Chapter 15
Cellular Ageing and the Actin Cytoskeleton

David Amberg, Jane E. Leadsham, Vasillios Kotiadis, and Campbell W. Gourlay

Abstract For some time the view that the actin cytoskeleton acts primarily as a scaffold, to be assembled in response to a signaling cascade as an end point in the pathway, has prevailed. However, it is now clear that the dynamic nature of the cytoskeleton is linked to downstream signaling events that further modulate cellular activity, and which can determine cell fate. Examples of this lie within the regulation of programmed cell death, the maintenance of homeostasis and the process of cellular ageing. In yeast the actin cytoskeleton has been shown to interact directly with signaling pathways known to be important in the regulation of both ageing and cell death. For example it has been discovered that the level of damage sustained by the actin cytoskeleton under conditions of oxidative stress is directly linked to apoptosis. Further evidence comes from the finding that actin based propulsion mechanisms are required for the inheritance of mitochondria and anti-ageing factors into newly formed cells. In addition to this actin is known to directly influence the formation of protein aggregations. In this chapter we will discuss these points and postulate as to their significance with respect to the maintenance of cellular homeostasis.

Keywords Actin · Mitochondria · Protein aggregation · Oxidative stress · ROS · Cytoskeleton · Ras/cAMP/PKA · Autophagy · Mitophagy

Introduction

The actin cytoskeleton ActinCytoskeleton is a major eukaryotic cellular component that is involved in a variety of essential functions. It is also a crucial part of the signaling networks that link processes such as polarisation, organelle movement, motility and division to environmental signals. Actin has been shown to be a target of proteolytic cleavage by caspases during the process of apoptosis (Mashima et al. 1995), and endures oxidative damage under conditions of cellular stress (see section "Actin as a Target and Effecter of the Oxidative Stress Response" below). In fact recent studies have shown that the actin cytoskeleton has an important role to play in the regulation of cell death pathways in diverse organisms from plant, animal and

C.W. Gourlay (✉)
Kent Fungal Group, School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK
e-mail: C.W.Gourlay@kent.ac.uk

fungal kingdoms (reviewed in Franklin-Tong and Gourlay (2008)). It is therefore of interest to consider the effects of actin damage within the context of cellular activity and the stochastic process of ageing. The budding yeast *Saccharomyces cerevisiae* has proven to be a useful tool in elucidating the role of actin in cell death and ageing, facilitated in part by the degree of conservation that exists with regards to the fundamental principles of actin dynamics. Additionally, as actin in yeast is encoded by a single gene, *ACT1* (Shortle et al. 1982) strains expressing mutant alleles as the sole source of G-actin for filament construction can be generated (Whitacre et al. 2001; Ayscough and Drubin 1996). This is not possible in many higher eukaryotic systems as multiple actin genes are present whose products are known to exhibit functional redundancy.

The Budding Yeast Actin Cytoskeleton

The monomeric (G) and filamentous (F) forms of actin co-exist in a dynamic equilibrium. Actin filaments possess a fast growing, or barbed, and a slower growing, or pointed, end which imparts polarity upon the structure. Actin filaments are assembled in a head to tail manner, with monomers of ATP-bound actin preferentially added to barbed ends of filaments (Pollard 1986). The subsequent hydrolysis of the ATP to ADP + P_i (inorganic Phosphate), promotes a conformational change in the actin filament structure which acts to promote instability. This is also aided by the release of P_i to yield ADP-bound actin at the filaments pointed end (Pollard et al. 2000). Upon dissociation from the pointed ends, resulting ADP-bound actin monomers are manipulated by proteins that co-operate in to replenish the filament assembly competent pool of ATP actin. This is done by promoting the exchange of ADP for ATP on actin monomers released from existing filaments (Moseley and Goode 2006; Wolven et al. 2000). The net addition and dissociation of actin subunits is termed treadmilling and can impart force in the direction of the barbed end. Because the initial step in filament assembly is intrinsically slow (Sept and McCammon 2001) – with the first stable structure being an actin trimer – there is a requirement for factors to promote, and initiate, nucleation. The Arp2/3 complex and members of the formin family (Pollard 2007), are the most well characterised of these nucleation factors (see below). There are also factors that further promote filament nucleation (**N**ucleation **P**romoting **F**actors) (Welch and Mullins 2002), for example Las17p, the yeast homologue to mammalian WASp, is required to stimulate Arp2/3 activity and actin branching (Naqvi et al. 1998). Furthermore, because steady state release of actin monomers from filaments is not sufficient to keep up with physiological requirements, ADP-actin subunits must be dissociated from filament pointed ends at a faster pace with the aid of severing and disassembly inducing factors such as cofilin, Actin Interacting Protein 1 (Aip1p), twinfillin, and Srv2/CAP (Moon et al. 1993; Nicholson-Dykstra et al. 2005). However, in order for cells to exploit actin filaments and their properties, they need to assemble them into higher order structures (Moseley and Goode 2006). In yeast the prominent higher order structures are cortical actin patches, actin cables and the cytokinetic contractile ring.

