Comprehensive analysis of the human ESCRT-III-MIT domain interactome reveals new cofactors for cytokinetic abscission

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Abstract

The 12 related human ESCRT-III proteins form filaments that constrict membranes and mediate fission, including during cytokinetic abscission. The C-terminal tails of polymerized ESCRT-III subunits also bind proteins that contain Microtubule-Interacting and Trafficking (MIT) domains. MIT domains can interact with ESCRT-III tails in many different ways to create a complex binding code that recruits essential cofactors to sites of ESCRT activity. Here, we have comprehensively and quantitatively mapped the interactions between all known ESCRT-III tails and 19 pure recombinant human MIT domains. We measured 228 pairwise interactions, quantified 58 positive interactions, and discovered 16 previously unreported interactions. We also report the crystal structure of the SPASTIN MIT domain in complex with the IST1 C-terminal tail. Three MIT enzymes were studied in detail and shown to: 1) localize to cytokinetic midbody membrane bridges through interactions with their specific ESCRT-III binding partners (SPASTIN-IST1, KATNA1-CHMP3, and CAPN7-IST1), 2) function in abscission (SPASTIN, KATNA1, and CAPN7), and 3) function in the “NoCut” abscission checkpoint (SPASTIN and CAPN7). Our studies define the human MIT-ESCRT-III interactome, identify new factors and activities required for cytokinetic abscission and its regulation, and provide a platform for analyzing ESCRT-III and MIT cofactor interactions in all ESCRT-mediated processes.

Introduction

To complete cell division, a cell must equally and fully partition a faithfully duplicated genome into two nascent progeny cells through mitosis, and these cells must physically separate through cytokinesis. In animal cells, cytokinesis initiates when an actomyosin contractile ring ingresses to create a cleavage furrow between the two spindle poles once the chromosomes are fully segregated(Addi et al., 2018). The plasma membrane furrow ultimately narrows into an intercellular membrane bridge with a dense central array of microtubules (the midbody), which is then abscised to create two new cells. Premature abscission in the presence of aberrantly segregated DNA can lead to tension-induced double strand breaks in chromatin bridges that traverse the cleavage furrow(Janssen et al., 2011), chromosome instability(Umbreit et al., 2020), and formation of micronuclei and extensive DNA damage through chromothripsis(Zhang et al., 2015, Crasta et al., 2012). Hence, abscission is required for cell proliferation and its misregulation can contribute to DNA damage and even cancer development(Pharoah et al., 2013, Sadler et al., 2018).

The ESCRT (Endosomal Sorting Complexes Required for Transport) pathway mediates abscission in cultured mammalian cells(Carlton and Martin-Serrano, 2007, Morita et al., 2007), and in neural progenitor cells *in vivo*(Tedeschi et al., 2020, Little et al., 2021). Approximately 30 human ESCRT factors comprise five subcomplexes: ALIX, ESCRT-I, ESCRT-II, ESCRT-III, and VPS4, which assemble sequentially to constrict and sever membranes(McCullough et al., 2018). During cytokinesis, ESCRT complexes are recruited to the central Flemming body within the midbody by the CEP55 adaptor protein. CEP55 binds the early-acting ESCRT protein, ALIX (Lee et al., 2008, Christ et al., 2016, Morita et al., 2007, Carlton and Martin-Serrano, 2007), which in turn recruits ESCRT-III subunits of the CHMP4 class(Christ et al., 2016, McCullough et al., 2008, Katoh et al., 2003, Katoh et al., 2004, Kim et al., 2005). In a parallel pathway, CEP55 binds the TSG101 subunit of ESCRT-I/II complexes(Morita et al., 2007, Carlton and Martin-Serrano, 2007, Christ et al., 2016, Lee et al., 2008), again leading to ESCRT-III recruitment. ESCRT-III recruitment is also promoted by the midbody protein SEPT9(Karasmanis et al., 2019), and other CEP55-independent pathways(Merigliano et al., 2021, Addi et al., 2020). Humans express 12 homologous ESCRT-III proteins that fall into eight different families, termed CHMP1-7 and IST1. The different ESCRT-III subunits co-polymerize to form spiraling filaments within the midbody(Guizetti et al., 2011, Mierzwa et al., 2017, Pfitzner et al., 2021, Nguyen et al., 2020, Elia et al., 2011). These filaments recruit VPS4 AAA+ ATPases that in turn promote dynamic ESCRT-III subunit exchange, midbody constriction, membrane fission, and daughter cell separation(Pfitzner et al., 2021, Mierzwa et al., 2017, Elia et al., 2012, Pfitzner et al., 2020).

