. 2006a). Within the PAR complex, this sequence of events leads to the exchange of Lgl for Bazooka, which in turn enables phosphorylation of the cell fate determinant Numb and its subsequent segregation into the differentiating daughter cell, the GMC (Wirtz-Peitz et al. 2008).

These data from *Drosophila* provide a direct link between asymmetric protein localisation and mitotic spindle orientation. A linkage between apical cortex and mitotic spindle was previously identified with the Mud/NuMa protein and its role in regulating neuroblast self-renewal via proper spindle-orientation. However, previous mutant studies showed that Mud does not alter cortical polarity (Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006), whereas mutant AurA does (Lee et al. 2006b; Wang et al. 2006b; Wirtz-Peitz et al. 2008); yet, both proteins localise to the centrosomes and tissue mutant for AurA and Mud exhibits similar defects in spindle orientation (Berdnik and Knoblich 2002; Giet et al. 2002; Izumi et al. 2006; Siller et al. 2006; Bowman et al. 2006; Lee et al. 2006b; Wang et al. 2006b; Wirtz-Peitz et al. 2008). Genetic interaction data in dividing neuroblasts indicate that AurA controls mitotic spindle orientation by regulating the asymmetric localisation of Mud (Wang et al. 2006b). Moreover, AurA seems not only to act on Mud and DmPar6, but also on Notch signalling. Mutational inactivation of AurA leads to ectopic activation of Notch (Wang et al. 2006b), which in its cleaved, intracellular form is able to promote self-renewal and to suppress differentiation of neural stem cells in the larval central brain of *Drosophila*.

In addition to the Mud/NuMA and AurA axis involved in mitotic spindle orientation and asymmetric stem cell division, recent studies have identified a complementary role of the centrosome and centrioles in the regulation of stemness. These data suggest that the centrosome and centrioles of the dividing "mother stem cell" are inherited by the differentiating daughter cell (Januschke et al. 2011), whereas the newly formed centrosome and centrioles are retained by the self-renewed stem cell, a process which requires centrosomin (Cnn) function (Conduit and Raff 2010).

Taken together, data from *Drosophila* suggest that AurA acts via Mud to orient mitotic spindles required for the establishment of a proper division plane (Fig. 4.3), which is a prerequisite for the unequal segregation of cell fate determinants during neural stem cell cytokinesis (Fig. 4.3b). Simultaneously, asymmetric protein localisation is achieved, at least in part by AurA acting on DmPar6 and in turn via phosphorylation of DaPKC followed by that of Lgl. Such a dual role of AurA linking asymmetric protein localisation and mitotic spindle orientation may explain to some extend why in AurA and Mud, but also in DaPKC and Lgl mutants, the net result is the same: supernumerary neural stem cells at the expense of differentiating neurons. Moreover, recent results suggest that AurA also links Pins and Dlg to the mitotic spindle orientation pathway (Jonhston et al. 2009).

In mammals, comparable mechanisms have been observed that are involved in the establishment of apical cell polarity and mitotic spindle orientation. The mammalian cerebral cortex and retina contain multipotent neuroepithelial progenitor cells with pronounced apical/basal polarity. Their apical domain or "apical endfoot" contains a complex of Cdc42 – Par-3 – aPKC – mPar-6, similar to *Drosophila*, as well as the transmembrane protein Prominin/CD133 (for review, see Götz and Huttner 2005; Farkas and Huttner 2008; see also Chap. 12 by Huttner). Moreover, a mammalian homologue of Pins, termed LGN, can bind NuMA and links NuMa to heterotrimeric G-proteins, thereby regulating mitotic spindle orientation (Du et al. 2001; Du and Macara 2004). Mouse Inscuteable (mInsc) has been shown to play a critical role in spindle reorientation in cortical progenitors of the mouse neocortex: both loss and gain of mInsc mutations affect correct mitotic spindle positioning, which in the wildtype appears to be essential for generating the correct numbers of neurons in all cortical layers (Postiglione et al. 2011). In addition, mammalian Par-3 (mPar-3) specifies the polarity of dividing radial glial cells in the developing mouse neocortex and differentially regulates Notch signalling activity in the resulting daughter cells (Bultje et al. 2009). In mouse skin progenitor cells, recent data provide evidence that the switch from symmetric to asymmetric divisions concomitant with stratification relies on LGN, NuMA and dynactin (Dctn1) activity (Williams et al. 2011). These data suggest that at least some of the mechanisms underlying apical polarity formation and mitotic spindle alignment are evolutionary conserved and essential prerequisites for asymmetric stem cell division.