Cortical Actin Patches

Patches are highly dynamic structures that exhibit rapid movement and facilitate internalisation of early endosomes (Kaksonen et al. 2003) (Fig. 15.1). Studies using electron microscopy (EM) were able to demonstrate that actin-rich structures surrounded invaginations of the plasma membrane before internalisation (Mulholland et al. 1994). The polymerisation of actin to form the patch ultrastructure is considered to be the driving force for generating endocytic vesicles by pulling the membrane inwards whilst also serving as a structural scaffold (Kaksonen et al. 2003; Ayscough et al. 1997; Huckaba et al. 2004). Once formed, vesicles bound to actin patches are driven inwards from the cortex using the force generated by actin polymerisation (Kaksonen et al. 2003). As the vesicle is internalised, actin
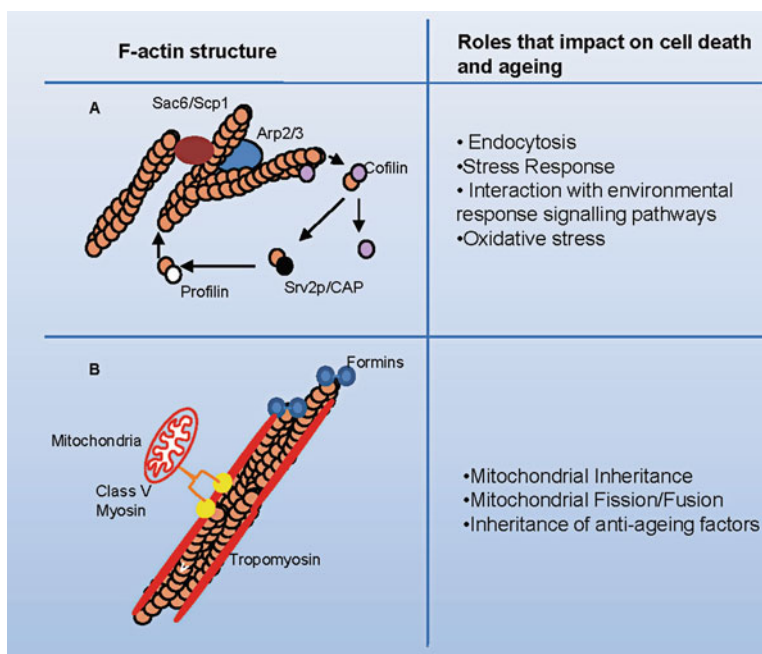
Fig. 15.1 Actin polymerization in cortical patches (**a**) and cables (**b**) and their impact on processes linked to ageing. Actin monomers exist in a dynamic equilibrium and are present in monomeric (G) or filamentous (F) form. F-actin is organized into higher order structures such as cortical patches and cables. These structures perform different tasks within the cell, a number of which contribute to cellular homeostasis and have an impact on ageing and cell death. In patches the Arp2/3 complex nucleates branched networks. The dynamic nature of patches is essential for the process of endocytosis and is facilitated by actin binding proteins such as cofilin, Srv2p/CAP and profilin. Stability in the patches is maintained by the bundling proteins Sac6p and Scp1p (**a**). Actin cables serve as tracks for the movement of organelles by the myosin motor proteins. They are nucleated by members of the formin family and stabilized by the tropomyosins (**b**). Inheritance of mitochondria into the newly forming cell, as well as the retention of damaged proteins within the mother cell relies on their function (Boldogh and Pon 2006)

polymerisation induced by the Arp2/3 complex is inhibited to help promote vesicle release (Smythe and Ayscough 2003, 2006). The release of vesicles requires the motor function of myosins Myo3p, Myo5p as well as additional proteins such as Rvs161p, and Rvs167p that interact with the Arp2/3 complex and F-actin. Mutants defective in these proteins exhibited an inability to achieve scission of endocytic vesicles from the cell cortex (Kaksonen et al. 2005). Once scission of the endocytic vesicles is achieved, the actin patch/vesicle structures are transported to endosomes. This is achieved by a passive transport system utilising the retrograde flow of actin cables from the cell cortex inwards (Huckaba et al. 2004).

Actin Cables

The actin cable network first described by Adams and Pringle (1984) is utilised by yeast cells for polarised growth, organelle segregation, and intracellular transport (Bretscher 2003) (Fig. 15.1). Cables are comprised of shorter actin filaments overlapping each other in a unidirectional conformation of barbed and pointed ends. Maintenance of cell polarity by actin was initially observed in temperature sensitive mutants of the major yeast tropomyosin isoform, *Tpm1* (Pruyne et al. 1998). This was further confirmed by temperature sensitive mutants of the type V myosin, Myo2p, which is required for the movement of cargo in to the growing bud (Johnston et al. 1991). Other factors involved in cable formation and stability such as capping protein (Amatruda et al. 1990), the formin Bni1p (Evangelista et al. 1997), and profilin (Haarer et al. 1990), also confer cell polarity defects when mutated.

The transport and segregation of organelles during bud formation and growth is achieved through the action of type V myosin coupled to actin cables. This has been shown by the GFP tagging of post-Golgi transport vesicles which can be visualised moving towards newly forming bud tips (Schott et al. 2002). Additionally organelles such as the vacuole, nucleus, cortical ER, and peroxisomes have been shown to be transported in a Type V myosin dependent manner to growing buds in *S. cerevisiae* (Pruyne et al. 2004). In addition mitochondria appear to be transported on cables, this phenomena is discussed in detail in section "The Role of Actin in Regulating Mitochondrial Function" of this chapter. The formation of actin cables, unlike patches, relies on the nucleation activity of formins (Evangelista et al. 2002; Sagot et al. 2002). The formin-based initiation of cable assembly was confirmed by studies in which fragments of the formin Bni1p were shown to nucleate actin in vitro (Pruyne et al. 2002). Formin assembly can be regulated by Rho1p, Rho3p, Rho4p, and cdc42p, which are members of the Rho-GTPase family (Dong et al. 2003). Rho3p and Rho4p have been shown to activate formins under normal growth, whereas Rho1p does so during stress conditions. Interestingly, although Cdc42p is not required to initiate cable filament assembly, its absence leads to improper cable assembly organisation during bud growth.

Once nucleated, actin filaments need to be organised in order to form stable cable ultrastructures. Actin cables are targeted specifically to polarity sites which are used to co-ordinate cables during bud formation and growth (Casamayor and Snyder 2002), as well as within other cellular events such as endocytosis and exocytosis (Pruyne and Bretscher 2000). Polarity sites are essentially assemblies of proteins that direct cell growth by and comprise components such as Spa2p, Pea2p, and Bud6p that can regulate both the actin cytoskeleton and signalling pathways involved in polarisation (Sheu et al. 1998).