Abscission timing and progression are regulated by the evolutionarily conserved abscission/NoCut cell cycle checkpoint(Norden et al., 2006, Steigemann et al., 2009). This checkpoint pauses abscission progression so that upstream mitotic events can be completed correctly before the cell irreversibly separates. Several different conditions are known to sustain abscission checkpoint activation, including incompletely segregated

DNA within the midbody(Norden et al., 2006, Mendoza and Barral, 2008, Amaral et al., 2016), nuclear pore subunit depletion(Mackay et al., 2010), and DNA damage resulting from replication stress(Mackay and Ullman, 2015), and midbody tension(Lafaurie-Janvore et al., 2013, Strohacker et al., 2021, Caballe et al., 2015). These conditions all converge on phosphorylated Aurora B (pAurB) kinase, which maintains checkpoint signaling and inhibits ESCRT activity. AurB is targeted to the intercellular bridge as a component of the multi-protein Chromosomal Passenger Complex (CPC), where it phosphorylates multiple substrates, including the regulatory ESCRT-III protein CHMP4C (pCHMP4C)(Carlton et al., 2012, Capalbo et al., 2012, Steigemann et al., 2009). pCHMP4C associates with ANCHR, and this complex sequesters VPS4 AAA+ ATPases from abscission sites, thereby inhibiting abscission progression(Thoresen et al., 2014). pAurB and pCHMP4C also localize to cytoplasmic Abscission Checkpoint Bodies (ACBs), where pro-abscission machinery, including ALIX and ESCRT-III proteins, are sequestered from the midbody(Strohacker et al., 2021). The importance of the abscission checkpoint and its regulation by CHMP4C is underscored by the discovery that a missense mutation in the penultimate CHMP4C residue (A232T) disrupts ALIX binding, inactivates the checkpoint, and predisposes carriers to several types of cancers(Sadler et al., 2018, Pharoah et al., 2013)

In addition to constricting the midbody, ESCRT-III filaments also recruit cofactors that contribute to abscission and checkpoint regulation. Human ESCRT-III proteins have conserved helical core domains that mediate filament formation(McCullough et al., 2018, Pfitzner et al., 2021, Bajorek et al., 2009b), and variable C-terminal tails that contain short peptide elements called MIT-Interacting Motifs (MIMs) (Figure 1A). MIMs bind cofactors that contain MIT (Microtubule-Interacting and Trafficking) domains(Hurley and Yang, 2008). MIT domains are simple three helix bundles, but they can bind MIM elements in at least seven distinct ways(Skalicky et al., 2012, Obita et al., 2007, Scott et al., 2005, Stuchell-Brereton et al., 2007, Kieffer et al., 2008, Yang et al., 2008, Solomons et al., 2011, Fujioka et al., 2014), which we have termed Type 1-7 binding modes (Figure 1C).

Humans express more than 20 MIT domain-containing proteins, which also contain a variety of associated activities that could function in cytokinesis (Figure 1B). Well-characterized examples of MIT domain-containing proteins that bind ESCRT-III proteins and perform important midbody functions include: 1) VPS4. The archetypal MIT domains of the related VPS4A and VPS4B ATPases bind promiscuously to different ESCRT-III filaments. These interactions promote assembly of VPS4 hexamers and activate ATPase activity(Han et al., 2015, Azmi et al., 2008, Norgan et al., 2013). VPS4 MIT domains can alternatively bind a MIM element within ANCHR, which sequesters the ATPases from the abscission zone(Thoresen et al., 2014). 2) ULK3 kinase. The second ULK3 MIT domain binds IST1, which localizes this kinase to the midbody where it phosphorylates ESCRT-III proteins and inhibits membrane fission(Caballe et al., 2015). 3) SPASTIN. The MIT domain of the microtubule severing AAA+ ATPase SPASTIN binds CHMP1B(Yang et al., 2008), which reportedly localizes the enzyme to the midbody where it clears spindle microtubules to allow abscission(Connell et al., 2009). 4) MITD1. The MIT domain of

MITD1 binds preferentially to CHMP1A, CHMP1B, CHMP2A and IST1 and localizes this phospholipase-D family member to midbodies where it stabilizes the bridge, preventing abscission failure (Hadders et al., 2012, Lee et al., 2012). 5) SPARTIN. The MIT domain of the hereditary spastic paraplegia protein SPARTIN preferentially binds IST1, which localizes the protein to the midbody to support abscission (Renvoise et al., 2010).