4.3.4 Basal Polarity and Cell Fate Determinants

As a result of polarity formation, two opposite sides within a stem cell are generated: an apical side and a basal side. In conjunction with polarity formation (see above), mitotic spindles are aligned and a future axis of division is established, along which apical and basal cell fate determinants are segregated during cell division (Fig. 4.2d, e). Apical cell fate determinants are involved in stem cell self-renewal, whereas basal cell fate determinants are involved in differentiation processes. This dichotomy is most obvious in the developing nervous system of *Drosophila* where self-renewal and differentiation is not only regulated in proliferating neuroblasts but also in the intermediate progenitor cells, the GMCs.

In *Drosophila*, GMCs usually are destined to exit the cell cycle by terminal, symmetric division, thereby generating the majority of neurons that constitute the adult CNS. The destiny of GMCs is determined by the exclusive inheritance of key differentiation factors such as the Notch repressor Numb (Uemura et al. 1989), the NHL-domain protein Brain tumour (Brat) (Arama et al. 2000) and the homeodomain transcription factor Prospero (Vaessin et al. 1991; Doe et al. 1991; Matsuzaki et al. 1992) (see Fig. 4.3). Basal targeting of these cell fate determinants in dividing neuroblasts is achieved via their adaptor proteins, Partner of Numb (Pon) (Lu et al. 1998) and Miranda (Shen et al. 1997; Ikeshima-Kataoka et al. 1997). Previous

experiments in *Drosophila* showed that segregation of Numb into GMCs is regulated by Pon in a cell-cycle-dependent manner, and recent data provide evidence that Polo, a key cell cycle regulator itself, is critically required for this event by direct phosphorylation of Pon (Wang et al. 2007). Accordingly, mutant polo affects the asymmetric localization of Pon, Numb and DaPKC and supernumerary neuroblast-like cells are produced at the expense of neurons. Over-expression of Numb in polo mutant lineages is able to suppress over-proliferation, indicating that Polo inhibits progenitor cell self-renewal by regulating the localization and function of Numb. As is the case for AurA, polo function therefore provides another link between cell cycle regulation and asymmetric protein localization. However, the mechanism by which Numb directly or indirectly regulates cell cycle activity and proliferation is poorly understood.

Ganglion mother cell fate is also determined by Prospero (Pros). Pros mRNA and protein is already detectable in dividing neuroblasts where it is transported via its adaptor Miranda to the basal side (Shen et al. 1998; Schuldt et al. 1998; Broadus et al. 1998; Matsuzaki et al. 1998). Cytokinesis segregates Prospero solely into the GMC where Mira degrades, thereby releasing Prospero from the cortex, which then translocates into the nucleus (Hirata et al. 1995; Spana and Doe 1995). Prospero acts as a transcription factor in the GMC nucleus, where it has a dual role. Pros inhibits cell cycle progression by repressing cell cycle regulators such as cyclin A, cyclin E and the *Drosophila* cdc25 homologue, string, as well as by activating the expression of dacapo, a cyclin-dependent kinase inhibitor, ultimately leading to terminal differentiation of the GMC into two post-mitotic neurons/or glial cells (Li and Vaessin 2000; Liu et al. 2002). Moreover, genome-wide expression profiling using *prospero* loss and gain-of function embryos as a template indicate that Prospero represses neuroblast-specific apical polarity genes like inscuteable, bazooka and DaPKC, and activates expression of neural differentiation genes such as fushi tarazu and even skipped (Choksi et al. 2006). In addition, mutant analyses provide in vivo evidence that loss of *pros* results in enlarged neuroblast lineages essentially devoid of differentiating, post-mitotic neurons (Bello et al. 2006; Lee et al. 2006c; Betschinger et al. 2006). Instead, the vast majority of cells within these mutant clones show sustained expression of stem cell markers and increased mitotic activity, eventually leading to neoplastic tumor formation (Bello et al. 2006). These data indicate that loss of *pros* causes a transformation of GMCs into stem-like cells that are unable to exit the cell cycle and continue to proliferate. Based on these experimental observations, it is reasonable to consider Prospero as a gate-keeper in regulating self-renewal and differentiation in GMCs.

