

Deep Insight Section

The role of ESCRT-III in cell division

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Summary

During cell division the genome is duplicated and segregated along with organelles into the daughter cells with high fidelity. The physical separation of the two daughter cells topologically resembles virus budding and multivesicular endosome (MVE) formation in the sense that they all involve outward budding from cytosol. According to the crucial role of the *Endosomal Sorting Complex Required for Transport* (ESCRT) machinery that pinches off membranes in MVE biogenesis and virus budding, extensive work from several laboratories during the last six years has now confirmed an essential role of ESCRT members also in cytokinesis. This Deep Insight highlights the current views explaining how the ESCRT machinery and associated proteins collaborate to drive abscission of the intercellular bridge at the correct time when DNA and organelles are successfully segregated. Even though the ESCRT machinery seems to play a central role for proper cytokinesis, it is important to emphasize that alternative mechanisms for abscission have been proposed. Before the role of ESCRTs in cell division was discovered, secretory vesicles and recycling endosomes were hypothesized to provide the molecular machinery required for abscission (Neto et al., 2011). However, this hypothesis is lately debated based on electron microscopy studies showing busy vesicle trafficking in the intercellular bridge early in cytokinesis but not when abscission occurs. Rather these vesicles seem to play important roles prior to abscission, in the thinning of the intercellular bridge, which will be described later.

Introduction

During mitosis the cell undergoes profound morphology changes, it rounds up and finally divides into two equal daughter cells (Green et al., 2012). Cell division is a highly regulated process in time and place to perfectly coordinate duplication of DNA and the physical division of the cell, implying precise regulation to coordinate entry and exit of the sequential phases. This fine-tuned control is employed by phosphorylation and de-phosphorylation of crucial mitotic factors. The master cytokinetic kinases Cyclin dependent kinase 1 (Cdk1), Polo like kinase 1 (Plk1) and Aurora B are active and modify essential proteins during initial, middle and late steps of cell division, respectively (Ma and Poon, 2011). Interestingly, lipids and phosphoinositides also seem to regulate cell division, in addition to provide extra membrane material required for the increase in cell surface required for the extensive morphology changes (Atilla-

Gokcumen et al., 2010; Brill et al., 2011; Emoto et al., 2005).

During the last steps of cell division, called cytokinesis, physical cleavage of the dividing cell occurs (Fededa and Gerlich, 2012). Cytokinesis can be divided into three major steps, the first ingression when the contractile actomyosin ring contracts and forms an intercellular bridge approximately 2 μm wide. The second ingression follows when the intercellular bridge is further constricted down to 200-100 nm in diameter. Finally, the intercellular bridge is pinched off during abscission, releasing two genetically identical daughter cells. Before this final cut, actin filaments and microtubules stabilizing the intercellular bridge must be severed. Recently, electron microscopy imaging has revealed that late in cytokinesis the intercellular bridge is decorated with spirals of thin filaments thought to represent the actual abscission complex (Guizetti et al., 2011). Yet it remains to characterize these spirals, but most likely they are made of ESCRT proteins given their potential to oligomerize and their important role in

cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007).

The ESCRTs

Originally the ESCRT proteins were found to be crucial for efficient sorting of transmembrane cargo destined for the lysosomes (vacuole) in yeast (Katzmann et al., 2001), and their role in lysosomal sorting is conserved in humans (Bishop et al., 2002). Endosomal sorting of cargo involves inward budding of the endosomal limiting membrane, sequestration of cargo in intraluminal vesicles (ILVs) whose content is degraded proteolytically when MVEs fuse with lysosomes. This special topology of budding resembles release of viruses and abscission in cytokinesis. The role of ESCRTs in these membrane fission processes has been investigated, and indeed the ESCRT machinery has been proven to support proper release of retroviruses such as HIV (Garrus et al., 2001; VerPlank et al., 2001) and cytokinetic abscission (Carlton and Martin-Serrano, 2007; Morita et al., 2007), in addition to support MVE formation. Extensive investigations have progressively dissected the detailed action of the ESCRT machinery (Henne et al., 2011). To date more than 20 different human proteins arranged in four separate ESCRT complexes exist - ESCRT-0 (Hrs and STAM), ESCRT-I (Tsg101, Vps37A-D, Vps28, Mvb12A/B, UBAP1), ESCRT-II (EAP20, EAP30, EAP45) and ESCRT-III (CHMP2A-B, CHMP6, CHMP3, CHMP4A-D, IST1). These multiprotein complexes are sequentially recruited to membranes and facilitate protein sorting, membrane deformation and scission. ESCRT-0 is thought to recognize and anchor the rest of the ESCRT machinery to the endosomal membrane and to its specific cargo, such as ubiquitinated growth factor receptor. As highlighted later, the nature of the substrate-recognition complex is membrane fission-specific (i.e. ESCRT-0 in MVE formation, Alix in virus budding, and Alix together with CEP55 in abscission) thus explaining why ESCRT-0 is less evolutionary conserved compared to ESCRT-I-II and -III. Nevertheless, when the specific endocytic cargo is recognized, early-acting factors, ESCRT-I followed by ESCRT-II, are recruited and facilitate accumulation of cargo, membrane bending and massive recruitment of ESCRT-III. Monomers of ESCRT-III are recruited from cytosol onto membranes, where they upon conformation changes are activated and form spirals of filament which facilitates constriction of membrane necks, thus pinching off ILVs, new viruses from the cell surface or intercellular bridges. This final cut is dependent on the AAA ATPase Vps4 which is specifically recruited to the membranes by interacting with ESCRT-III proteins (Hill and Babst, 2012; Howard et al., 2001). The number of proteins interacting with and assisting ESCRT family members in these similar but still diverse cellular events is steadily increasing (Roxrud et al., 2010). Even so, specific isoforms of ESCRT-I and

