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Animal cell cytokinesis: the role of dynamic changes in the plasma membrane proteome and lipidome.

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Summary

In animal cells, cytokinesis is characterised by the formation of the mitotic spindle that signals the assembly of an actomyosin ring between the spindle poles. Contraction of this ring drives ingression of the cleavage furrow, and culminates in the formation of a thin intercellular bridge between the daughter cells. At the centre of this bridge is the midbody, which is thought both to provide a site of attachment for the plasma membrane furrow and act as foci for the spatial and temporal control mechanisms that drive abscission. This review will focus upon recent studies that offer new insight into these events, in particular studies that elaborate on the mechanism of attachment between the furrow plasma membrane and the underlying cytoskeleton, and how dynamic changes in membrane composition might underpin key aspects of cytokinesis.

Key words:

Cytokinesis/centralspindlin/anillin/ESCRT complex/abscission.

Abbreviations.

ALIX, apoptosis-linked gene-2 interacting protein X. ANCHR, abscission/NoCut Checkpoint Regulator. CHMP, charged multivesicular body protein. CPC, chromosome passenger complex. ESCRT, Endosomal Sorting Complex Required for Transport. GFP, green fluorescent protein. GUV, giant unilamellar vesicles. LBPA, lyso-bisphosphatidic acid. PI3P, phosphatidylinositol 3-phosphate. PI4P, phosphatidylinositol 4-phosphate. PI4,5P₂, phosphatidylinositol 4, 5-bisphosphate. PI3,4,5P₃, phosphatidylinositol 3, 4, 5-trisphosphate. RBD, Rho binding domain.

1. Introduction.

Cytokinesis drives the physical scission of the daughter cells at the completion of mitosis. In animal cells, cytokinesis begins in anaphase with the reorganisation of the mitotic spindle and proceeds via the formation of a contractile ring at a site positioned equatorially between the two spindle poles; contraction of this ring drives the formation of a cleavage furrow. After completion of furrowing, a thin intercellular bridge, that has at its centre the midbody, separates the daughter cells. The midbody provides both a means to anchor the ingressed furrow and acts as a platform for the regulated assembly of the abscission machinery. Abscission involves regulated membrane trafficking into the intercellular bridge followed by the step-wise assembly of the Endosomal Sorting Complex Required for Transport (ESCRT)-machinery that is believed to catalyse the final scission event [1-4].

It is apparent, even from an overly simplified synopsis such as the above, that cytokinesis involves structural upheavals and dynamic membrane remodelling events at many stages, from early furrow ingression, through stable anchoring of the furrow to scission. We are fortunate from a range of genetic screens and proteomic analyses to have a reasonably detailed idea of the genes/proteins involved, and many excellent reviews have over the past few years provided a wide-ranging and clear analysis of the steps and players in animal cell cytokinesis. These include central spindle assembly, cleavage plane specification, the assembly and constriction of the actomyosin ring and the mechanics and regulation of abscission [4-6].

By way of contrast, this review will focus mainly on events at the plasma membrane during cytokinesis; as most of the experimental work I will discuss has utilised mammalian cells in culture, this review will focus largely on animal cell cytokinesis. Specifically I will discuss: how the membrane of the furrow may be anchored to the intracellular machinery during furrowing and abscission and how this interaction is regulated. How does RhoA get activated at specific spatial coordinates to drive the equatorial furrow? How do the physico-chemical properties of the plasma membrane change and control aspects of furrowing and abscission? And how is the temporal control of cytokinesis achieved? Recent studies have provided fascinating insight into these areas.

2. How does the plasma membrane engage the furrow machinery?

2.1 *Centralspindlin – a multi-tasking complex that links the plasma membrane to the spindle and spatially activates RhoA in the furrow.*

Centralspindlin, a heterotetramer comprised of a dimer of the kinesin-6 motor protein MLKP1 and a dimer of a Rho-GTPase activating protein MgcRacGAP (Cyk4), is a well-established regulator of cytokinesis [7]. MLKP1 is composed of an amino-terminal motor domain joined to a carboxy-terminal globular domain via a parallel coiled-coil and a long linker domain [8]. This long linker domain interacts with the amino terminus of MgcRacGAP that is in turn assembled from an amino terminal coiled-coil domain, a putative C1 domain (see below) and a carboxy-terminal RhoGAP domain. This complex localises to the centre of the spindle midzone in anaphase where, together with other proteins it participates in the formation of antiparallel arrays of microtubules and the midbody (for detailed review, see [7]). Phosphorylation of MgcRacGAP/Cyk4 by Polo-like kinase-1 facilitates the interaction of this complex with the Rho guanine nucleotide exchange factor ECT2. This drives the activation of the small GTPase RhoA and initiates the formation of the furrow and the assembly of the actomyosin ring [7].

Centralspindlin is also involved in the recruitment of some of the abscission machinery prior to the final cleavage event [9]. Thus, the multi-functional centralspindlin complex has come to be regarded as the ‘conductor of the cytokinetic orchestrator’ [7, 10]. The constriction of the actomyosin ring proceeds until the daughter cells remain separated only by a thin intercellular bridge, rich in microtubules, with the midbody at its centre. One of the mysteries of this process is how the plasma membrane of the furrow is linked to the midbody. Recent studies have identified a key role for the centralspindlin complex in this association.