Another recently identified cell fate determinant is Brain Tumor (Brat). *brat* encodes a member of the conserved NHL family of proteins (Arama et al. 2000; Reymond et al. 2001; Sardiello et al. 2008). Similar to *pros*, *brat* mutation results in over-proliferating neuroblast lineages at the expense of differentiating neurons (Bowman et al. 2008; Bello et al. 2006; Lee et al. 2006c; Betschinger et al. 2006). Brat mutant neuroblast clones show cortical mis-localisation of Miranda and the loss of nuclear *pros* (Lee et al. 2006c), suggesting that these proteins may play a

role in the same molecular pathway. This is supported by genetic experiments showing that ectopic expression of Pros can rescue the tumour formation in Brat mutants in the larval central brain (Bello et al. 2006). However, Brat localisation remains unaffected in Pros mutants, whereas in Mira mutants Brat and Pros are mislocalised.

These results indicate that Mira is essential for the asymmetric localisation of the cell fate determinants Brat and Pros. This is in line with the fact that Pros can bind to the central Pros-binding domain of Miranda (Fuerstenberg et al. 1998), and Brat binds to the coiled-coil cargo binding domain of Miranda (Betschinger et al. 2006). Moreover, the interaction between the NHL domain of Brat and the C-terminal domain of Mira (Lee et al. 2006c) appears to be essential for promoting asymmetric localisation of Pros to the GMC, where it is required for cell cycle exit and neuronal fate determination. Thus, it is conceivable that Mira and its cargo proteins Brat and Pros maybe transported across the dividing NB as a complex. But what drives basal protein targeting of adaptor proteins and their respective cell fate determinants?

Previous studies suggested that the localisation of Mira and Pros appear to be dependent on actin (Broadus and Doe 1997), as well as on motor proteins, Myosins in particular (Ohshiro et al. 2000; Petritsch et al. 2003). These studies indicated an interaction between Lgl with a plus-end directed motor, myosin II (Ohshiro et al. 2000). Subsequent experiments showed that Spaghetti Squash (Sqh), the regulatory light chain of Myosin II, is required in embryonic neuroblasts both, to organize the actin cytoskeleton, thereby enabling determinants to localize to the cortex, and to confine determinants to the basal side (Barros et al. 2003). These data suggested that Myosin II is one of the motor proteins involved in basal localisation of the cell fate determinants. In line with this, Mira was also found to physically interact with Zipper, the heavy chain of myosin II (Ohshiro et al. 2000). Thus, non-phosphorylated Lgl can negatively regulate Myosin II in embryonic NBs by directly binding to it.

In Myosin II mutant studies, cell fate determinants failed to form a basal crescent in embryonic neuroblasts (Ohshiro et al. 2000), notably Mira is mis-localised uniformly around the cortex (Erben et al. 2008). Similarly, reduced Myosin VI (Jaguar) activity in embryos, leads to a failure in basal crescent formation as well, with Mira mis-localising to the cytoplasm in patches (Petritsch et al. 2003). Myosin VI transiently accumulates in the basal cortex, partially co-localises with Mira during metaphase, and in vitro studies using *Drosophila* embryonic extracts also showed physical interaction with Mira. The distinct phenotype, mode of action, and sub-cellular localisation of Myosin II and Myosin VI suggests that they may act at consecutive steps in a single pathway to localise Mira and its cargo proteins to the basal side of dividing NBs. However, it is currently not know how exactly Mira is transported to the basal side of a dividing neural stem cell. Yet, recent experimental evidence suggests that direct phosphorylation of Mira by aPKC leads to exclusion of Mira from the apical cortex (Atwood and Prehoda 2009), which is a prerequisite for its basal targeting, and in turn the unequal segregation of cell fate determinants that are transported by Mira.