ESCRT-III proteins build complexes specifically engaged in abscission rather than virus budding and MVE formation, for instance (Morita, 2012). Moreover, recent data indicate that the action of ESCRTs in cytokinetic abscission is more complex regarding regulation, exemplified by the high number of proteins specifically involved in this ESCRT dependent process.

ESCRTs and cell division

The initial clue indicating that ESCRTs are required for proper cell division came actually from plant biology, where ELC mutants, the plant homolog of Tsg101 of ESCRT-I, gave multinuclear cells (Spitzer et al., 2006). A year later the role of ESCRT-I in cell division was confirmed in human cells when depletion of Tsg101 or the ESCRT-associated protein Alix, resulted in abscission defects and multinuclear cells (Carlton and Martin-Serrano, 2007; Morita et al., 2007). Furthermore, homologs of ESCRT-III were proved to support normal cell growth in *Archae* (Lindas et al., 2008; Samson et al., 2008), yeast (Köhler, 2003) and human cells (Carlton et al., 2008; Morita et al., 2007). Very interestingly ESCRT-III function in cell division seems to be more highly conserved in evolution compared to ESCRT-I function, since *Archae* do not express homologs of ESCRT-0, I or II (Samson et al., 2008). Also the involvement of Vps4 is conserved from *Archae* (Samson et al., 2008) to humans (Morita et al., 2007). Since endomembrane structures are absent in *Archae* it is thought that ESCRT proteins originally developed to function in cell division. Multinuclear cells are generally caused by cytokinesis failure (Lacroix and Maddox, 2012), further indicating that the ESCRT machinery mediates normal cytokinesis. This raises the question of how this machinery promotes cytokinesis.

Recruitment of ESCRTs to the intercellular bridge

A key player inducing cytokinesis is the centrosomal protein Centrosomal Protein 55 kDa (CEP55), which localizes to the intercellular bridge by associating the Mitotic Kinesin-Like Protein 1 (MKLP1) of the centralspindlin complex (Fabbro et al., 2005; Zhao et al., 2006). Plk1 prevents hasty onset of cytokinesis by phosphorylating MKLP1 and thus preventing its interaction with CEP55 (Bastos and Barr, 2010). Prior to the second ingression, CEP55 is present at the midbody recruiting factors required for the final abscission such as Alix and Tsg101 of ESCRT-I (Carlton et al., 2008; Carlton and Martin-Serrano, 2007; Morita et al., 2007). In the central hinge-domain separating the two coiled coil domains, CEP55 exhibits a so-called ESCRT and Alix Binding Region (EABR) domain shown to interact with GPPX₃Y motifs in Alix and Tsg101 (Lee et al., 2008). Efficient recruitment of Alix and ESCRT-I is ensured since CEP55 appears as dimer at the intercellular bridge (Zhao et al., 2006). The

significance of Alix and Tsg101 for complete abscission is further highlighted by the mechanism underlying formation of ring canals in male germ cells (Haglund et al., 2011). These ring canals connecting neighboring cells are formed by incomplete cytokinesis, mediated by Testis Expressed gene 14 (TEX14) which interacts stronger with CEP55 than Alix and Tsg101, and thus prevents ESCRT-mediated abscission (Greenbaum et al., 2009; Iwamori et al., 2010).

Alix and ESCRT-I recruit ESCRT-III members to the midbody (Carlton et al., 2008; Dukes et al., 2008; Morita et al., 2007). The BRO1-domain in Alix binds specifically to CHMP4, exhibiting an unique spacing sequence between the amphipathic helices compared to CHMP1-3 (Fisher et al., 2007; McCullough et al., 2008). During lysosomal sorting ESCRT-II is a crucial player bridging ESCRT-I and ESCRT-III (Babst et al., 2002; Langelier et al., 2006; Malerød et al., 2007). However, ESCRT-II seems to be dispensable for cytokinesis, and binding between ESCRT-I and III is conceivably circumventing the need for ESCRT-II in this process (Pineda-Molina et al., 2006). Indeed direct interaction between Vps28 of ESCRT-I and CHMP6 of ESCRT-III has been reported, but its significance for proper cytokinesis remains unclear. The ordered recruitment of CHMPs has been deduced from yeast and cell culture studies and *in vitro* assays using giant unilamellar vesicles. Upon recruitment to membranes the conformation changes and the open active monomers are able to associate with other CHMPs by interacting head-to-tail building long filaments which has been characterized in detail using electron microscopy (Henne et al., 2012). CHMP6 recruits CHMP4 which oligomerizes forming long filaments capped by CHMP3 and CHMP2 (Bajorek et al., 2009; Fabrikant et al., 2009; Ghazi-Tabatabai et al., 2008; Teis et al., 2008). Importantly, CHMP2A/CHMP3 remodels the flat CHMP4 spirals into three-dimensional cone-shaped spirals (Henne et al., 2012) which conceivably resembles the abscission spirals detected on the intercellular bridge by electron microscopy (Guizetti et al., 2011). The CHMPs are arranged to expose their membrane-associating domains on the outside of the filaments and their Vps4-interacting domain on the inside (Bajorek et al., 2009; Lata et al., 2008). Accordingly, electrostatic interaction between negatively charged lipids such as phosphatidylserine and phosphatidylinositol-3-phosphate (PI3P) in the membranes seems to further promote curling of CHMP4-filaments into spirals (Henne et al., 2012). The relevance of lipid composition in the intercellular bridge for ESCRT function is highly interesting and potentially subject of future investigation.