The observation that actin cables are highly dynamic suggests that the usual factors involved in actin turnover should be present. Indeed studies implicate cofilin together with Aip1p in cable filament turnover (Okada et al. 2006). The current model suggested is a mechanism by which Aip1p, cofilin, and tropomyosin cooperate in order to appropriately regulate cable filament turnover, and steadily "prune" them along their length. In order to observe actin cable dynamics in real time, yeast cells with an Abp140p-GFP fusion were generated (Yang and Pon 2002). Studies utilising Abp140p-GFP have revealed that one end of each cable is associated with the budding tip or the mother cell neck region. Polymerisation then pushed filament growth into the mother or bud. The force driving this movement of cables maybe solely due to the polymerisation of actin, however recent studies have implicated myosins in actin cable dynamics (Huckaba et al. 2006). This study demonstrated reduced rate of cable transfer with the loss of the Myo1p motor activity.

Contractile Ring

During cytokinesis in *S. cerevisiae*, the bud is separated from the mother cell by the combined action of the contractile ring (which is principally comprised of actin and myosin) and the formation of a septum at the mother daughter cell interface. Although Adams and Pringle (1984) had observed the clustering of actin on the bud neck, it was not confirmed until much later that the actomyosin rings contracts specifically during cytokinesis to facilitate separation (Bi et al. 1998; Lippincott and Li 1998). Septins are the first structural components to be assembled, comprising Cdc3p, Cdc10p, Cdc11p, and Cdc12p and form a scaffold for the actin ring (Longtine et al. 1996). The septin ring recruits factors such as myo1p, formins and IQGAP (IQ motif containing GTPase activating protein) proteins which facilitate the formation of the actin ring (Lippincott and Li 1998). Once all of the essential components (e.g. organelles, genetic material, etc) are transported into the forming daughter cell, it must be separated from the mother through the concerted action of the actomyosin ring and septum formation (Bi et al. 1998). To achieve this, the actomyosin ring constricts driving the formation of the septum, which provides a physical diffusion barrier for both mother and daughter cell until membrane and cell wall synthesis is complete.

Actin Cytoskeletal Function and Toxicity

Nutritional Sensing and Stress Signaling

The actin cytoskeleton is highly responsive to environmental change. In yeast this can be visualized when cells are subjected to a range of stressful conditions such as glucose withdrawal, heat shock, osmotic shock or centrifugal force. Under these conditions dividing cells, which usually exhibit a polarized actin cytoskeleton (Fig. 15.2), rapidly de-polarise their actin patches throughout the cell. This phenomenon can also be seen under conditions of nutritional depletion when cells enter the diauxic phase of growth (Fig. 15.2). Recent studies have demonstrated that during the diauxic shift the dynamic status of the actin cytoskeleton is linked to the activity of the Ras/cAMP/PKA signaling pathway, which co-ordinates cell growth and proliferation with sensation of the nutritional environment (Rolland et al. 2002; Thevelein and de Winde 1999). Within this signaling cascade, production of the secondary messenger cAMP is carried out by the adenylate cyclase, Cyr1p, and can be stimulated by either the G protein coupled receptor GPR1-GPA2 system, or through binding of GTP-bound Ras and adenylyl cyclase-associated (Srv2p/CAP) proteins.
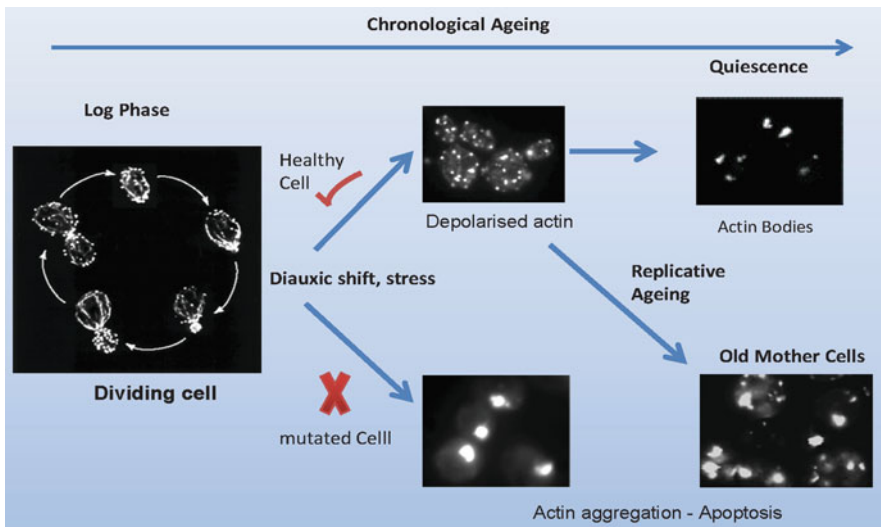
Fig. 15.2 F-actin re-arrangements in stressed, apoptotic and ageing cells. During the cell cycle actin structures are polarized to facilitate the movement of new cell material to the growing cell. Actin patches are found to accumulate in emerging buds and actin cables direct movement of organelles and vesicular cargo towards the new cell. The sensation of cellular stress, or nutritional depletion, is accompanied by depolarisation of actin patches and a reduction in the prominence of actin cables. As cells chronologically age actin forms into "bodies" which are aggregations of fully functional actin. These bodies act as reservoirs of actin that are used to rapidly engage cell growth when nutrition returns. In old cells, or in mutants with reduced actin dynamics, actin aggregates form spontaneously and can induce apoptosis