A number of proteomic and genetic studies have identified a long list of proteins known to be involved in cytokinesis or associated with midbodies [11]. Lekomtsev and colleagues examined these components for the presence of membrane-association motifs, and identified a poorly characterised but evolutionary-conserved C1 domain in MgcRacGAP (shown schematically in Figure 1A) [12]; C1 domains are known to function as lipid-binding domains, raising the interesting possibility that a similar function may be integral to the function of MgcRacGAP. Mutations within this C1 domain, including point mutants within the structural ‘core’ of the protein, impaired cytokinesis,

suggesting that this C1 domain played a functional role [12]. Careful analysis of the myriad of functions of MgcRacGAP revealed that the C1 domain was not required for the formation of the centralspindlin complex nor did deletion of the C1 domain impair either localisation of the complex or the recruitment of other cytokinetic regulators, suggesting that this domain had hitherto played an unrecognised role in cytokinesis [12]. Using GST-fusion proteins, Lekomtsev and colleagues revealed that the C1 domain selectively interacted with phosphatidylinositol-4, 5-bisphosphate (PI4,5P₂) and phosphatidylinositol-4-phosphate (PI4P), lipids known to be enriched in the furrow of dividing cells (section 5 below). Importantly, depletion of both of these lipids using engineered selective phosphatases resulted in the release of a fluorescently tagged C1 dimer from the plasma membrane, and mutations in the putative lipid binding domains of MgcRacGAP impaired both cytokinesis and lipid binding *in vitro*. These observations suggest that the ability to bind to the plasma membrane may be a key function of the C1 domain.

In an elegant series of experiments Lekomtsev tested this hypothesis by replacing the atypical C1 domain of MgcRacGAP firstly with a typical C1B domain from protein kinase C α . Typical C1 domains are characterised by tight binding to phorbol esters (or diacylglycerol) that forms a hydrophobic surface that interacts with membranes. Depletion of endogenous MgcRacGAP impaired cytokinesis; expression of the MgcRacGAP-C1B hybrid rescued this effect, but only upon addition of phorbol esters [12]. A similar restoration of cytokinesis was observed if a distinct membrane targeting sequence was substituted. Such observations suggest that the atypical domain of MgcRacGAP is involved in membrane tethering of centralspindlin, and this tethering is essential for completion of cytokinesis [12]. This was supported by live-cell imaging, which revealed that in cells expressing wild-type MgcRacGAP, the plasma membrane remains associated with the midbody until abscission. By contrast MgcRacGAP mutants with defective C1 domain function, while still localising to the midbody, did not support association of the plasma membrane and the midbody; the plasma membrane was frequently observed to detach from the midbody, leading to furrow regression and cytokinesis failure [12]. These data implicate centralspindlin as a point of attachment of the plasma membrane to the midbody, and suggest a model in which the ingressing furrow can physically bind the midbody at a key step, stabilising the furrow and perhaps

allowing the assembly of the abscission machinery once this association is established (Figure 1B).

Interestingly, MKLP1 has been shown to interact with the GTPase Arf6 [13]. Like many small GTPases, Arf6 is associated with the membrane by means of post-translational modification, in this case the addition of a myristoyl group at the amino-terminus. This additional means of interaction of the membrane and centralspindlin may serve to increase the avidity of the association (Figure 1B). A further potential role for Arf6/centralspindlin interaction will be discussed below.

2.2 Anillin – hold on tight or let me go?

The constriction of the actomyosin ring during furrowing requires continuous RhoA signalling and involves multiple interactions between cytoskeletal proteins including actin, myosin II, anillin, septins and regulatory molecules such as citron kinase [6]. Once the ring has closed to a diameter of 1-2 μm , the cortical ring transforms into the midbody ring; the formation of a stable midbody ring is essential for proper cytokinesis and this process requires the scaffold protein anillin which, after its Rho-dependent recruitment to the equatorial zone, is proposed to act as a link between the actomyosin ring and the furrow membrane [14]. Consistent with this, the amino terminal domain of anillin binds to several components of the contractile machinery, including actin, myosin and septins [15] and the carboxy-terminus contains a PH domain that binds both septins [14] and phosphoinositides (mainly PI4,5P₂) [16]. In *Schizosaccharomyces pombe*, Mid1p (the anillin orthologue) is released from the nucleus, whereupon it becomes anchored at the equator and recruits a range of other proteins in a carefully choreographed series of steps [17, 18]. Unlike animal cells, no involvement of Rho has been identified in *S.pombe*; although there is a lipid-binding PH domain in mid1p, it does not appear to be required for membrane association [19].

Recent studies have provided new insight into anillin function in these processes, and again highlight dynamic interactions with the plasma membrane. Kechard and colleagues used high-resolution imaging to probe the role of anillin in different stages of furrowing and midbody ring formation in *Drosophila* S2 cells depleted of endogenous anillin by re-expressing distinct domains of the protein (Figure 2A) [14]. They found that the amino-terminus of anillin interacts with the contractile ring and was able to

form structures resembling midbody rings, but these rings were unable to anchor the plasma membrane. By contrast, they found that domains within the carboxy terminal region of anillin could be independently targeted to the equatorial cortex of S2 cells where they formed septin-dependent structures not associated with either the contractile ring or the midbody [14]. This latter observation is consistent with previous studies showing that a septin-based anchor engages with anillin to trim away excess membrane from the furrow by a mechanism that might involve membrane shedding (or blebbing - see 4.3 below) [20]. Hence, a model arises in which the amino-terminal domains of anillin interact with the contractile ring and assist in the establishment of the midbody ring; in tandem, the carboxy-terminal domain may organise septins into a membrane anchor and facilitate membrane remodelling during ingression. Despite the presence of the PH domain in anillin, it was unclear if this anchor involved direct interaction of anillin with the plasma membrane.