The ESCRT-III associated protein CHMP1 interacts with both CHMP2 and CHMP3 and links Increased Sodium Tolerance 1 (IST1) to the CHMP filaments (Dimaano et al., 2008; Xiao et al., 2009). Originally

IST1 was characterized as an ESCRT-III associated protein but was redefined as a true ESCRT-III protein (Bajorek et al., 2009). Importantly IST1 knock-down impaired cytokinesis but not retrovirus budding nor endosomal sorting in mammalian cells, only modestly in yeast (Agromayor et al., 2009; Bajorek et al., 2009; Dimaano et al., 2008; Rue et al., 2008; Xiao et al., 2009). Thus, IST1 is the first ESCRT-III protein which process-specifically associates the ESCRT machinery, further indicating that the ESCRT function during cytokinesis is more complex than in virus budding or endosomal sorting. Even though IST1 is shown to associate Vps37B of ESCRT-I, it seems more likely that this stabilizes rather than nucleates CHMP filaments at the midbody, given its late recruitment onto these filaments (Bajorek et al., 2009). Further IST1 possesses an important role in recruiting Vps4 to the midbody.

The role of Vps4 in ESCRT-mediated abscission

The AAA ATPase Vps4 is recruited from cytosol by ESCRT-III, and as many as 12 different interactions between Vps4 and ESCRT-III/associated proteins have been characterized, all involving the Microtubule Interacting and Trafficking (MIT) domain of Vps4 and MIT Interacting Motif (MIM) domains of ESCRT-III (Shestakova et al., 2010). Since the MITMIM bindings display modest affinity (K_d= 30 μM) (Obita et al., 2007; Stuchell-Brereton et al., 2007), numerous interactions are required for stable recruitment of Vps4 onto membranes. Despite the high number of interactions observed, *in vivo* analysis in yeast indicated that CHMP2 and CHMP4-interactions are superior compared to CHMP6 (Shestakova et al., 2010), in contrast to what observed by *in vitro* assays (Azmi et al., 2008; Kieffer et al., 2008). Upon closer examination it was shown that highest binding affinity to Vps4 was dependent on MIM2 which is only exposed in active, oligomerized CHMP6, in addition to the MIM1 at the C-terminus ubiquitously exposed regardless of conformation. By this delicate mechanism Vps4 is massively recruited to CHMP-filaments rather than inactive monomers in cytosol. High affinity binding between Vps4 and CHMP-filaments is further achieved since the Vps4-double ring complex composed of 24 subunits in total exhibit 24 MIT domains. Association of a pre-existing CHMP1-IST1-Vps4 complex with CHMP2 further ensure massive recruitment of Vps4 onto CHMP filaments (Bajorek et al., 2009), both directly as described above and indirectly. Membrane-attached and active Vps4 forms a dodecamer composed of two stacked hexamer rings (Gonciarz et al., 2008; Landsberg et al., 2009). Each ring consists of six Vps4 monomers arranged tail-to-tail. Cryo-electron microscopy showed that the upper ring exposes the MIT domains enabling it to interact with ESCRT-III whereas in the lower ring these domains are masked and not directly interacting with

ESCRT-III (Yu et al., 2008). Vps4 resembles a bowl since the upper ring is open whereas the lower ring is closed and seems to serve a structural or regulatory role rather than being important for substrate recognition. When ESCRT-III filaments interact with the upper Vps4 ring, they are possibly drawn through the 'Vps4-bowl' which mediates the disassembly upon ATP hydrolysis (Yu et al., 2008). During MVE formation and retrovirus budding, the ESCRT-III proteins are stripped off the membranes into cytosol and can be reused (Babst et al., 1998; Lata et al., 2008). In contrast the possible recycling of ESCRT components after cytokinesis seems unlikely, as discussed below.