The 12 ESCRT-III and >20 MIT human proteins have the potential to form a complex combinatorial network of ESCRT-III-MIT interactions that help mediate and regulate abscission. However, the ESCRT binding and cytokinetic functions of many human MIT proteins have not yet been tested, and prediction of MIT-MIM interactions can be difficult owing to their remarkable variety of different possible binding modes (Figure 1C). To address these limitations, we systematically quantified the binding of all 12 human ESCRT-III MIM elements to nearly all known human MIT domains. Three MIT enzymes with interesting activities and ESCRT-III binding patterns were further tested for midbody localization, and roles in cytokinetic abscission and the abscission checkpoint. These studies revealed that SPASTIN, KATANIN-P60 (KATNA1), and CALPAIN-7 (CAPN7) all localize to midbodies through specific MIT-ESCRT-III interactions and are required for efficient abscission, and that SPASTIN and CAPN7 are also required for abscission checkpoint maintenance. These studies define the human MIT-ESCRT-III interactome and identify new factors and activities required for cytokinetic abscission and its regulation.

Results

MIT-MIM interaction screen

We defined the ESCRT-III-MIT interactome by using fluorescence polarization anisotropy (FP) to quantify the binding interactions between recombinant human MIT domains and ESCRT-III MIM peptides. These experiments employed fluorescently-labeled C-terminal peptides (52 to 88 residues) that encompassed the known MIM elements of the 12 human ESCRT-III proteins (Figure 1A, Supplementary Table 2). Literature analyses were used to identify 21 candidate human MIT domains (Cicarelli et al., 2003, Rigden et al., 2009, Skalicky et al., 2012, Xiao et al., 2008, Fujioka et al., 2014) (Figure 1B; see Materials and methods), which were expressed as recombinant proteins and purified to homogeneity. Constructs for tandem MIT domains spanned both domains because paired MIT domains can interact and stabilize each other intramolecularly (Skalicky et al., 2012, Vild et al., 2015, Guo and Xu, 2015). 19/21 of the human MIT domain constructs could be purified in sufficient quantities for FP binding titrations (Figure 2). The two exceptions were the C-terminal tandem MIT domains of ULK1 and ULK2, which function in autophagy (Fujioka et al., 2014) and apparently do not bind ESCRT-III proteins (see below).

Pairwise FP binding isotherms were fit to 1:1 equilibrium binding models (Figure 2, Figure 2-figure supplements 1-4, Figure 3). Dissociation constants (K_D) for the 228 interactions tested are summarized in Figure 2 and color-coded based upon interaction strengths. The tightest MIM-MIT binding pair was LIP5-CHMP5 ($K_D = 2.5$ nM), which reflects the fact that CHMP5 tail helices 5 and 6 and adjacent linkers form an amphipathic “leucine collar” that wraps almost completely around the second LIP5 MIT domain (Figure 1C) (Skalicky et al., 2012, Yang et al., 2012). Our binding survey confirmed a series of previously reported ESCRT-III-MIT domain interactions (See Supplemental discussion), and also revealed 16 previously unreported interactions (Figure 2), including establishing new direct links between ESCRT-III proteins and the MIT domains from KATNA1, KATNAL1, RPS6KC1, RPS6KL1, SNX15, and AMSHLP. These new interactions should facilitate future investigations of ESCRT-associated activities.