A new structural comparison of anillin and the *S. pombe* orthologue Mid1p, has provided still greater insight into these functions [21]. The carboxy terminal domain of anillin, identified as a site of membrane attachment, contains an Anillin-Homology Domain (AHD). Analysis of the structure of the AHD revealed it to be comprised of two distinct domains: an anti-parallel coiled-coil domain and a β -sandwich domain (Figure 2B), both of which were well conserved in animal cells [21]. Using Dali structural alignment comparisons, Sun et al realised that the β -sandwich of anillin adopted a C2-like structure, similar to that found in Munc13; like many other C2 domains, this structure was found to tightly bind to anionic lipids, including PI45P₂ and phosphatidylserine *in vitro*. Mutation of this C2 domain disrupted anchorage of anillin at the cleavage furrow, and deletion of the lipid-binding loop of this domain impaired the ability of anillin to rescue cytokinesis in cells depleted of the endogenous molecule [21]. The same group also identified a RhoA binding domain (RBD) in anillin, and thus, when considered with the already identified PH domain, Sun et al reasoned that these three membrane association sites (RBD, AHD and C2 domains, cf. Figure 2A) might act synergistically to anchor anillin to the furrow (cf. Figure 1B). Indeed, mutation or deletion of any of these three membrane association domains disrupted the localisation of anillin to the furrow. Sun et al quantified the affinity of these domains for anionic lipids using surface plasmon resonance. They found that the AHD domain had a relatively weak affinity for PI4,5P₂ liposomes, with a K_d of $\sim 14 \mu\text{M}$. However, the addition of the PH domain to the AHD

domain increased the affinity of anillin to PI4,5P₂ and PS by a factor of 4. When considering the RBD/RhoA interaction (estimated as being $\sim 7 \mu\text{M}$), Sun et al concluded that the synergistic action of these three membrane-binding domains could result in up to a 50-fold enhancement of the affinity of anillin for PI4,5P₂ or PS containing membranes [21]. This multivalent interaction serving to increase the avidity of association is a theme we shall return to again below.

These data suggest that anillin might function to link the membrane and the contractile ring, with the amino-terminus of anillin binding the latter (Figure 2B and Figure 1B). In a delightfully simple experiment, Sun et al. showed that F-actin, when mixed with giant unilamellar vesicles (GUVs), does not associate with membrane structures. Addition of anillin resulted in the formation of actin bundles, consistent with the previously reported ability of anillin to bundle actin filaments. Addition of lipidated RhoA however resulted in a profound enhancement in the association of actin filaments with the GUVs, some of which became completely surrounded by actin [21]. These conceptually elegant experiments provide support for the hypothesis that anillin can link the actomyosin ring with the cleavage furrow membrane.

These analyses also offer an element of unification of cytokinesis in different organisms that were hitherto thought to utilise different means to anchor the contractile ring. Knockdown of anillin resulted in cytokinesis defects in HeLa cells. Expression of the C-terminal domain of anillin (AHD and PH domains) alone did not rescue this defect, but fusion of this domain with the either actin binding domains from other proteins or myosin regulatory light chain restored cytokinesis, suggesting that the main function of the amino terminal domain of anillin is stable binding to the actomyosin ring [21]. Remarkably the amino-terminal domain of Mid1p when fused to AHD-PH also rescued cytokinesis, supporting the notion that anillin and Mid1p are functionally equivalent [21]. This notion was further enhanced when Sun et al. solved the structure of the Mid1p carboxy terminal domain, which was found to be virtually superimposable with the corresponding region of anillin, despite only around 20% amino acid identity (Figure 2C). One notable difference was the lack of an apparent RBD in Mid1p, consistent with no role for RhoA in Mid1p localisation [19].

Unlike anillin, Mid1p was found to dimerise via the C2 domain. This dimeric Mid1p exhibits high affinity for PI4,5P₂, $\sim 0.12 \mu\text{M}$, but more modest affinity for

phosphatidylserine ($K_d \sim 1 \mu\text{M}$). Previous work (see below) has identified PI4,5P₂ in the equatorial region of *S. pombe* [22], prompting Sun et al to suggest that this could provide the equatorial anchor for Mid1p during cytokinesis [21]. This experimental *tour de force* has provided insight into the similarity of functions of anillin/Mid1p, and highlighted how subtle evolutionary modifications tailor the use of this protein to fulfil its function in closed or open mitosis.

The anillin story is, however, still far from complete, and a further as yet poorly examined function of anillin is worthy of mention: the transition from complete contraction of the actomyosin ring and the establishment of a stable midbody involves significant membrane remodelling events. El Amine and colleagues used high-resolution spinning disc microscopy to study the dynamics of GFP-anillin in S2 cells. During the formation of the midbody ring, the ring appeared to thin accompanied by extrusion of GFP-anillin into blebs or tubules that were often completely shed from the cell; GFP-anillin was also found to be internalised into intracellular vesicles [20]. This dynamic redistribution of anillin occurred primarily in the phase leading up to the formation of the stable midbody ring. Hickson and co-workers proposed a model whereby the stable retention of anillin (via the action of the citron kinase Sticky) was in competition with the dynamic removal of anillin, in a mechanism involving the septin Peanut [20]. These observations suggest that the dynamic control of membrane remodelling is involved in the formation of a stable midbody, not only in the abscission event.