ESCRT-mediated membrane fission

Constriction of the CHMP filaments is thought to pinch off membrane necks in related processes such as MVE formation, retrovirus budding and abscission. To date it is still unclear if membrane scission is mediated by identical mechanisms in these topologically similar processes. Recently live cell imaging studies provided new knowledge regarding ESCRT-III mediated membrane fission at the intercellular bridge. Firstly the sequential anchoring of CEP55, then almost simultaneously Tsg101 and CHMP4B and finally Vps4 to each side of the midbody dark zone was verified by live cell imaging (Elia et al., 2011). Secondly the appearance of a second CHMP4 pool at approximately 1 μm from the initial one illustrates that only a small fraction of the recruited CHMP-spiral is actually involved in the final abscission (Elia et al., 2012). Two likely scenarios have been suggested to explain how CHMP-filaments induce membrane-fission. Both models predict that constriction of the CHMP-spiral breaks off the outer spiral-segment, and the elastic energy released upon breakage pushes the tip outwards until equilibrium is achieved approximately 1 μm from the first pool. The energy required for this spiral constriction is according to the first model provided intrinsically within the spiral or alternatively by lateral movement of negatively charged lipids which CHMP filaments associate electrostatically. Alternatively, the second model assumes that Vps4 binding to the spiral cut off the outer segment according to the disassembly role of Vps4 and the energy is generated by ATP hydrolysis. Unfortunately it is still unknown whether

Vps4 appears in one or two pools at the midbody, and characterizing of Vps4 localization by live cell imaging will be valuable for further understanding its role in cytokinesis.

Looking into similar membrane fission processes in other organisms may provide valuable knowledge and hints when characterizing the mechanism for ESCRT mediated membrane scission. In bacteria membrane fission is facilitated by FtsZ proteins, which like ESCRT-III proteins, oligomerize into long filaments (Lu et al., 2000). Moreover the flat FtsZ filaments are re-arranged into protruding spirals by FzIA, resembling CHMP2/3 mediated transformation of the flat CHMP4-tubules (Goley et al., 2010). This architectural reorganization is thought to provide energy facilitating membrane bending and fission (Osawa et al., 2009). Interestingly FtsZ is also shown to be important for cell division, which clearly proposes that constriction of filaments is a conserved mechanism driving membrane fission in abscission (Osawa et al., 2009). One important difference is the source of energy, since FtsZ hydrolyzes GTP itself whereas ESCRT-III must associate Vps4 to generate the energy required for membrane fission. Thus introduction of Vps4 adds complexity to the mechanism in higher eukaryotes compared to in prokaryotes.

The peripheral CHMP pool was found to coincide with the actual abscission site, where intercellular bridge is only 100 to 200 nm in diameter (Elia et al., 2012). The ESCRT spirals were reported to be approximately 85 nm wide, which then defines the optimal diameter of membrane necks the ESCRT spirals are able to constrict (Henne et al., 2012).

Therefore it seems unlikely that the ESCRTs facilitate the second ingression, thinning the intercellular bridge from 2 μm to 100 nm. As described later fusion of FIP3-Rab11-recycling endosomes with the plasma membrane in the intercellular bridge might mediate this second ingression locally in proximity to the midbody (Schiel et al., 2012). The existence of anti-parallel microtubules in the intercellular bridge efficiently prevents abscission, giving the ESCRTs yet another problem to circumvent prior to the membrane scissioning.