ESCRT-III binding behaviors

MIT domains displayed a range of different ESCRT-III binding behaviors that we classified as promiscuous (>2 ESCRT-III binding partners) (Figure 2-figure supplement 1), specific (≤ 2 ESCRT-III binding partners) (Figure 2-figure supplement 2), non-specific (Figure 2-figure supplement 3), and non-binding (Figure 2-figure supplement 4). The seven promiscuous binders interacted with high ($K_D < 2$ μ M) or moderate ($K_D < 100$ μ M) affinities. The MIT domain of the deubiquitinase AMSH exhibited the greatest promiscuity, binding all but two of the 12 different ESCRT-III subunits. The eight specific binders interacted uniquely with either CHMP3 (KATNA1, KATNAL1, RPS6KL1, and AMSHLP), IST1 (RPS6KC1, CAPN7, SNX15, and SPARTIN), or CHMP1B and IST1 (SPASTIN). The predicted MIT domain of the catalytically inactive deubiquitinase, USP54 (Rigden et al., 2009), bound weakly ($K_D > 100$ μ M) to nearly all ESCRT-III proteins, indicating that these

interactions were probably non-specific in our assay (Figure 2-figure supplement 3). Finally, two MIT domains (NRBF2 and VPS9D1) did not bind any ESCRT-III proteins (Figure 2-figure supplement 4). The MIT domain of NRBF2 has been characterized structurally (PDB 4ZEY, 2CRB) and shown to interact with the VPS15 kinase to promote autophagosome biogenesis (Young et al., 2019). The putative VPS9D1 MIT domain was predicted through bioinformatics, has not been characterized structurally and has no known ESCRT connections (Rigden et al., 2009).

The ESCRT-III proteins IST1, CHMP3, and CHMP1B partner with the largest subset of MIT domains (Figure 2). IST1 bound 11/19 MIT domains with high affinity, and this promiscuity likely reflects the presence of two MIMs in the IST1 tail; a helical Type 1 or Type 3 (Type 1/3) element (residues 344-366) and an extended Type 2 element (residues 316-343) (Bajorek et al., 2009a, Guo and Xu, 2015) (Figure 3B). To distinguish the contributions of each IST1 MIM element to MIT-binding, we tested their binding separately to each of the IST1-interacting MIT domains, using shorter peptides comprising either MIM (Figure 3B, C). These experiments revealed that the IST1 Type 1/3 (MIM₃₄₄₋₃₆₆) element bound 10/11 MIT domains, and the Type 2 element (MIM₃₁₆₋₃₄₃) bound 5/11 MIT domains. Three binding patterns for the IST1 MIMs emerged, with MIT domains binding: 1) only the Type 1/3 element (SPASTIN, ULK3, USP8, and SPARTIN), 2) only the Type 2 element (RPS6KC1), and 3) both MIMs (VPS4A, VPS4B, CAPN7, SNX15, LIP5, and MITD1) (Figure 3C). Although we did not directly detect binding between the IST1 Type 2 element and the LIP5 and CAPN7 MIT domains, the IST1 Type 2 element appeared to contribute to LIP5 and CAPN7 binding because its absence reduced binding affinity >10-fold (vs. the full IST1 tail). Thus, both IST1 MIM elements can bind MIT domains, thereby contributing to promiscuity. Moreover, both MIM elements can simultaneously engage single MIT proteins in some cases, thereby increasing binding affinity (Osako et al., 2010, Bajorek et al., 2009a).

In contrast, a fluorescently labeled CHMP4C peptide did not bind detectably to any MIT domain tested. This was surprising, particularly as MITD1, USP8 and AMSH all bound the other two CHMP4 paralogs, CHMP4A and CHMP4B (Figure 2). CHMP4C is unique in containing a serine-rich insertion that is phosphorylated by AurB (Carlton et al., 2012, Capalbo et al., 2012) (Figure 2-figure supplement 5A). We hypothesized that this flexible insert might reduce the fluorescence anisotropy in bound complexes, thereby generating false negative results. To test this idea, we assayed whether the CHMP4C peptide could competitively inhibit AMSH, MITD1 and USP8 MIT binding to labeled CHMP4B (Figure 2-figure supplement 5B). These experiments revealed that CHMP4C bound AMSH MIT (K_i 14 μ M), but still failed to bind MITD1 or USP8. CHMP4C performs a unique role in abscission checkpoint regulation (Carlton et al., 2012, Capalbo et al., 2012), and this specialized function may have relieved the selective pressure to maintain some MIT binding interactions.