Elevation of cAMP levels leads to dissociation of the protein kinase A (PKA) regulator Bcy1p to yield active A kinases, which elicit alterations in processes such as cell cycle progression and stress responses. There are three A kinase catalytic subunits in yeast, encoded by *TPK1*, *-2*, and *-3*, which display overlapping and separable functions in response to activation by cAMP. The sensation of nutritional depletion leads to a reduction in Ras/cAMP/PKA pathway activity, allowing effective cell cycle exit and initiation of the cellular stress response. Published data suggests that the activity of the Ras/cAMP/PKA pathway is linked to mitochondrial activity as cells expressing the constitutively active *RAS2^{ala18val19}* allele exhibit an increase in respiration rate and elevation of ROS levels (Heeren et al. 2004; Hlavata et al. 2003). Our work has shown that the dynamic status of the actin cytoskeleton can act to regulate the activity of Ras. We were able to demonstrate Ras hyperactivity in mutants that exhibit reduced actin dynamics, leading to the accumulation of aggregates of F-actin, mitochondrial dysfunction resulting in an apoptotic cell death. It was concluded that the inappropriate activation of Ras was indeed induced as a result of the formation of F-actin aggregations. This conclusion was drawn as the addition of an actin binding drug, Latrunculin A, at concentrations which prevented the formation of aggregates also prevented ROS accumulation and cell death (Gourlay and Ayscough 2005, 2006). F-actin aggregations were shown to require the activity of the protein Srv2p/CAP, a highly conserved actin regulatory protein that binds preferentially to ADP–G-actin via its C-terminal domain (Balcer et al. 2003; Mattila et al. 2004). Srv2p/CAP can also associate with actin filaments through an interaction between a proline region and the SH3 domain of actin binding protein 1 (Abp1p) (Freeman et al. 1996). This protein is a good candidate to link Ras signaling to actin reorganization as The N terminus of Srv2p/CAP can bind to adenylate cyclase (Cyr1p) and facilitate cAMP/PKA activation (Gerst et al. 1991; Mintzer and Field 1994). Actin aggregation that triggered Ras/cAMP/PKA pathway activity was shown to require only the C-terminal actin binding region of Srv2p/CAP, however presence of the N-terminal cyclase binding domain enhanced PKA stimulation leading to higher levels of ROS production. The accumulation of ROS induced damage and subsequent death in actin aggregating cells was demonstrated to require the activity of the PKA subunit, Tpk3p, an enzyme known to play an important role in mitochondrial function. Yeast lacking Tpk3p exhibit altered mitochondrial enzymatic content, including reduced levels and activity of cytochrome c, a constituent of the electron transport chain (Chevtzoff et al. 2005). Our unpublished data suggests that Tpk3 acts to suppress respiration during growth on fermentable carbon sources such as glucose, and that excessive Tpk3 activity leads to a dysfunctional electron transport chain that is prone to producing ROS in large amounts which in turn accelerates the ageing process and promotes cell death.

   The appearance of aberrant actin formations in ageing and diseased cells, such as Hirano bodies and ADF/cofilin rods have been reported in neuronal populations from patients suffering from neurodegenerative diseases such as Huntington's and Alzheimer's. Hirano bodies are para-crystaline F-actin rich structures that contain a

number of associated proteins (Davis et al. 2008; Fechheimer et al. 2002; Maselli et al. 2002, 2003). Recent studies have concluded that the presence of Hirano bodies does not have an immediate toxic effect, however it may be the case that their presence or persistence leads to the disruption of actin-dependent processes. This in turn may have a detrimental effect on actin dependent signaling mechanisms under conditions of stress. In light of the evidence we propose that stabilization of actin structures can influence the apoptosis decision making process (Franklin-Tong and Gourlay 2008).

The budding yeast *S. cerevisiae* provides an interesting model system in which to study the effects of actin aggregation on cellular homestasis. Recent studies from Sagot and colleagues have investigated the nature of the F-actin cytoskeleton in chronologically aged, or in other words quiescent culture (Sagot et al. 2006). This research found that the actin cytoskeleton enters into what can be described as a holding state during quiescence (Fig. 15.2), presumably in order to provide a reservoir of actin that can be utilised by the cell when fresh nutrition becomes available. Indeed the re-introduction of fresh media was shown to lead to the breakdown of actin bodies in yeast cells and the rapid re-establishment of a dynamic cytoskeleton (Sagot et al. 2006). Although the mechanisms by which actin "bodies" form remains unclear, it appears to be largely a result of the reduction of the dynamic state of the cytoskeleton. It may be the case that efficient exit from quiescence requires the correct assembly of actin bodies, and that the accumulation of actin aggregates in ageing, or oxidatively stressed cells that abrogate signalling mechanisms represent a phenomenon that contributes to disease in higher organisms. Recent research from our group has tested this hypothesis using actin disrupting drugs, or mutant strains that modulate the dynamic status of the actin cytoskeleton. Actin aggregates can be induced to form in yeast through disturbance of an actin regulatory function, or by the treatment of cells with actin stabilizing drugs such as Jasplakinolide. It has recently been discovered that the stabilization of cortical actin structures induces apoptosis in yeast (Gourlay et al. 2004). A similar phenomena can also be observed in many mammalian and plant cells (Franklin-Tong and Gourlay 2008). Mutations in actin regulatory proteins that lead to the accumulation of aggregates of stabilized F-actin have been shown to trigger a process termed Actin mediated apoptosis (ActMAp) (Gourlay and Ayscough 2005, 2006; Gourlay et al. 2004). In actin aggregating cell lines, ActMAp leads to a loss of mitochondrial membrane potential and the production and release of ROS into the cell which results in an apoptotic cell death. Interestingly, mutations that lead to an increase in the dynamic nature of the actin cytoskeleton were shown to result in reduced levels of ROS (Gourlay et al. 2004). In addition, deletion of a gene encoding the actin bundling protein Scp1p, the yeast homologue of mammalian SM22/transgelin, which also destabilises cortical actin structures, reduced ROS levels and led to a significant increase in replicative lifespan (Gourlay et al. 2004). These data suggest that in yeast, control of actin dynamics is linked to processes that regulate mitochondrial function and in turn impact on lifespan.