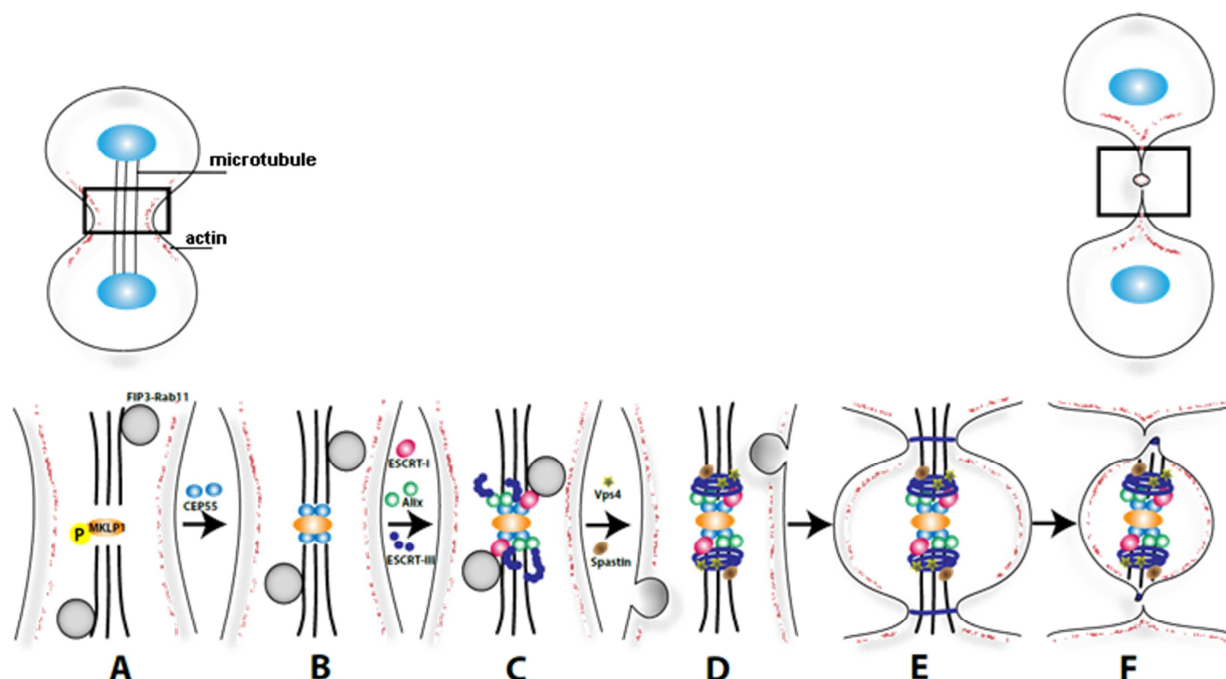


Figure 1. Possible role of ESCRT proteins in abscission. **A)** During mitosis the high expression of Plk1 phosphorylates (P) MKLP1 at the midbody. **B)** Reduced Plk1 expression in telophase generates unphosphorylated MKLP1 which recruits CEP55. **C)** Alix and ESCRT-I bind CEP55 at the midbody and recruit ESCRT-III monomers. FIP3-Rab11 recycling endosomes transported along microtubules in the intercellular bridge may anchor in proximity to the midbody dark zone by binding to Tsg101. **D)** The ESCRT-III monomers are activated and oligomerize into filaments which are rearranged into cone-shaped spirals. **E)** The AAA ATPase Vps4 interacts with the ESCRT-III filaments and is thought to generate the energy required to constrict this spiral, breaking off the outer segment observed as a peripheral pool of ESCRT-III in the intercellular bridge. Alternatively lateral sliding of negatively charged lipids may provide this energy. Simultaneously, microtubule severing facilitated by Spastin recruited by ESCRT-III occurs locally adjacent to the secondary pool of ESCRT-III. Further waves in the intercellular bridge due to busy vesicle trafficking promotes mechanical breakage of the microtubule. Also FIP3-Rab11 endosomes stimulate actin depolymerization by transporting p50RhoGAP to the midbody. In sum, vesicle fusion and depolymerization of actin and microtubules locally decreases the diameter of the intercellular bridge from 2 μm to 100-200 nm. **F)** In these thin parts of the intercellular bridge ESCRT-III filaments are able to constrict the bridge further until membrane fission occurs spontaneously. **G)** After abscission is completed, the midbody remnant containing the ESCRT machinery, is either retained by one daughter cell or released into the extracellular space.

Moreover it is unclear whether the midbody is cleaved once or twice, hence giving rise to a midbody remnant retained to one of the daughter cells or released into the extracellular space (Chen et al., 2012).

Interestingly it was reported decreased release of midbody remnants when CEP55, Alix or Tsg101 were knocked down, in favor of ESCRT-mediated double cleavage of the intercellular bridge (Ettinger et al., 2011).

Accordingly the fate of this midbody remnant varies between studies, perhaps reflecting cell type specific preferences for clearance of this 'organelle'. Autophagic degradation, either of the attached midbody or released midbody endocytosed from the extracellular space, seems to constitute the major route for clearance although future investigation might shed light on alternative mechanisms.

In either case, recycling of ESCRTs after cytokinesis seems unlikely since the proteins are trapped within the midbody remnant, again illustrating the differences between the role of ESCRT in cell division compared to in MVE formation and retroviral budding.

The current knowledge regarding the roles of ESCRT and FIP3-Rab11 endosomes during abscission is summarized in Figure 1, which represents one mechanism for abscission during cell division.

ESCRT-III mediates severing of cytoskeleton filaments in the intercellular bridge

The intercellular bridge contains actin-filaments from the actomyosin-ring mediating the first ingression and antiparallel microtubule filaments which stabilizes the bridge.

Clearly, removal of these cytoskeleton filaments is required before the final membrane fission. Recent reports indicate an essential role for FIP3-Rab11 recycling endosomes in the actin disassembly at the intercellular bridge (Schiel et al., 2012).

Upon closer examination these endosomes contained p50RhoGAP which most likely facilitate the required actin disassembly.

Further, Secretory Carrier Membrane Protein 2/3 (SCAMP 2/3) was found in FIP3-Rab11-recycling

endosomes, thought to stimulate the second ingression during cytokinesis.