As noted above, we were unable to express and purify the ULK1 or ULK2 tandem MIT domains in sufficient quantities for direct binding assays. Nevertheless, we felt it was

important to test whether the MIT domains from these related kinases could bind ESCRT proteins because: 1) the MIT domains of yeast and human ULK1/2 kinases bind ATG13 within autophagosome initiation complexes, making interactions that are reminiscent of ESCRT-III MIT interactions (Chan et al., 2009, Fujioka et al., 2014), and 2) the MIT domain of the related ULK3 kinase binds tightly to IST1, and weakly to CHMP1A, CHMP1B, CHMP2A, and CHMP3 ((Caballe et al., 2015) and Figure 2-figure supplement 6A, B). We therefore performed pulldown assays from human HEK239T cell extracts to test whether ULK1(MIT)₂ could bind ESCRT-III proteins (Figure 2-figure supplement 6A, B). Positive control pulldown assays recapitulated the known ULK3(MIT)₂-ESCRT-III interactions, as well as ULK1(MIT)₂-ATG13 binding. However, ULK1(MIT)₂ did not bind detectably to any of the ESCRT-III binding partners of ULK3, implying that these ESCRT-III interactions are not conserved in the ULK family of protein kinases.

In summary, most MIT proteins can engage ESCRT-III proteins and their interactions span a range of specificities and affinities, indicating that they couple to ESCRT pathway functions in a variety of different ways.

SPASTIN, KATNA1, and CAPN7 as paradigms for MIT-ESCRT-III interactions

Three MIT proteins with specific ESCRT-III interactions and interesting associated enzymatic domains were selected for further characterization: 1) SPASTIN (IST1 and CHMP1B binding), 2) KATNA1 (CHMP3 binding), and 3) CAPN7 (IST1 binding). ESCRT-III interactions with SPASTIN (Reid et al., 2005, Yang et al., 2008, Agromayor et al., 2009) and CAPN7 (Yorikawa et al., 2008, Osako et al., 2010) have been described previously, whereas KATNA1 binding to CHMP3 has not. As noted above, the single MIT domain of SPASTIN bound tightly to the IST1 MIM₃₄₄₋₃₆₆ (Type1/3) element. The CAPN7 tandem MIT domain binds both MIM elements (Osako et al., 2010), and in this case multiple different binding modes are possible. SPASTIN and KATNA1 are microtubule severing AAA+ ATPases (Sharp and Ross, 2012). Both have been implicated in clearing midbody microtubules prior to abscission (Yang et al., 2008, Connell et al., 2009, Benz et al., 2012, Matsuo et al., 2013), but neither has been linked to abscission checkpoint activity. CAPN7 is an understudied cysteine protease that has not previously been linked to any cytokinetic functions.

Structure and analysis of the SPASTIN MIT-IST1 complex

The SPASTIN MIT domain is unusual in binding specifically to two different ESCRT-III proteins, CHMP1B and IST1. The SPASTIN MIT-CHMP1B complex has been characterized structurally (Figure 1C) (Yang et al., 2008), whereas the SPASTIN MIT-IST1 interaction has not. We therefore determined a high resolution (1.15 Å) crystal structure of this complex (Figure 4A, and Figure 4-figure supplement 1A, B, and Supplemental Table 7)). The structure revealed that the IST1 MIM₃₄₄₋₃₆₆ adopts an amphipathic helix that buries its hydrophobic side in the SPASTIN MIT H1/H3 groove (Type 3 binding, Figure 4A, Figure 4-figure supplement 1). The IST1 and CHMP1B MIM elements exhibit similar binding modes (Figure 4A-D), but with two significant differences (Figure 4-figure supplement 1C): 1) The longer CHMP1B helix extends three additional turns beyond the

1 IST1 N-terminus, and 2) the MIT H1/H3 groove expands to accommodate the longer
2 CHMP1B helix, with a maximal displacement of 2.5 Å at the N-terminus of helix 3. The
3 structure of SPASTIN-IST1 is nearly identical to SPARTIN-IST1, which also adopts a
4 Type 3 interaction(Guo and Xu, 2015)(Figure 4-figure supplement 1D).

5 Based on our structural analyses, we created SPASTIN MIT mutations designed to
6 disrupt the binding of both CHMP1B and IST1 (F124D, red in Figure 4A-D) and to disrupt
7 only CHMP1B binding without affecting IST1 binding (L177D, cyan in Figure 4A-D). As
8 shown in Figures 4E and 4F, these mutations behaved as designed, thereby providing a
9 set of mutants that we could use to compare the biological effects of disrupting SPASTIN
10 binding to both CHMP1B and IST1 vs. specifically disrupting binding to CHMP1B alone.