Protein Aggregate Formation

A common observation in a variety of ageing related and neurodegenerative disorders is the accumulation of protein aggregates. Although there is widespread debate, it is generally thought that the formation, propagation and persistence of protein aggregations, such as those caused by the presence of expanded polyglutamine (Poly-Q) tracts, is important in the progression or onset of diseases such as Huntington's and prion associated encephalopathy. A current popular hypothesis is that the initial oligomers, or "seeds" harbour the highest potential for toxicity, while the consequential larger protein aggregations may be inert or even offer a protective role for the cell. It has long been the counter argument that aggregations may represent a method by which cells can safely store unfolded polypeptides and thus protect cells from potential disruption. Intriguingly, evidence derived from research on the budding yeast *S. cerevisiae* demonstrates a role for the actin cytoskeleton, and associated machinery important for endocytosis, in the formation of a variety of protein aggregates implicated in human disease (Bailleul et al. 1999; Meriin et al. 2003). A fragment of human Huntingtin followed by the normal 25-Q repeat failed to aggregate when expressed in yeast, but one containing a 103-Q region readily aggregated. The expression of 103-Q in yeast also proved to be toxic in yeast cells, while the presence of 25-Q had no effect. Therefore yeast cells form Poly-Q length dependent aggregates in vivo that are associated with an increase in cellular toxicity, analogous to that observed in higher eukaryotes. Interestingly the cell death associated with 103-Q aggregate formation was shown to exhibit a number of phenotypes associated with apoptosis (Sokolov et al. 2006). Research points to a conserved role for actin and the endocytosis machinery in the regulation of prion and poly-Q aggregate formation (Bailleul et al. 1999; Meriin et al. 2003). For example in yeast, the actin patch and endocytosis regulator Sla1p had been shown to interact with an extended Poly-Q sequence by yeast 2 hybrid studies (Bailleul et al. 1999). In addition the deletion of a large number of genes involved in the regulation of the actin cytoskeleton and endocytosis were found to promote an increase in sensitivity to the presence of Poly-Q aggregates (Meriin et al. 2003). The close relationship between these two processes is highlighted by the discovery that Poly-Q aggregate formation also leads to a severe disruption of endocytosis (Meriin et al. 2003). 103Poly-Q aggregates formed in yeast were shown to contain several proteins known to be involved in the regulation of endocytosis, namely Pan1p and the E3 ubiquitin ligase Rsp5p, as well as filamentous actin. The apparent interaction between Poly-Q aggregates and the actin/endocytosis machinery led to the suggestion that the loss of endocytic function may underlie the associated cellular toxicity. Interestingly the formation of 103-Q aggregates in a mammalian cell culture model also led to defects in endocytosis, suggesting some conservation of the underlying mechanism. As many of the proteins involved in endocytosis have been conserved, then this may well be the case, promoting yeast as a useful model in which to study the toxicity associated with PolyQ aggregation. This likelihood is further reinforced by the fact that huntingtin has been shown to localise to sights of endocytosis and

interact with components of the endocytic machinery (Velier et al. 1998; Metzler et al. 2001; Singaraja et al. 2002). Further evidence to support a common mechanism comes from experiments that show that in both yeast and human cells the disruption of actin with the monomer sequestering drug Latrunculin-A leads to an increase in the presence of poly-Q aggregates. Interestingly recent evidence suggests that the removal of aggregated proteins from the daughter cell into the mother relies on a functional actin cytoskeleton (Liu et al. 2010). It is therefore likely that studies in yeast will yield important information as to the role that actin dynamics and endocytosis plays in the regulation of protein aggregation in higher eukaryotes.

Yeast may also prove a good model for understanding the effects of toxic protein aggregates on the function of mitochondria, whose tightly regulated activity is essential to the well being of the cells (see below). Reduced movement of mitochondria is seen in injured neurons where damaged mitochondria cluster around the site of injury preventing the arrival of healthy mitochondria which can lead to neuronal degeneration. This is similar to what is seen in Alzheimer's disease where axonal swelling may block mitochondrial movement. The presence of huntington aggregates have also been shown to impair mitochondrial movement in neuronal cells (Chang et al. 2006). In yeast it has been shown when the actin cytoskeleton was disrupted by polyQ aggregate formation, reduced mitochondrial respiration and increased ROS production were observed (Solans et al. 2006). This demonstrates a link between protein aggregation, the integrity of the actin cytoskeleton and mitochondrial function. Further studies may lead to insights into the interplay between protein aggregation, actin integrity and mitochondrial function that impact upon diseases such as Huntington's, Parkinson's and ALS.

The Role of Actin in Regulating Mitochondrial Function

It has been speculated that the presence of mitochondria in eukaryotes originates from engulfment of aerobically respiring bacteria 1.5×10^9 years ago. The resulting symbiotic relationship gave eukaryotes efficient energy production in return for the use of its protein synthesis machinery (Gray 1993). Thus mitochondria have evolved from a common ancestor, and therefore, we may expect that some common mechanisms used by eukaryotic cells for maintaining, controlling and organising mitochondria may be conserved, despite the now large diversity of eukaryotic cell types from unicellular to multi-cellular organisms.

The primary function of mitochondria in cells is the production of ATP via oxidative phosphorylation. However other mitochondrial functions include synthesis of lipid, heme, some amino acids and nucleotides. Mitochondria are also clearly important organelles within programmed cell death pathways and have been broadly promoted as important factors in the process of cellular ageing. The maintenance and movement of mitochondria to facilitate function requires the presence of a transport network. The cytoskeleton is ideally placed to carry out this function. Good examples of this can be found within the transport and compartmentalization of mitochondria within nerve cells. Within neurons there is the need for elevated

ATP production at sights of high electrical signal transduction (Chang and Reynolds 2006). Mitochondria need to be concentrated in these areas due to the poor diffusion of ATP in the cytoplasm. Mitochondria must therefore be moved to and from the cell body effectively (Chang and Reynolds 2006; Davis and Clayton 1996). The microtubular molecular motors kinesin (plus end directed) and dynein (minus end directed) appear to be responsible for anterograde and retrograde transport of mitochondria respectively. However, there is some evidence of actin based transport in neurons which it is proposed may mediate transport over short distances (Morris and Hollenbeck 1995). Actin has also been implicated in responsive mitochondrial localisation (Morris and Hollenbeck 1995; Evans and Bridgman 1995). Chada and Hollenbeck (2004) showed that mitochondria localise in response to nerve growth factor (NGF) along an axon. In these experiments cells pre-treated with latrunculin-B, which abolishes F-actin, failed to accumulate mitochondria in response to NGF (Chada and Hollenbeck 2003, 2004).