The second ingression is locally thinning the intercellular bridge from 1.5 μm to 100 nm, and might be driven by fusion of these FIP3-SCAMP2/3-p50RhoGAP vesicles with the plasma membrane since SCAMP2/3 originally was found to regulate exocytic membrane fusion (Liao et al., 2008; Liu et al., 2005). Interestingly, Tsg101 interacts with both SCAMP2/3 (Aoh et al., 2009; Falguières et al., 2012), FIP3 and FIP4 (Horgan et al., 2012). Even though it is tempting to speculate that Tsg101 may be translocated to the intercellular bridge in FIP-positive endosomes, this seems unlikely since Tsg101 localization at the midbody was unaffected by dominant negative FIP4-mutant (Horgan et al., 2012) and live cell microscopy revealed heavy trafficking of FIP endosomes when CEP55 and Tsg101 were already present at the midbody (Schiel et al., 2012). On the other hand, the opposite scenario would be interestingly to investigate further. What if Tsg101 anchors FIP3-SCAMP2/3-p50RhoGAP-endosomes close to the midbody? This would define the place for local actin-severing by p50RhoGAP, followed by second ingression mediated by SCAMP2/3-mediated exocytic fusion which narrows the intercellular bridge enough for the ESCRT-III filaments to pinch off the membrane. By this means CEP55 co-localizes factors mediating actin depolymerization and second ingression via Tsg101-recruitment of FIP3-SCAMP2/3-p50RhoGAP-vesicles and abscission machinery via Alix-recruitment of ESCRT-III. This mechanism would ensure coordinated localization to a restricted area of the intercellular region defining the abscission site, but this hypothesis remains yet to be tested. Not only recycling endosomes but also secretory vesicles are transported into the intercellular bridge to support proper cell division (McKay and Burgess, 2011). Therefore it is interesting to notice that Tsg101 facilitates midbody localization of Tom1L1/SCRASM which is thought to function in post-Golgi trafficking (Yanagida-Ishizaki et al., 2008). Thus one intriguing possibility is that Tsg101 anchors vesicles at the midbody, although this remains to be addressed in future studies.

Proper abscission depends on clearance of the anti-parallel microtubule filaments in the intercellular bridge. The microtubule severing enzyme Spastin is localized to the intercellular bridge by specifically interacting with CHMP1B (Yang et al., 2008), IST1 (Agromayor et al., 2009) or NA14 (Errico et al., 2004). The importance of IST1 in microtubule severing was exemplified by intercellular bridges filled up of microtubules upon IST1 depletion but not CHMP1 depletion (Bajorek et al., 2009). The fact that the Spastin-IST1 interaction exhibits the highest MIT-MIM binding affinities reported ($K_d = 4.6 \mu\text{M}$) further suggests a major role for IST1 in recruiting Spastin compared to CHMP1 (Renvoisé et al., 2010). Future experiments will reveal the relative importance of

ESCRT-III and NA14, and elucidate whether they represent cell specific or parallel mechanisms. Discrepant observations regarding the outcome of Spastin depletion such as microtubule-filled intercellular bridges and abscission failure, indicates that such a crucial event as microtubule severing during cytokinesis is backed up by alternative mechanism(s) (Connell et al., 2009; Schiel et al., 2011).

An ESCRT-III-independent mechanism for microtubule severing involving mechanical buckling and breakage (Schiel et al., 2011). The mechanism is still elusive but the busy trafficking of FIP3-endosomes in the intercellular bridge fusing with the plasma membrane may create waves resulting in microtubule severing. Live cell imaging showed that microtubule-depolymerization in the intercellular bridge occurred before the second ingression (Schiel et al., 2012). Since this seemed to occur before ESCRT-III appeared in the intercellular bridge, it may perhaps argue against the original model that only ESCRT-III supports microtubule-severing. Interestingly, the mechanisms of microtubule depolymerization seems to be cell type dependent and depend on the length of the intercellular bridge (Connell et al., 2009; Schiel et al., 2012). This is exemplified by that epithelial cells are more dependent on spastin-dependent microtubule depolymerization whereas highly motile cells as fibroblasts are more dependent on mechanical-stimulated depolymerization. This further explains why spastin depletion just delays and not completely blocks cytokinesis.

Spatial and temporal regulation of ESCRT-III in cell division

The ultimate goal of cell division is to ensure segregation of DNA and organelles between daughter cells with high fidelity, implying tight control of the different steps in cytokinesis. Our knowledge of this crucial control is progressively emerging, yet much is still unclear. The initial recruitment of CEP55 and thereby ESCRT-I and Alix to the intercellular bridge is regulated by Plk1-mediated phosphorylation of MKLP1 (Bastos and Barr, 2010). To date ESCRT-I has not been observed to be regulated, but Alix is thought to associate with the midbody as a dimer (Pires et al., 2009; Zhou et al., 2008). The inactive Alix exhibits a closed conformation thus masking the Bro1-domain shown to bind CHMP4B, but exactly how this autoinhibited conformation is relieved upon membrane-attachment remains mysterious and represents a putative regulation step (Zhou et al., 2009). Likewise the conformation-induced activation of CHMP-monomers upon membrane-attachment is likely subject of tight regulation. Recently, CHMP4C was found to directly associate the Chromosomal Passenger Complex (CPC) composed of Aurora B, INCENP, Borealin and Survivin in both flies and human cell lines (Capalbo et al., 2012; Carlton et al., 2012). Upon binding to the N-terminus of CHMP4C, Borealin preserves it locked in the inactive state and prevents