11 **KATNA1 and CAPN7 binding to ESCRT-III proteins**

12 We also screened for MIT point mutations that could block KATNA1 binding to CHMP3
13 and CAPN7 binding to IST1. In the KATNA1-CHMP3 case, a point mutation in the MIT
14 H1/H3 groove (V55D) eliminated CHMP3 binding (Figure 4G). The CHMP3 MIM element
15 is predicted to be helical, and our data therefore indicate a Type 3 interaction. CAPN7
16 has tandem MIT domains, and we found that IST1 binding was significantly reduced (~30-
17 70-fold) by point mutations in the H1/H3 groove of either the first (V18D) or second (F98D)
18 CAPN7 MIT domains (Figure 4H). Together with our MIM mapping experiments (Figure
19 3C), these data indicate that both CAPN7 MIT domains engage both IST1 MIM elements,
20 employing Type 2 (MIM₃₁₆₋₃₄₃) and Type 3 (MIM₃₄₄₋₃₆₆) binding modes.

21 **SPASTIN, KATNA1, and CAPN7 localize to midbodies**

22 As an initial screen for cytokinetic functions, we examined whether endogenous
23 SPASTIN, KATNA1, and/or CAPN7 localize to the midbodies of dividing cells (Figures
24 5A-C respectively). Midbodies were co-stained for microtubules and CEP55, which is
25 recruited to either side of the Flemming body where it initiates ESCRT complex
26 assembly(Fabbro et al., 2005, Carlton and Martin-Serrano, 2007, Morita et al., 2007).
27 Antibody staining specificity was confirmed in parallel experiments in which each MIT
28 protein was separately depleted using siRNA (Figure 5A-C; right panels).

29 In midbody-containing cells, all three MIT proteins co-localized with CEP55 ring structures
30 on either side of the Flemming body (Figure 5A-C). Uniquely, KATNA1 also distributed
31 along the midbody arms. Arm staining was particularly noticeable in early midbody-stage
32 cells (Figure 5B), and a similar KATNA1 staining pattern has been reported in rat cell
33 lines(Matsuo et al., 2013). Our observations of SPASTIN and KATNA1 Flemming body
34 localization are also in good agreement with previous reports(Yang et al., 2008, Connell
35 et al., 2009, Matsuo et al., 2013). Importantly, however, this is the first report that CAPN7
36 localizes to midbodies.

37 MIT protein localization was also examined in cells in which abscission checkpoint
38 signaling was sustained by nucleoporin depletion(Strohacker et al., 2021) (Figure 5D-F).
39 In these experiments, cells were synchronized using thymidine treatment and release,

together with siRNA depletion of NUP153 and NUP50, which sustains the abscission checkpoint. In all cases, the MIT proteins again localized to Flemming bodies, although in each case abscission checkpoint signaling led to more diffuse staining and spreading of the signals to midbody arms. Thus SPASTIN, KATNA1, and CAPN7 localize to midbodies under both unperturbed and checkpoint active conditions, and they are therefore poised to function in abscission and/or the abscission checkpoint.

SPASTIN, KATNA1, and CAPN7 function in abscission

To test for abscission functions, we depleted SPASTIN, KATNA1, and CAPN7 and quantified abscission failure, as reflected by increased numbers of multi-nucleated cells and cells with persistent intercellular bridges (Figure 5G and Figure 5-figure supplement 1). Specific depletion and abscission phenotypes were confirmed using two different siRNA oligonucleotides in each case, and successful target protein depletion was confirmed by Western blot (Figure 5-figure supplement 1).

Cells lacking KATNA1 and CAPN7 both showed strong abscission failure phenotypes, with near doubling of the percentages of cells with midbodies or multiple nuclei (vs. control cells transfected with non-targeting (NT) siRNA). These pronounced phenotypes resembled those of cells lacking the essential IST1 protein (positive control)(Bajorek et al., 2009a, Agromayor et al., 2009). Cells lacking SPASTIN also exhibited significant abscission defects, although the effects were weaker. Our results are consistent with previously reported roles for KATNA1 and SPASTIN in promoting abscission by clearing midbody spindle microtubules from abscission sites(Matsuo et al., 2013, Yang et al., 2008, Connell et al., 2009). The observation that CAPN7 is required for efficient cytokinetic abscission is a new discovery.