4.3.5 Cell Cycle Progression and Growth Control

These data coming from studies of the developing CNS of *Drosophila* provide compelling evidence that one strategy to regulate stem cell-self-renewal and differentiation is asymmetric segregation of cell fate determinants in a dividing cell. This is achieved, in part, by asymmetric protein localisation and related mitotic spindle orientation, thereby providing a template for unequal distribution of key regulators such as AurA, DaPKC, Numb and Pros (Fig. 4.3b). Interestingly, however, such a cascade of events does not explain why mutant stem cells continue to proliferate, thereby self-renewing for an extended period of time without progressive volume decline. This is particularly evident in the case of continued proliferation in *pros* mutant neuroblast clones in *Drosophila*. There, continued cell division cycles appear to be accompanied by compensatory cell growth. Thus, *pros* mutant cells display sustained symmetric divisions without shrinkage in cell size (Bello et al. 2006), a phenomenon that is usually accompanied with neuroblast division in the embryonic CNS. Thus, in *pros* mutant clones, a constant cell size appears to be maintained over many rounds of self-renewing divisions, indicating that Pros may also act as a transcriptional repressor on genes involved in growth control. However, genome-wide expression profiling did not identify growth control genes as potential targets of *pros*, maybe because embryos had been used as a template (Choksi et al. 2006). A possible link between asymmetric protein localisation, cell cycle progression and growth control may be provided by Brat.

Previous studies in *Drosophila* had shown that *brat* is a translational repressor (Sonoda and Wharton 2001) which also functions in the regulation of cell growth and ribosomal RNA synthesis (Frank et al. 2002). Growth and proliferation of *brat* mutant cells might be perpetuated by dis-inhibited dMyc activity (Betschinger et al. 2006), a transcription factor regulating cell growth and proliferation (Eilers and Eisenman 2008). The available data however suggest that Brat activity regulates a large number of direct and indirect targets involved in cell cycle progression and growth control. This notion is supported by genome-wide expression studies using adult wildtype and *brat* mutant brain tissue as a template (Loop et al. 2004). These studies identified several potential target genes of Brat, most prominent among them genes involved in cell cycle regulation and translation control, as well as RNA binding/processing, all being up-regulated in *brat* mutant tissue (Loop et al. 2004). In addition, *brat* gain of function can inhibit cell growth and ribosomal RNA accumulation, and slowdown cell division cycles (Frank et al. 2002). Considering its mutant lineage phenotype, these data suggest that *brat* may inhibit cell growth by limiting the rate of ribosome biogenesis and protein synthesis.

Comparable data have been found in *C. elegans* where Brat homologues regulate PAR protein-dependent polarity and asymmetric cell division (Hyenne et al. 2008). In addition, homologues of Brat have been identified in mammals where they are also involved in progenitor cell proliferation control. Recent genetic evidence in mice suggests that the Brat homolog TRIM32 can bind Ago1, a protein involved in microRNA processing. TRIM32 functions both by degrading c-Myc as well as by activating certain microRNAs, among them the stem cell regulator Let-7a (Gangaraju

and Lin 2009). TRIM32 activity thereby suppresses self-renewal in dividing cortical progenitor cells, and induces neuronal differentiation (Schwamborn et al. 2009). These findings indicate that Brat/TRIM-NHL proteins regulate self-renewal and differentiation of stem/progenitor cells by modulating microRNA activity as well as ribosome biogenesis and protein synthesis.

These data also suggest that deregulated stem/progenitor cell division can lead to uncontrolled cell growth and tumor formation (Caussinus and Hirth 2007). Indeed, recent experimental evidence suggests that so-called cancer stem cells drive the growth and metastasis of human tumors and cancer stem cells have already been identified in leukemia, and in solid tumors of the breast and brain (for review, see Reya et al. 2001; Pardal et al. 2003; Al-Hajj and Clarke 2004; Fomchenko and Holland 2005; Stiles and Rowitch 2008; Visvader and Lindeman 2008; Schatton et al. 2009). Moreover, inappropriate activation of the WNT, sonic hedgehog (SHH), Notch, PTEN, and BMI1 pathways have all been shown to promote the self-renewal of somatic stem cells, and their dysregulation can lead to neoplastic tissue formation (for review, see Pardal et al. 2003; Jiang and Hui 2008).