3. Oligomerisation of centralspindlin is important for distinct facets of its function.

Given the extraordinary range of functions of the centralspindlin complex, the Glotzer group tested the hypothesis that the membrane binding capacity of this complex described in 2.1 may be important for different facets of its biology [23]. It was already known that centralspindlin hetero-tetramers could assemble into further higher order structures via an oligomerisation signal in MKLP1. This oligomerisation is under active control; it is inhibited by 14-3-3 proteins and promoted by the mitotic kinase Aurora B, which phosphorylates MKLP1, releases 14-3-3 and thus facilitates centralspindlin binding to the spindle [24].

New work from Glotzer and colleagues has revealed that a similar mechanism underpins the cortical activation of RhoA [23]. In support of this hypothesis they showed that the 14-3-3 protein, PAR-5, inhibits membrane association of centralspindlin and prevents oligomerisation. This function of PAR-5 is antagonised by the Chromosome Passenger Complex (CPC) kinase, Aurora B. Activation of Aurora B in restricted spatial coordinates - at the central spindle and the equatorial plasma membrane - antagonise the function of PAR-5, and allow oligomerisation of centralspindlin; this in turn facilitates RhoA activation so driving furrow ingression in a spatially-specific manner. These data suggest that the main function of the CPC is to activate centralspindlin in space and time, and so trigger the formation of the furrow (Figure 3) [23].

This assembly of oligomers at the plasma membrane furrow region, in addition to activating RhoA at this site, could also offer a platform for membrane remodelling. In this regard it is important to note that the avidity of the interaction of the MgcRacGAP C1 domain with lipids is greatly increased by its presence in tandem repeats [12]– hence the oligomerisation of centralspindlin is likely to significantly enhance its membrane binding, although it should be noted that absence of the C1 domain in human MgcRacGAP did not inhibit furrowing (only stable association of the membrane with the midbody – see 2.1) [12]. This may serve both as an anchor during furrow ingression, and as mechanism for the stable association of the furrow with the midbody, as described above (Figure 3). A further clue as to the importance of this mechanism comes from the Mishima group, who showed that 14-3-3 proteins compete with Arf6 for association with centralspindlin [25]. Expression of centralspindlin mutants that can bind 14-3-3 proteins but not Arf6 results in the formation of unstable midbodies that collapse before abscission (cf. Figure 3). Hence, the release of 14-3-3/PAR-5 may reveal a cryptic Arf6-binding site in centralspindlin and help initiate or maintain its plasma membrane association. Using 14-3-3 proteins to control the aggregation state of proteins is not a new phenomena – nature is remarkably parsimonious; 14-3-3 mediated oligomerisation has been implicated in the regulation of p53 activity [26], potassium channels [27], Tau proteins [28] and even plant ATPases [29]. The multiplexing of different associations may well underlie the control of centralspindlin oligomerisation at the central spindle and the plasma membrane, and is reminiscent of that discussed above for anillin. When harnessed to specific changes in lipid composition at defined spatial coordinates, these

kinds of mechanisms can be a powerful force for tight and specific interactions of proteins with membranes.

This membrane association might also regulate the delivery of other membrane compartments into the region of the ingressing furrow and midbody. Prekeris and colleagues identified an interaction between MgcRacGAP and FIP3, a component of recycling endosomes that accumulate in the intercellular bridge in late telophase [9]. It is tempting to speculate that the coordinated action of Aurora B, centralspindlin and membrane associations might serve as an important control node in the switch from furrowing to abscission. Dissecting the relative contributions of these different facets of centralspindlin biology will present an interesting experimental objective for the next few years.

4. Organisation and physico-chemical properties of the plasma membrane in cytokinesis.

4.1. Corrals, pickets and fences: domains of the plasma membrane in cytokinesis.

The idea of diffusional barriers within cell membranes was given impetus from the pioneering studies of Kusumi and co-workers who have re-defined our understanding of the plasma membrane [30, 31]. Small-scale (nano-) domains generated from associations of lipids and proteins in a non-ideal mixture with varying degrees of mutual miscibility, such as those exemplified by cholesterol-rich lipid 'rafts', are an established biological phenomena; but Kusumi and colleagues also defined larger (meso-) scale domains, between 2-300 nm in size that serve to compartmentalise membrane function [30, 31]. These include spatial restriction for zones of endo- and exocytosis, the formation of tight junctional zones and the generation of cilia [32]. These processes harness a range of mechanisms, some still ill-defined, to generate areas of membrane tailored to specific functionalities. These domains may be short-lived (10 μ sec for Rhodopsin dimers) or longer lasting (10 ksec for cytoskeletal assemblies) [33]. The mechanisms that create these barriers remain relatively poorly studied, but include the concept of membrane corrals, in which proteinaceous fences act to 'corral' proteins within a specific domain and so-called 'picket fences' in which the underlying actin cytoskeleton could generate boundaries for the diffusion of membrane proteins with cytosolic domains [34]. Evidence for the latter includes dramatic increases in the

barrier-free walk distances of integral membrane proteins, measured using single molecule approaches, upon deletion of their cytosolic domains.

In the context of cytokinesis, Dobbelaere and Barral investigated membrane sub-compartmentalisation in yeast, and identified the formation of septin rings, adjacent to the actomyosin ring, as a key step in cytokinesis in *Saccharomyces cerevisiae* [35].