In yeast actin dependent movement of mitochondria has also been demonstrated. Initial evidence was achieved using temperature sensitive actin mutants which lose actin cables at the non-permissive temperature, concomitantly the mitochondria were seen to aggregate (Lazzarino et al. 1994). Live cell imaging of fluorescently labelled mitochondria and actin cables has also revealed that mitochondria are actively moved around the cell by the cytoskeleton (Fehrenbacher et al. 2004). In yeast binding of mitochondria to actin fibres requires a complex of linking proteins that can form a bridge between the actin cable and the mitochondrial outer membrane. The mitochore is a complex of three mitochondrial transmembrane proteins (Mdm10p, Mdm12p and Mmm1p) (Boldogh et al. 2003). Mdm10p/12p are located in the outer mitochondrial membrane while Mmm1p transcends both the inner and outer mitochondrial membranes, probably interacting with the mtDNA nucleoid in the matrix. A *Δmmm1* strain prevented binding of mitochondria to actin (Boldogh et al. 1998) and mutants in Mdm10p/12p also show loss of antero and retrograde mitochondrial movement. This structure has also been implicated in the stability of mtDNA. mtDNA nucleoids in a temperature sensitive *mmm1-1* mutant collapsed into aggregates and inheritance of mtDNA was compromised (Boldogh et al. 2003). The Arp2/3 complex, which binds to actin fibres and nucleates actin branching links actin to mitochondria via two accessory proteins Puf3p and Jsn1p. These proteins interact with the mitochore and loss of these genes effects mitochondrial morphology and movement, presumably leading to unequal energy distribution within the cell (Garcia-Rodriguez et al. 2007).

For some years a focus of research has been to determine the driving force which pushes mitochondria along the actin fibre during anterograde movement. In mammalian systems actin motor proteins, myosins, are involved. Until recently the class V myosin Myo2p had been implicated in mitochondrial inheritance into the bud tip and retention of mitochondria in the new daughter cell (Boldogh et al. 2004). Myo2p and its associated myosin light chain (Mlc1p) has now been shown to be required for mitochondrial binding to actin in vitro. A reduction of Myo2p or Mlc1p activity results in a loss of anterograde mitochondrial movement, inheritance in to the bud tip and reduced mtDNA content (Boldogh et al. 2004).

Inheritance of Mitochondria and Anti-ageing Factors

An important function of the actin-mitochondrial bridge is the inheritance of mito-chondria into buds, which appears to be selective for healthy mitochondria (Klinger et al. 2010) and essential if the daughter cell is going to be born at a young age. Boldogh and Pon have suggested a model by which the actin cytoskeleton could be used to achieve inheritance of the fittest mitochondria (Boldogh and Pon 2006). Normally functioning mitochondria produce a membrane potential by pumping pro-tons from the matrix to the intermembrane space. This proton motive force drives ATP generation. It also is required for the correct import of nuclear encoded and cytoplasmically synthesised mitochondrial proteins. A number of these proteins appear to be involved in the complex required for the interaction with the myosin motor required for anterograde transport into the daughter cell. Lack of these pro-teins, it is postulated, would leave the effected mitochondria either attached to the actin fibre but unable to be moved in the anterograde direction, or they would fall off the actin fibre thus the mitochondria would be unable to undergo retrograde trans-port and would remain in the mother cell. Thus the actin cytoskeleton may be able to function as a quality control mitochondrial sorter during cell division. Newly emerg-ing cells have also been found to possess a greater capacity to reduce ROS levels than their progenitor mother cells. The mechanism of daughter cell ROS clearance was shown to utilise the actin cytoskeleton and the function of the Sir2 protein, a member of the Sirtuin family that have been shown to be a key regulators of aging in a variety of organisms (Longo and Kennedy 2006; Blander and Guarente 2004). Treatment of dividing cells with the actin disrupting drug Latrunculin-A prevented the protection of newly forming cells from ROS accumulation, implicating actin in this process. Therefore actin is involved in the segregation of mitochondria and ROS detoxification mechanisms into newly forming yeast cells, further strengthen-ing the suggestion that this part of the cytoskeleton is important in the regulation of processes linked to cellular ageing.

Actin and Mitophagy

The distribution and quality control of mitochondria is important for the healthy lifespan of dividing cells. Despite inbuilt detoxification systems mitochondria are exposed to the danger of ROS induced damage, which in turn may lead to the accu-mulation of damaged mitochondria and threaten cell health. The maintenance of a healthy population of mitochondria is ensured by a specialized autophagic mecha-nism called mitophagy (Kanki and Klionsky 2008). A set of approximately 20 ATG (autophagy) genes have been identified in yeast, those involved in selective degra-dation of mitochondria ATG9 and ATG11 require a functional actin cytoskeleton. This has been observed in the *act1-159* mutant, which exhibits reduced ATPase activity and so a less dynamic cytoskeleton. The reduced dynamic status of this mutant leads to aberrant localisation of Atg11p, and to defects in Atg9p cycling and the Cytosol to vacuole targeting (Cvt) pathway (He et al. 2006). Atg9p localises

to the mitochondria and interacts with Atg11p which appears to target them in an actin dependent manner to the pre-autophagosomal structure, which then fuses with the vacuole (He et al. 2006). A further study observed the movement of Atg9 using real-time fluorescence microscopy in living cells (Monastyrska et al. 2008). A component of the Arp2/3 complex, Arp2p, was found to co-localise with Atg9 and to regulate the dynamics of Atg9 movement. The authors proposed that the Arp2/3 complex and actin are involved in the regulation of Atg9 transport for specific types of autophagy (Monastyrska et al. 2008).

Maintenance of mitochondria in peak condition and at optimal levels is vital to the cells well being. The research outlined above clearly suggests that the accumulation of damaged mitochondria and a failure to remove these aberrant organelles is linked to the integrity of the actin cytoskeleton. This relationship places actin at the centre of cellular homeostasis and processes that are tightly linked to the ageing process. In addition actin is directly influenced by the oxidative burden on a cell (discussed in the next section), so setting up a positive feedback loop that may contribute to cellular decline during the ageing process.