oligomerization of ESCRT-III filaments and thereby abscission. The suggested regulatory role of Borealin is evolutionary conserved from flies to humans, but it is currently unknown if this negative regulation is mediated solely by Borealin or with assistance from accessory proteins. Interestingly, when comparing the amino acid sequence between the different CHMP4 paralogs, CHMP4C exhibits a unique insertion region enriched in serines and threonines at its C-terminus containing a putative Aurora B phosphorylation site. Aurora B-mediated phosphorylation of S210 at CHMP4C was proven to support proper cell division without affecting its midbody-recruitment. Thus, it seems that although CHMP4C is recruited to the midbody, it is kept inactive and prohibited from completing abscission as long as CPC is active. Lagging chromosomes in the intercellular bridge activates the so-called NoCutcheckpoint dependent of Aurora B, which delays complete abscission until the lagging chromosomes are completely segregated (Carmena, 2012; Norden et al., 2006; Stauffer et al., 2001). Precisely, how Aurora B recognizes the lagging chromosomes and halt abscission is unclear but it is tempting to speculate that the mechanism involves Aurora B and CHMP4C. This ESCRT-NoCut-checkpoint association seems to be most important in higher eukaryotes like mammals, since Aurora B is unable to phosphorylate CHMP4 in flies even though Borealin of CPC interact with CHMP4 proteins, and the S210 residue is poorly evolutionarily conserved (Capalbo et al., 2012; Carlton et al., 2012). In addition, the centrosomal protein TTC19 has been proposed to regulate the oligomerization of CHMP4B at the midbody (Sagona et al., 2010). It seems that TTC19 in complex with FYVE-CENT is recruited to the midbody by interacting with CHMP4A/B and PI3P, respectively (Sagona et al., 2010; Tsang et al., 2006).

Regulation of Vps4 activity has been extensively studied in context of cytokinesis but also MVE biogenesis and virus budding, and revealed five important regulators of Vps4, namely Vta1/SBP1, CHMP1, CHMP2, IST1 and CHMP5. In contrast to CHMP2, Vta1 and IST1, CHMP5 and CHMP1 indirectly regulate Vps4 activation by recruiting Vta1 and IST1 as described below (Azmi et al., 2008). Vps4 is recruited to ESCRT-III filaments by directly interacting with CHMP2 and leading to Vps4 activation in all cytokinesis, endosomal sorting and virus budding (Azmi et al., 2008). The Vps4 co-activator Vta1 interacts with the lower of the two hexameric rings of Vps4 and is thought to facilitate activation by efficiently tethering Vps4 to ESCRT-III filaments by directly associating CHMP5 and CHMP1 (Azmi et al., 2008; Bowers et al., 2004; Stuchell-Brereton et al., 2007; Yang and Hurley, 2010; Yang et al., 2012). The recruitment of Vta1 to Vps4 is negatively regulated by ISG15 (Interferon Stimulated Gene 15) (Pincetic et al., 2010). The role of ISG15 has so far only been investigated in HIV budding and future studies will

reveal if it is able to regulate Vta1-Vps4-function in MVE formation and cytokinesis as well (van Balkom et al., 2009; Ward et al., 2005; Yeo et al., 2003). Especially interesting is it that Vta1 functions as a dimer, representing yet another putative regulation step which awaits further investigation (Xiao et al., 2008; Yang and Hurley, 2010).

IST1 was found to negatively regulate Vps4 activity *in vitro* (Dimaano et al., 2008). Possibly the strong binding of the MIT-Domain containing protein 1 (MITD1) to IST1 but also CHMP2A and less extent CHMP1, could prevent their interaction with Vps4 at the midbody (Hadders et al., 2012; Lee et al., 2012). Interestingly, MITD1 is specifically involved in cytokinesis and not growth factor receptor degradation as previously reported for IST1.

In yeast Vps4-promoted disassembly of endosomal ESCRT-III was found to be regulated by Bro1 which associated CHMP4 (Wemmer et al., 2011). Careful characterization of the interaction-motif in CHMP4 rules out a competitive binding between Bro1 and Vps4 to CHMP4 filaments. The mechanism is presently not elucidated neither is the potential role of Bro1 in regulating retroviral budding or cytokinesis. Bro1 seems to exert numerous roles related to the ESCRT function. In addition to promote de-ubiquitination of cargo in endosomal sorting (Luhtala and Odorizzi, 2004; Richter et al., 2007), it is also thought to stabilize ESCRT-III filaments on membranes as its closest human orthologue Alix.

In general it seems that the ESCRT role in cytokinesis, in contrast to retrovirus release and MVE formation, is more complex regarding the number of auxiliary proteins and regulation. This is further illustrated by the discovery of Vps4 regulators like MITD1 which specifically modulates Vps4 in cell division.

Ubiquitination during cell division

During cell division ubiquitinated proteins accumulate at the spindle midzone (Mukai et al., 2008). To date we can just speculate the purpose and nature of enzymes stimulating this modification. Perhaps ubiquitination facilitates interaction and activity, but also regulates expression levels of proteins implicated in cell division. Proteasomal degradation of mitotic proteins normalize their expression levels at the end of cytokinesis (Min and Lindon, 2012). Accordingly CHMP1, IST1 and to less extent CHMP4B/C, CHMP2 and CHMP7 were reported to recruit Calpain-7 onto membranes via MIM-MIT interactions (Maemoto et al., 2011; Osako et al., 2010; Yorikawa et al., 2008). Even though CHMP1 and IST1 stimulate the proteolytic activity of this cysteine protease, it remains to elucidate its putative substrates and if it is important for proper cytokinesis. Several of the ESCRT members are shown to directly bind to ubiquitin-modifying enzymes. For instance Tsg101 interacts with the E3 ubiquitin ligase Nedd4L, which most likely controls cell division since depleting Nedd4L give multinuclear cells (Chung et al., 2008).