Septins form a ring at the neck of the growing bud, and were shown to recruit actomyosin components, which form the contractile ring that subsequently constricts between the two rings of Septins. Dobbelaere and Barral found that these septin rings act to restrict protein movement by establishing a cortical domain within which proteins could diffuse but not exit [35]. Examples of proteins localised within these structures were components of the exocyst complex (Sec3p), the polarizome (Spa2p) and an integral membrane protein called Ist2p; the latter will be returned to below. This work in yeast was subsequently extended into an analysis of the behaviour of proteins associated with the inner and outer leaflets of the cleavage furrow of dividing mammalian cells. These studies revealed that there was a block in diffusion of proteins with a cytosolic domain, but no barrier to the free diffusion of either lipid analogues or proteins anchored to the outer leaflet; again, a role for septins in the maintenance of this diffusional barrier was posited [36]. Such studies lead to the idea that specialised domains of the inner leaflet of the plasma membrane adjacent to the actomyosin ring contribute to cytokinesis.

This is consistent with the localisation of sterol-rich domains to the furrow notably in fission yeast, zebrafish and mammalian cells [37-39], dynamic alterations in plasma membrane phospholipid distributions (described in 5 below), and the accumulation of phosphoinositides; phosphatidylinositol 3-phosphate (PI3P), PI4P and PI4,5P₂ have all been shown to accumulate in the furrow membrane and act to recruit a range of key cytokinetic molecules (for review, see [40]). So, what might these domains represent, and how could the lipids in such domains influence this process? Some recent studies have begun to offer new insight into these elusive structures.

4.2. Ist2p – an ER-based microdomain at the bud neck?

mRNA encoding Ist2 is transported into the growing bud during *Saccharomyces cerevisiae* division. Ist2p accumulates in the bud, and the polarised distribution of this

protein is maintained by the septin rings described above [41]. The alignment of new views of membrane compartmentalisation coupled with more detailed analysis of Ist2p has offered a different view. Ist2p is in fact localised almost exclusively within the endoplasmic reticulum (ER) membrane, where it acts to tether the ER to the plasma membrane at so-called cortical-ER sites [42]. The close apposition of plasma membrane and ER membranes is not a new phenomenon and it has a well-established role in non-vesicular transport of lipids, and also in cell signalling [43]. Ist2p was found to act as a tether, linking ER to the plasma membrane, perhaps via association with phosphoinositides [42]; hence the role of septins might be to act as a barrier to the movement of the ER-membrane associated material across the bud neck [34]. This has two remarkable consequences. First, the Barral lab have recently shown that this diffusional barrier applies to ER-membrane associated proteins, not ER-lumen proteins, and that mis-folded proteins in the ER do not pass from mother to daughter cell, thereby confining ER 'stress' to the mother [44]. This barrier was septin-dependent, as one would have predicted from the studies alluded to above, but also was found to be dependent upon sphingolipids and required the activity of sphinganine C4-hydroxylase (Sur2p). This led to a proposal that the role of septins at the bud neck might therefore be to scaffold the formation of ER-plasma membrane contact sites to control the production and/or delivery of specific lipids to these domains [44]. Support for this notion comes from a recent report revealing localisation of a phosphatidylinositol/phosphatidic acid exchange protein at ER-plasma membrane contact sites [45]; this exciting observation suggests that these specific domains may serve as a focal point for the modulation of plasma membrane lipid composition. We shall return to this issue in section 5.

4.3. Membrane blebs and tension release – new control points for furrowing and abscission?

Membrane blebs have been observed at the poles of dividing cells for many years [46], where they have been proposed to act as release valves for cortical contractility, based largely on mathematical modelling. The idea is that these blebs can act as 'pressure sinks' to reduce contractile tension. Bleb nucleation can be driven by localised detachment of the membrane from the underlying cortex, and may be regulated by changes in lipid composition or the disengagement of protein anchors via active

modification of the cell membrane inner leaflet [46]. Although first studied in the context of polar blebs, the recent identification of blebs and shedding events in the midbody [20] offers the potential that such a mechanism could engage with or regulate the abscission machinery. Support for this idea comes from the demonstration that abscission is regulated by release of tension in the intercellular bridge, at least in animal cells. Prompted by observations that abscission times decrease when cells are closely packed, Lafaurie-Janvore et al found, somewhat counter-intuitively, that the more cells could move apart, the longer abscission was delayed [47]. Using traction force Microscopy and laser-ablation approaches, they found that there was a strong correlation between intercellular bridge tension and the speed at which the daughter cells separated; faster separating cells exerted a strong pull on their intercellular bridge. Careful measurement of bridge forces showed that tension in the bridge delays abscission. This group then extended this analysis by showing that the force on the intercellular bridge is transmitted to the abscission machinery, specifically the *rate* of recruitment of the ESCRT-III complex (but not the *extent* of ESCRT-III recruitment) at the abscission sites (ESCRTs are discussed in 6 below) [47]. Stronger forces delay ESCRT-III recruitment, suggesting that forces that regulate intercellular bridge tension directly impact the abscission machine. This is consistent with a mathematical analysis that suggests that ESCRT-III assembly/function in the intercellular bridge may be controlled directly by interaction with the membrane [48], and implies that forces acting on the membrane, perhaps mediated by changes in the mechanical properties of the membrane, can influence abscission via the physio-chemical properties of the intercellular bridge plasma membrane. Consistent with this, the forces required to penetrate the membrane of dividing cells are substantially greater than non-dividing cells [49]. While this may in part be a consequence of changes to the sub-cortical cytoskeleton, changes in the mechanical properties of the membrane itself are also likely to be important (see 5).