Actin as a Target and Effecter of the Oxidative Stress Response

The Relevance of Actin Oxidation in Mammalian Cells

It has been known for some years that in mammalian cells the actin cytoskeleton is regulated by oxidative stress and can be a target of oxidative damage, in particular through the oxidation of its cysteine residues (Dalle-Donne et al. 2001). Most of what we know about the cellular response of mammalian cells to oxidative stress come from studies of cell lines in culture treated with an exogenous source of reactive oxygen species (ROS), usually H_2O_2, menadione, or diamine. Despite the fairly diverse origins of these cells lines the responses are fairly common and include membrane blebbing, a rise in intracellular calcium levels, cell rounding with loss of cell adhesion, the formation of phalloidin stainable actin aggregates, and a general increase in cellular F-actin content (Mirabelli et al. 1988; Hinshaw et al. 1986, 1988; Omann et al. 1994). Although the term aggregates is commonly used, this is misleading as there is no reason to believe that these actin structures contain unfolded actin, quite the opposite: since they stain with phalloidin they must be constructed of actin filaments. In fact, in one case electron microscopy suggests that these filaments are well organized into parallel bundles of actin filaments (Hinshaw et al. 1988).

Examination of actin from oxidatively stressed cells on non-reducing gels shows the presence of molecular weight species consistent with the formation of dimers and even higher molecular weight forms (Mirabelli et al. 1989; Bellomo et al. 1990). These are presumed to reflect disulfide cross-links since in reducing gels the actin of the same samples run as a single species of monomer size. Interestingly, from menadione treated hepatocytes a faster migrating form of G-actin can be observed in non-reducing gels. We have made a similar observation for purified

yeast actin and have shown that the faster migrating form has a C285-C374 intra-molecular disulfide bond (D. Amberg, unpublished observations). In addition to disulfide cross-links, several studies have reported the glutathionylation of actin on cysteine 374. In one case glutathionylation occurs in response to oxidative stress (Rokutan et al. 1994), in the second case de-glutathionylation occurs upon epidermal growth factor stimulation of quiescent human epidermal A431 cells (Wang et al. 2000) while in the third case it is induced by activation of the respiratory burst in human neutrophils (Chai et al. 1994). In general, it appears that glutathionylation of actin on Cys374 does not favor filament assembly or stability. Glutathionylated actin prepared in vitro has increased ATPase activity, an increased critical concentration and forms filaments that are sensitive to shear stress (Drewes and Faulstich 1990; Stournaras et al. 1990; Dalle-Donne et al. 2003). These results agree with in vivo observations of EGF-induced de-glutathionylation of actin that is followed by an increase in F-actin content (Wang et al. 2001). My laboratory has been unable to detect glutathionylation of yeast actin in vivo (D. Amberg, personal observations) and therefore it will not be discussed further in this review.

The properties of actin filaments assembled from oxidized actin subunits provide insight into ROS-induced alterations in the organization of the actin cytoskeleton. For example, treatment of actin in vitro with H_2O_2 renders 2 of the 5 cysteines non-reactive to maleimide and the resulting actin has a slower rate of polymerization, a longer lag phase, an increase in critical concentration and the resulting filaments do not cross-link well with proteins such as filamin (Dalle-Donne et al. 1995). Disulfide bonded actin dimers generated in vitro have been shown to assemble into F-actin and induce filament cross-links (Tang et al. 1999), while in other cases H_2O_2 treatment of actin was found to induce C374-C374 dimers that could not assemble into actin filaments (Faulstich et al. 1992), could not interact with profilin and induced the disassembly of existing actin filaments (Lassing et al. 2007).

Perhaps the best understood, and most relevant example of actin oxidation is the C285-C374 intra-molecular disulfide bond that has been found to accumulate in the actin of sickle red blood cells. In many ways, sickle cell anemia is a disease of oxidative stress (Hebbel et al. 1982); the mutant hemoglobin releases free Fe^{++} that in Fenton reactions can react with oxygen to generate high levels of ROS (Hebbel et al. 1988). For this reason, RBCs of sickle cell patients are in a state of chronic oxidative stress that is compounded by low levels of reduced glutathione (Lachant et al. 1983). In sickle cell patients that are in crisis, a form of the sickle RBC accumulates called the irreversibly sickle red cell (ISC) that is locked into the sickle shape (Kaul et al. 1983). The ISCs are of interest as they are believed to contribute to vaso-occlusion because of their unusual lack of morphological plasticity. This lack of plasticity was shown to be due to oxidative damage to the membrane cytoskeleton (Lux et al. 1976), specifically the accumulation of C285-C374 intra-molecular disulfide bonded actin (Shartava et al. 1995; Bencsath et al. 1996) such that it accounts for upwards of 90% of the actin in these cells. When purified, this form of oxidized actin was found to form filaments that disassemble unusually slowly and incompletely (Shartava et al. 1997) thereby explaining the lack of morphological plasticity of these cells. These studies are particularly relevant in that cysteines

285 and 374 are universally co-conserved in all actin isoforms, including yeast actin suggesting that this form of actin oxidation is likely to occur in all eukaryotes.

Actin Oxidation in the Yeast Model

Compared to work in mammalian cells, analysis of the roles of actin oxidation in yeast is in its infancy. However, the system has much to offer and that has allowed new insights to be made in a relatively short time frame. First of all, yeast (*S. cerevisiae*) has a single, essential ß-actin gene. This fact coupled with the ease of genetic manipulation means that oxidation resistant mutants can be rapidly made in the normal chromosomal locus and their phenotypic effects quickly analyzed. Furthermore, yeast actin is highly conserved with 87% identity and 100% similarity to human ß-actin. Yeast actin has 4 of the 6 cysteine residues found in human actin and these are the most highly conserved cysteine residues within the family of conventional actin isoforms: C17 and C217 are found in nearly all actin isoforms while C285 and C374 are universally co-conserved. Given the rather devastating effects of C285-C374 oxidation in sickle cell crisis, it begs the question as to why natural selection would favor retention of these cysteine residues. Mutation of either or both C285 and C374 to alanine in yeast is well tolerated under normal growth conditions (Haarer and Amberg 2004) and when these mutants are purified they display normal polymerization behavior (unpublished). Therefore, these cysteines do not appear to be involved in core actin functions but have perhaps been retained for the purpose of regulation.