Moreover IST1 binds Spartin via MIM-MIT-interaction and recruits it to the midbody to support proper cytokinesis (Renvoisé et al., 2012). At the midbody Spartin may act as a platform recruiting the E3 ubiquitin ligases AIP4 and AIP5 (Bakowska et al., 2007; Eastman et al., 2009; Edwards et al., 2009). Although Spartin knock-down gave multinuclear cells implying that it is essential for complete cytokinesis, a putative role for AIP4 or AIP5 in cytokinesis remains unexplored (Renvoisé et al., 2010).

In agreement with the importance of ubiquitin for optimal cell division (Pohl and Jentsch, 2008), depletion of different deubiquitinases, such as UBPY and AMSH, results in multinuclear cells (Agromayor et al., 2009). The deubiquitinating enzymes UBPY and AMSH exhibit MIT domains shown to associate MIM-domains in CHMP1,2,7 and CHMP1A, 3, respectively (Mukai et al., 2008). Additionally, IST1 binds both AMSH and UBPY via MIM-MIT interactions (Agromayor et al., 2009).

ESCRT-III ensures normal cell division by controlling centrosome duplication and thus spindle formation

Even though the main focus to date has been on the role of ESCRTs during abscission, it seems that this machinery may control earlier steps such as centrosome duplication and thus spindle formation, at least in mammalian cell lines. Cells depleted of either CHMP proteins or Vps4 exhibited increased number of centrosomes (from 5 and to 20) giving multipolar mitotic spindles and abscission failure (Morita et al., 2010). On the other hand, CHMP2A or CHMP5 depleted cells exhibited only one centrosome giving rise to monopolar spindles, and arrested abscission due to lagging chromosomes in the intercellular bridge. Exactly how ESCRTs control centrosome duplication is so far unknown

although CHMP4 interacts with CC2D1A (Tsang et al., 2006), which is implicated in centrosome duplication (Nakamura et al., 2009). Another intriguing possibility is that ESCRTs may be important for centrosome clustering forming two mitotic organizing centre and hence bipolar spindles. In line with this hypothesis the CHMP5 ortholog in *Drosophila* was picked up in a centrosome clustering siRNA screen (Kwon et al., 2008).

Conclusion and future perspectives

Even though several cellular processes rely on the ESCRT machinery it seems that the entry of each pathway is specific; during lysosomal sorting the ESCRT-I-II-III is engaged by ESCRT-0, whereas in retrovirus budding Gag proteins attract the ESCRTs and during cytokinesis CEP55 recruits Alix and ESCRT-I-III to the intercellular bridge. Interestingly several isoforms exist of Vps37 in ESCRT-I and

CHMP4 in ESCRT-III, and our knowledge regarding fission-specific involvement of these isoforms is emerging. For instance UBAP1, together with Vps37A, was proven to be an endosome-specific component of ESCRT-I affecting EGFR-degradation and not cytokinesis (Stefani et al., 2011).

Among the CHMP4 proteins, CHMP4C in difference to CHMPA-B, seems to be more important for checkpoint control during cytokinesis (Carlton et al., 2012). Moreover IST1 of ESCRT-III seems to be specifically involved in abscission, exerting numerous roles such as bridging ESCRT-I and III by binding Vps37B, recruiting Vps4 and its regulators in addition to anchoring ubiquitin modifying enzymes and microtubule-severing enzymes to the midbody. Interestingly, the discovery of MITD1 as the first, but unlikely last, cytokinesis-specific ESCRT-associated protein has further shed light on the complexity in ESCRT-mediated abscission. Future proteomic-analyses and siRNA screens will provide us even deeper insight into the network of proteins required for completing cytokinesis. Also, the spatial- and temporal control of abscission awaits future characterization.

Still the precise mechanism by which ESCRT-III facilitates membrane fission remains elusive, but it is conceivable that this will be elucidated by high resolution microscopy and advanced live cell imaging. We know that negatively charged lipids modulate ESCRT-III oligomerization, but still the relationship between lipidcomposition and abscission is vague and highly interesting to pursue. Finally the interplay between Rab11-FIP3 recycling endosomes and the ESCRT machinery during cell division is emerging but still requires further investigation.

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Abbreviations

Cdk1: Cyclin dependent kinase 1
CEP55: CEntrosomal Protein 55 kDa
CHMP-: Chromatin Modifying Protein:Charged Multivesicular body Protein
CPC: Chromosomal Passenger Complex
EABR: ESCRT and Alix Binding Region
ESCRT: Endosomal Sorting Complex Required for Transport
ISG15: Interferon Stimulated Gene 15
IST1: Insensitive to
MIM: MIT Interacting Motif
MIT: Microtubule Interacting and Trafficking
MITD1: MIT Domain containing protein 1
MKLP1: Mitotic Kinesin Like Protein 1
MVE: MultiVesicular Endosome
PI3P: PhosphatidyloInositol-3-Phosphate

Plk1: Polo like kinase 1
SCAMP2/3: Secretory Carrier Membrane Protein 2/3
TEX14: Testis EXpressed gene 14

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