5. Cytokinesis and the wonderful world of lipids.

Cytokinesis is accompanied by changes in cell shape and the dynamics of trafficking pathways to and from the cell surface. These changes are predicated on a dynamic plasma membrane, and likely are underpinned by changes in the distribution and/or

composition of membranes, at both a gross (total) and micro (e.g. within the furrow) scale. The idea of lipid-based control of membrane protein function is well-established; recent work has identified lipid binding domains that can regulate membrane trafficking via direct interaction with coat proteins [50], lipid species acting as nucleating species in the assembly of membrane remodelling complexes [51] and lipids acting to control membrane protein interactions by regulating surface charge [52]. This, coupled with the examples described in section 2 in which the association of key cytokinesis proteins with membranes is revealed as being of functional importance, emphasises the need to understand the cells 'lipidome', and how this relates to cell function. Recent work from Riki Eggert's group has provided a remarkable insight into the influence of lipids on cytokinesis.

5.1. Specific lipids accumulate during cytokinesis.

Using electrospray ionization and tandem mass spectroscopy, Eggert and colleagues compared the lipidome of HeLa cells in cytokinesis with populations in S-phase, and also used a modified midbody preparation to examine the lipidome of the midbody-associated membranes [49]. Their data revealed a number of surprising results. Firstly, of thousands of different lipids detected by their mass spectroscopic analysis, only eleven were found to accumulate >4-fold in dividing cells. These were specific species from multiple families of lipids, suggesting that these changes are genuine, selective changes that accompany cell division. These included a novel sterol derivative and an ether/ester-linked phosphatidic acid; these latter molecules are thought to drive alterations in the arrangements of lipids within the membrane as a consequence of their (relatively) more hydrophobic head groups, but their functions remain somewhat elusive. Eight of the 11 species identified as accumulating in cytokinesis were sphingolipids derivatives, in particular a number of dihydroceramides [49]. Expressed at very low levels in non-dividing cells, the selective increase in dihydroceramides is suggestive of a specific role of this class of molecules during division, and is consistent with data showing that inhibition of glycosylceramide synthase caused cytokinesis failure [53].

Lipid bilayers display phase behaviour dependent on distinct, but coupled, order-disorder events: the first involves lipid chain packing and the other lipid chain

conformational ordering [54]. Different lipid species can exert profound effects on these properties, such as the well-described effects of cholesterol that can result in the formation of distinct equilibrium phase, the liquid-ordered phase, in which the bilayers are liquid but have translational disorder in which the lipid acyl chains are conformationally ordered [55]; this has profound implications for membrane function, and underlies the notion of 'lipid rafts' [55]. The accumulation of ceramides represent only a modest changes in the overall lipid profile of HeLa membranes (total ceramides represent <2% of HeLa lipids). Nonetheless, the small changes reported, when recapitulated in supported lipid bilayers *in vitro*, resulted in significant changes in both the topography of the lipid bilayers and their mechanical properties [49]. This is entirely consistent with the observations noted above that dividing cells are much more resistant to mechanical stress than their non-dividing counterparts, and suggests that the specific up-regulation of certain classes of ceramides may underlie this response.

5.2. The lipid composition of the midbody is distinct.

Previous studies, reviewed elsewhere [56], have revealed an important role for PI4,5P₂ and phosphatidylinositol 3, 4, 5-trisphosphate (PI3,4,5P₃) localisation to the furrow of dividing cells, probably linked to the regulation of actin polymerisation and membrane trafficking [56]. Using a modified procedure for the isolation of midbody membranes, Eggert and colleagues revealed that the lipidome of the midbody is subtly distinct to that observed in total membranes isolated from cells in cytokinesis [49]. Nine species of lipid accumulate in the midbody of dividing cells; five of these also accumulate globally in cytokinesis (5.1 above), and all are sphingolipids-derivatives, prompting suggestion that long-chain dihydroceramides play a role specifically at the abscission site. Interestingly, a number of species were identified to be markedly up-regulated in the midbody that were not up-regulated in dividing cells, suggesting that their *distribution* is subject to dynamic regulation during furrowing/formation of the intercellular bridge. These include a rare triacylglyceride and C24 hexosylceramide which are increased >30-fold and >50-fold, respectively. The importance of these molecules to the fidelity of cytokinesis is supported by an RNAi screen of 244 lipid biosynthetic enzymes performed as part of the same study. 23 genes exerted an effect on cytokinesis (increased the

frequency of binucleate cells), 11 of these are involved in sphingolipid metabolism, and another significant 'hit' was triacylglycerol synthase [49].

Phosphatidic acid and phosphatidylinositol also accumulate in the midbody [49], consistent with an important signalling role for these species and as targets for the localisation of proteins at different points in cytokinesis, a point discussed in section 2 for anillin and MgcRacGAP. The potential that the avidity of these lipid-protein interactions may be fine-tuned by the physico-chemical properties of the furrow membrane is an area for future investigation.

One final point of note from this study is the accumulation of a specific phosphatidylserine (18:0/20:4; stearoyl, arachidonyl) in the midbody [49].

Phosphatidylserine is synthesised in the ER and transported to the plasma membrane in part via vesicular transport. The localisation of ER to the bud neck might represent a distinct means of delivering specific lipid species into the abscission zone [42], a notion given credence by the specific localisation of phosphatidylinositol/ phosphatidic acid exchange proteins at these sites [45]; relatives of this protein could be responsible for the midbody-specific localisation of phosphatidic acid and phosphoinositides.