Recently, we have found evidence that actin's cysteines function in part to help protect cells from acute exogenously applied oxidative stress. Treatment of yeast with H_2O_2 induces a rapid change in the organization of the actin cytoskeleton: actin cables rapidly disappear, cortical patches cease to move and begin to fuse to form large foci of F-actin that we refer to as oxidized actin bodies or OABs. In agreement with a protective role, mutation of actin's cysteines blocks the formation of the OABs and makes the cells very sensitive to exogenous oxidative stress. Therefore, it appears that a function of actin's cysteines is to protect the cells from severe oxidative stress by sequestering actin and associated proteins into OABs. We are currently unsure of the protective mechanism of the OABs but it may relate to the prevention of continued polarized cell growth and/or endocytosis until the cell is able to repair oxidative damage to its protein, lipid and nucleic acid constituents (D. Amberg, unpublished observations).

Lastly, although the data is preliminary at this time, an apparent actin redox cycle may act as a ROS buffer. Untreated cultures expressing either the C285A or C374A actin mutants have slightly increased numbers of ROS positive cells and elevation of endogenous ROS levels by up-regulation of respiration has a strong negative impact on the growth of C285A or C374A mutants versus wild type cells. This model is attractive as actin is a very abundant protein in most if not all cells and could therefore have a very high ROS buffering capacity. At this time no one knows what systems keep actin reduced but we have found some evidence that in yeast the

glutaredoxins may also contribute to actin redox regulation. The glutaredoxin system is well conserved and known to participate in the reversal of oxidation induced protein disulfide bonds (Herrero et al. 2010). Furthermore, reduced glutaredoxins are replenished by reduced glutathione which is scarce in oxidatively stressed sickle RBCs thereby possibly explaining the accumulation of C285-C374 actin in ISCs.

Actin is an abundant and arguably the most functionally diverse protein found within cells where it participates in a myriad of cytoplasmic processes as well as core nuclear functions (Bettinger et al. 2004). Therefore, it is not surprising to find that it may play an important role in the yeast oxidative stress response. Although it is yet to be determined if the same is true in mammalian cells, it seems likely to be so given the high amount of functional conservation within the cytoskeleton. Our current model for actin's roles in the oxidative stress response is that under normal stress conditions, the metabolic production of ROS can lead to actin oxidation but this is rapidly reversed by the action of cellular redox systems and this actin/redox system is partly responsible for the inactivation of ROS in these cells. Under extreme conditions of oxidative stress, most of the actin is trapped into a non-dynamic and protective pool of F-actin aggregates (the oxidized actin bodies). These OABs assist in allowing at least a subset of cells to survive the oxidative insult.

Future Perspectives

The importance of cytoskeletal damage and the downstream consequences for cell populations, or tissues, is likely to be of particular importance during the process of yeast ageing (summarized in Fig. 15.3). This conclusion is drawn from the fact that actin is sensitive to the oxidative damage that occurs as a consequence of cellular ageing and that the dynamic status/damage level of the cytoskeleton appears to modulate downstream signaling events. It also seems to be clear that cell fate is linked to the stability of the cytoskeleton, with actin's dynamic nature being closely linked to apoptosis in plant, fungal and animal kingdoms. The close relationship that appears to exist between actin and the function of mitochondria is likely to play a crucial role. As this relationship appears to have been maintained in divergent eukaryotic systems, further studies in yeast should help to further our understanding as to how actin and the mitochondria communicate. Perhaps, as rapid actin dynamics are reliant on high cellular levels of ATP, there exists energy based signaling between these systems. Additionally, actin offers structural and positional support to the mitochondria, which are both likely to be significant in maintaining cellular homeostasis. Actin has also been shown to play a role in the regulation of translational accuracy (Kandl et al. 2002) which may feed into the production of inaccurate, or aggregating, proteins as cells age and the cytoskeleton accumulates damage. Of particular interest for future studies will be to increase our understanding of how accumulated damage to the cytoskeleton is perceived within tissues and cell types linked to age-related disease, for example within post-mitotic populations, such as neurons, and what the consequences are for cell fate. Are there particular cells that are particularly sensitive to, or cannot easily repair, the actin
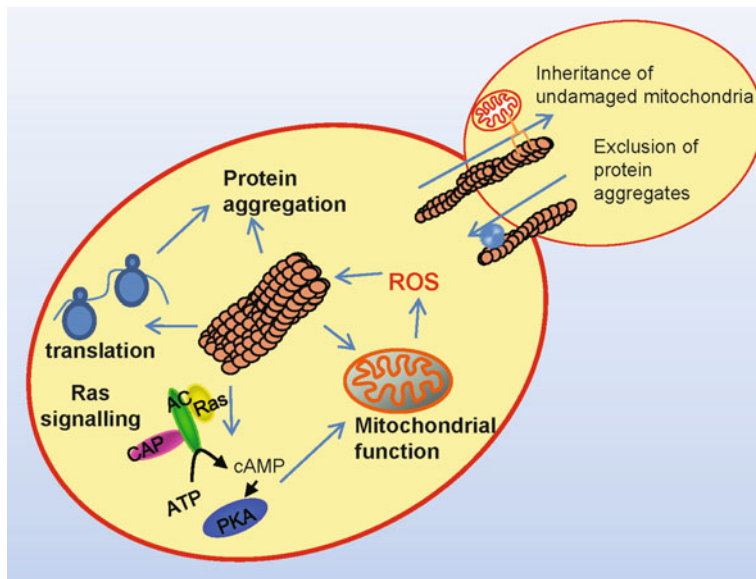
Fig. 15.3 Actin's role in the stochastic process of ageing. Actin has been shown to play a role in various cellular processes that have an impact on the ageing process. The formation of actin aggregates in aged or mutated cells is thought to trigger mitochondrial dysfunction and concomitant ROS production via inappropriate activation of the Ras/cAMP/PKA signaling cascade. Actin has also been shown to play a role in the regulation of translational accuracy and the formation of protein aggregations with an amyloid configuration such as prions and expanded poly-Q repeat proteins. The actin cytoskeleton also appears to be required for the inheritance of so called anti-ageing factors and for the inheritance of mitochondria into newly forming cells

damage incurred as cells age? If so, is the cytoskeletal dysfunction within such cells linked to increased cellular senescence, elevated levels of programmed cell death or disease pathologies associated with ageing?

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