Based on these observations, it is conceivable that similar to the situation in *Drosophila*, the machinery promoting asymmetric cell division may play an evolutionary conserved role in cell cycle control and tumor suppression. Indeed, mammalian homologues of Baz, Par6, DaPKC, Lgl, Numb and Brat have been shown to regulate asymmetric cell fate determination and tumor suppression. Thus, mammalian aPKC, Par3, and LGN are involved in asymmetric division of basal epidermal progenitor cells of the skin and their dysregulation can lead to skin cancer (Lechler and Fuchs 2005). The Brat homologue TRIM3 has been identified as a candidate brain tumor suppressor gene (Boulay et al. 2009), indicating that Brat/TRIM-NHL proteins act in a conserved genetic pathway regulating stem/progenitor cell self-renewal and differentiation. Moreover, there is evidence for the asymmetric segregation of vertebrate NUMB homologues (Wodarz and Huttner 2003) that seem to act as asymmetric cell fate determinants. Double knockouts of Numb and Numb-like in the mouse dorsal forebrain have been found to lead to impaired neuronal differentiation, hyper-proliferation of neural progenitors, and delayed cell-cycle exit (Petersen et al. 2002, 2004; Li et al. 2003). In addition, loss of Lgl1/Mlgl/Hugl, one of the two Lgl homologues in mice, results in failure to asymmetrically localize the fate determinant Numb and leads to severe brain dysplasia as neural progenitor cells fail to exit the cell cycle (Klezovitch et al. 2004). Reciprocally, a well-characterized human tumor suppressor, the kinase Lkb1, whose loss-of-function phenotype results in Peutz-Jeghers syndrome, regulates cell polarity in worms, flies and humans and might be involved in asymmetric cell division as well (Marignani 2005). In addition, recent data provide compelling evidence that also mammalian homologues of Notch, NuMa and dynactin (Williams et al. 2011) as well as Inscuteable (Postiglione et al. 2011) contribute to maintain a proper balance between neuronal proliferation and differentiation in the developing mouse neocortex. Thus, similar to the situation in *Drosophila*, asymmetric cell division in mammals appears to be involved in the regulation of stem and progenitor cell self-renewal, and the regulation of cell cycle progression and growth control.

4.4 Conclusions and Perspectives

Studies using model organisms, including the *C. elegans*, *Drosophila melanogaster*, and mice have revealed insights into the molecular mechanisms underlying asymmetric stem cell division. These studies identified key essential, consecutive steps of asymmetric cell division that are characterised by symmetry break, polarity formation, mitotic spindle orientation and segregation of cell fate determinants; these processes are mediated by evolutionary conserved molecules, including Aurora-A, aPKC, Mud/NuMa, Lgl, Numb and Brat/TRIM-NHL proteins. Asymmetric stem cell division lies at the interface of stem cell self-renewal and differentiation and therefore regulates the number and identity of differentiating progeny. Therefore, asymmetric cell division is of major therapeutic interest in regenerative medicine as asymmetrically dividing stem cells provide a powerful source for targeted cell replacement and tissue regeneration. For therapeutic applications, it will now be important to determine further details of the machinery involved, in order to be able to manipulate asymmetric stem cell division in vitro for the unlimited generation of differentiated cells at will. Several key questions need to be addressed and answered in order to achieve these goals. These include elucidation of the mechanisms and molecules that define and maintain stemness; to identify molecules that regulate the binary switch between self-renewal and differentiation; to determine the mechanisms that direct cell type specific differentiation; and to determine ways how an in vitro generated cell can integrate into an existing cellular context while remaining differentiated. Elucidating the molecular mechanisms regulating asymmetric stem cell division will significantly contribute to the successful application of stem cells in regenerative medicine.

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