5.3. *Sphingolipid domains.*

Sphingolipids play a pivotal role in the organisation of the diffusion barrier in the ER of the bud neck in *Saccharomyces cerevisiae*, as described above [44]. Sphingolipids are thought to reside mainly in the plasma membrane yet the enzymes responsible for their biosynthesis are ER-localised. The realisation that ER and plasma membrane often reside in close proximity, for example in the bud neck in *Saccharomyces cerevisiae*, might offer a potential to marry the observations from several of the studies described here. Clay and colleagues suggest that the active, signal-dependent formation of a sphingolipid-enriched domain in the ER at the bud neck plays a fundamental role in division in budding yeast, offering a mechanism to confine ER stress to the mother cell [44]. In their model, septins at the plasma membrane organise Bud1 and Cdc42, two small GTPases that in turn organise ER-localised sphingolipids perhaps via Bud6p [44]. The possibility that this confinement is a secondary advantage of an as yet uncharacterised function of these sphingolipids should be considered, particularly given the striking changes in localisation and levels of sphingolipids/ceramides observed

during cytokinesis and in the midbody. It is important to add the caveat that the localisation of vesicles containing ceramide to the midbody has been reported [49]; hence cells may utilise more than one means to actively modulate ceramide localisation/levels.

6. Timing the final cut.

The molecules and mechanisms involved in regulating abscission, the final stage of cytokinesis, have been excellently reviewed in a number of recent articles, and as such will not be extensively reviewed here [1, 4, 6, 57]. Key to abscission is the orderly assembly of the abscission machine, beginning with the delivery of vesicles to the intercellular bridge to drive secondary ingression ('thinning') of the bridge and the delivery of Cep55 to the midbody which acts to nucleate the formation of the ESCRT machinery, the key membrane scission machine that results in separation of the daughter cells [1, 4, 6, 57]. A key question for the field is how abscission is temporally controlled with earlier events in cell division. It is known that in budding yeast, Aurora B inhibits abscission when chromosomes are present in the cleavage area via the 'NoCut' pathway [58]. Similar abscission delay mechanisms exist in animal cells, and are also dependent upon Aurora B [59]. It is now well accepted that Aurora B acts as an inhibitor of abscission; consistent with this hypothesis, chromosome bridges result in prolonged activation of Aurora B and consequently delayed abscission [4]. This is a logical extension of the important role of Aurora in earlier events in cytokinesis (centralspindlin oligomerisation and anillin function for example – sections 2 and 3 above); high levels of active Aurora B drive early events in cytokinesis and at the same time act to prevent late stages (abscission). Recent work has begun to illuminate the molecular detail of Aurora B regulation of abscission.

Late in telophase, midbody-localised Cep55 drives the regulated assembly of the ESCRT-III complex. CHMP4C is a subunit of the ESCRT-III complex; although not required for cytokinesis, CHMP4C acts as a negative regulator of abscission. Expression of GFP-tagged CHMP4B had little effect on abscission timing, and was found to transiently localise to the midbody before abscission. By contrast, expression of GFP-CHMP4C significantly delayed abscission [60]. A significant difference between CHMP4B and 4C is the insertion of a short serine-threonine-rich sequence in CHMP4C that contains a

putative Aurora B phosphorylation site. Carlton and colleagues revealed that phosphorylation of S210 of CHMP4C by Aurora B activated the NoCut abscission checkpoint in HeLa cells, and delayed abscission; when Aurora B activity falls, dephosphorylation of this site triggers abscission [60]. A direct interaction between Snf7, a component of the ESCRT-III complex and Borealin (a CPC component) was revealed by D'Avino and colleagues, who also showed that Aurora B phosphorylated an Snf7 paralogue, CHMP4C [61]. Agromayor and Martin-Serrano suggest that phospho-CHMP4C may act to compete with other ESCRT-III components, preventing either the correct localisation or assembly in the midbody until such time as Aurora B levels fall [57].

The key role for Aurora B in abscission control has been further emphasised by the recent identification of a PI3P binding protein called ANCHR (Abscission/NoCut Checkpoint Regulator) [62]. ANCHR associates with the ESCRT-III regulatory ATPase VPS4; in cells in which the NoCut pathway is activated ANCHR, VPS4 and CHMP4C all co-localise at the midbody ring, whereas in normally dividing cells, VPS4 localises to abscission sites adjacent to the midbody ring [62]. Stenmark and his colleagues postulated that ANCHR serves to spatially restrict VPS4 away from the abscission site until such time as Aurora B levels fall, when VPS4 moves to the abscission sites adjacent to the midbody ring. Consistent with this, VPS4, ANCHR and CHMP4C form a ternary complex in dividing cells, and levels of this complex are reduced upon chemical inhibition of Aurora B. The presence of a PI3P binding domain within ANCHR again emphasises the integral role of membrane association in these events, and further supports the key role of phospholipid species in the control of cytokinesis [62].

This idea has gained further traction with the identification of a novel ESCRT-III interacting protein, MITD1, which also regulates abscission [63, 64]. This protein has a microtubule-interacting and trafficking (MIT) domain at the amino terminus, and a unique phospholipase D-like domain at the carboxy terminus that shows a broad specificity to phosphoinositide-containing membranes [63]. MITD1-depleted cells fail cytokinesis either as a consequence of midbody instability or abscission arrest suggesting MITD1 may be yet a further means to anchor the cleavage furrow to the mitotic machinery [63]. MITD1 disruption was also found to lead to blebbing and premature abscission in cells that complete cytokinesis [63]. As noted in 4.3, blebbing

may serve as a means to regulate tension in the intercellular bridge; increased blebbing may reflect cortical instability and thus drive faster abscission. The key role of the lipid-binding domain of MITD1 is established; whether the PLD-domain has membrane remodelling activity remains unclear, but Agromayor and Martin-Serrano raise the interesting idea that if MITD1 were to modulate midbody levels of PI4,5P₂, this may drive midbody instability and membrane blebbing. Further work on this fascinating protein is warranted [57].

6.1. *Lipids and the control of ESCRT function.*

It has been established from a range of *in vitro* reconstitution experiments that ESCRT-III subunits can form polymers capable of driving membrane scission [65]. Present models of abscission involve an element of interaction between ESCRT-III components and the membrane. ESCRT-III proteins exhibit a preference of acidic membranes in *in vitro* binding studies [66] and have been shown to associate with PI3P- or cholesterol-enriched membranes during multivesicular body formation and HIV budding, in which analogous ESCRT-III-dependent membrane scission events occur [67-69].

The idea that the presence of a particular lipid in the midbody could trigger activation of the ESCRT machinery should be carefully considered in the light of an intriguing paper from Gruenberg's group in which a specific interaction of ALIX (apoptosis-linked gene-2 interacting protein X) with lysobisphosphatidic acid (LBPA) was revealed [51]. ALIX interacts with the ESCRT-I subunit Tsg101 and the ESCRT-III subunit CHMP4 (see section 6) and plays a role in viral budding. ALIX was found to interact with LBPA on endosomes via a Bro1 domain in a calcium-dependent manner and so triggers viral nucleocapsid release into the cytosol during viral infection [51]. They suggest that upon binding the membrane via LBPA and calcium, ALIX undergoes a conformational change, leading to the insertion of a hydrophobic loop into the cytoplasmic leaflet of the membrane, whereupon it engages the ESCRT-I and ESCRT-III machinery, perhaps forming a localised domain on the surface of endosomes that can regulate ALIX dimerization and CHMP4 assembly.

This idea that a specific lipid change can drive the functional assembly of the ESCRT machinery could perhaps be imagined to also underpin the assembly of the abscission zones late in cytokinesis, either via the chemical nature of the lipid physically interacting

with the ESCRT proteins, or by changing the physical nature of the membrane, perhaps via induced curvature or the presence of more 'fusogenic' lipids [70].

7. Concluding remarks.

The complex interplay of multiple proteins, multiple domains within proteins, and a defined subset of membrane lipids offer a bewildering array of mechanisms to control and fine-tune the cytokinetic apparatus. Challenges for the future include the design of lipid-specific probes to interrogate the function of the individual species and unravelling how these and other facets of the cytokinetic apparatus are coupled to spatial and temporal control systems.

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Figure Legends.

Figure 1 MgcRacGAP and the plasma membrane connection machinery.

A shows the domain structure of MgcRacGAP. The N-terminal domain is involved in interaction with MLKP1, a coiled-coil domain (cc) shown in red mediates dimerization, and the GTPase activating domain (GAP domain) is shown in grey. The C1 domain, discussed in the text, is shown in blue, and mediates interaction with phosphoinositides. **B** summarises several potential mechanisms for plasma membrane anchoring to the intercellular bridge microtubule network. MgcRacGAP/MKLP1 interactions offer one potential means to couple the plasma membrane in the furrow to microtubules; the phosphoinositide-rich furrow PM couples to MgcRacGAP via the C1 domain. Alternative coupling mechanisms could involve direct binding of MLKP1 to the Arf6 GTPase, which is myristoylated (shown as a red tail in the figure), or via interaction with anillin/citron kinase (the structural domains of anillin are colour-coded as shown in Figure 2A); Septin/RhoA binding localises Anillin to the plasma membrane which is linked to microtubules via citron kinase/MKLP1 (see text for details).

Figure 2 Anilin and Mid1p share structure similarities.

A shows the domain structure of anillin and Mid1p. The amino terminal region (shaded pink) comprises the formin binding domain, the myosin-binding domain and the actin-binding domain (FBD, MBD and ABD, respectively). These are followed by anillin-homology domain (AHD) comprised of the Rho-binding domain (blue) and the C2 domain, shown in orange. A PH domain is present at the carboxy terminus, and is shown in yellow. The domain structure of Mid1p is shown for comparison. **B** shows the structure of the AHD domain, with the RBD in blue and the C2 domain in orange; in the structure determined by Sun et al., the lipid binding domain is disordered and is represented by a dashed line. **C** shows a structural alignment of the C2 domains of human anillin (orange) and *S. pombe* Mid1p (cyan). **B** and **C** are reproduced from Sun et al, with permission.

Figure 3 Membrane association of centralspindlin drives localised RhoA activation during furrowing.

Centralspindlin hetero-tetramers (shown in yellow) can assemble into further higher order structures via an oligomerisation signal in MKLP1. This oligomerisation is under active control; it is inhibited by 14-3-3 proteins (pale yellow structures) such as PAR-5 and promoted by the mitotic kinase Aurora B, which phosphorylates MKLP1, releases PAR-5 and thus facilitates centralspindlin clustering (dark yellow clusters). Hence, one model suggests that a zone of active Aurora B in the equatorial regions could drive the formation of centralspindlin clusters; this in turn facilitates RhoA activation so driving furrow ingression in a spatially-specific manner. This assembly of oligomers at the plasma membrane furrow region, in addition to activating RhoA at this site, could also offer a platform for membrane remodelling. The avidity of the interaction of the MgcRacGAP C1 domain with lipids is increased by its presence in tandem repeats [12]– hence the oligomerisation of centralspindlin is likely to significantly enhance its membrane binding. This may serve both as an anchor during furrow ingression, and as mechanism for the stable association of the furrow with the midbody, as described above. Adapted from [23].