SPASTIN and CAPN7 are required for abscission checkpoint maintenance

We also tested whether SPASTIN, KATNA1, or CAPN7 were required to maintain the AurB-mediated abscission checkpoint. As described above, co-depletion of the nuclear pore proteins NUP153 and NUP50 sustains abscission checkpoint activity, leading to an accumulation of midbody-connected cells(Mackay et al., 2010). However, simultaneous co-depletion of either SPASTIN or CAPN7 significantly reduced midbody accumulation, indicating roles for both proteins in abscission checkpoint maintenance (Figure 5H, Figure 5-figure supplement 1). Indeed, the effects of depleting either SPASTIN or CAPN7 were at least as penetrant as depleting CHMP4C (positive control), which plays a well-characterized role in inhibiting abscission in response to checkpoint signaling(Capalbo et al., 2012, Carlton et al., 2012). Unlike SPASTIN or CAPN7 depletion, KATNA1 depletion did not significantly alter midbody numbers, although a second oligo targeting KATNA1 showed a modest but significant decrease in cells undergoing an abscission checkpoint arrest (Figure 5H, Figure 5-figure supplement 1). Thus, our data indicate that SPASTIN and CAPN7 are required to sustain abscission checkpoint arrest, and KATNA1 may also contribute weakly.

ESCRT-III proteins recruit SPASTIN, KATNA1, and CAPN7 to midbodies

To unite our binding and functional data, we tested whether ESCRT-III interactions were responsible for recruiting SPASTIN, KATNA1 and CAPN7 to function at midbodies. This was done by generating cell lines that expressed doxycycline (DOX)-inducible, siRNA-resistant mCherry constructs fused to wildtype (WT) and mutant MIT proteins. We then treated with siRNA to deplete the respective endogenous MIT proteins (Figure 6-figure supplement 1), while inducing expression of the mCherry fusion proteins, and imaged the cells to test for midbody localization. As in Figure 5, cells were also synchronized with sustained checkpoints to maximize the number of midbody-stage cells (Strohacker et al., 2021). Importantly, all three WT mCherry fusion constructs recapitulated localization of the endogenous MIT counterpart within the midbody. The percentages of “arms only” localization patterns were higher for the endogenous constructs in every case, however, potentially reflecting reduced antibody epitope accessibility at the protein-rich Flemming body.

A WT mCherry-SPASTIN construct localized to more than half of all midbodies, as did the L177D mutant. In contrast, the F124D mutant did not localize to midbodies (Figure 6A, B). These data imply that ESCRT-III binding is required to localize SPASTIN to the midbody. The SPASTIN F124D mutation disrupts binding to both CHMP1B and IST1, whereas the L177D mutation selectively disrupts only CHMP1B binding (Figure 4E and 4F). Therefore, our data imply that CHMP1B binding is dispensable for midbody localization when IST1 binding capability is maintained.

The KATNA1 MIT domain binds the C-terminal domain of the KATNB1 subunit to form the Katanin holoenzyme (Faltova et al., 2019, Rezabkova et al., 2017). KATNB1 activates and localizes KATNA1 to centrosomes where it severs mitotic spindle microtubules (Matsuo et al., 2013). However, KATNB1 does not appear to be responsible for localizing KATNA1 for cytokinesis function, as the two proteins reportedly do not co-localize within the midbody (Matsuo et al., 2013). It has therefore not been clear how KATNA1 is recruited to the midbody to function in abscission. Our localization data demonstrate that WT mCherry-KATNA1 localizes to Flemming bodies and midbody arms, but the V55D mutant does not (Figure 6 C,D). CHMP3 is the sole ESCRT-III binding partner of KATNA1, and the V55D mutation disrupts this partnership (Figure 3), implying that CHMP3 localizes KATNA1 to the midbody for abscission.

The structure of the KATNA1 MIT-KATNB1 heterodimer reveals that V55 sits adjacent to the KATNB1 binding site (Rezabkova et al., 2017) (Figure 6-figure supplement 2A), and we therefore tested the possibility that the V55D mutation might also disrupt the canonical KATNA1-KATNB1 partnership. This was not the case, however, as KATNB1 co-precipitated with full length KATNA1 WT and V55D mutant proteins equally well from HEK293T cell extracts (Figure 6-figure supplement 2B). In contrast, a KATNA1 MIT mutation known to disrupt KATNB1 binding, R14A (Rezabkova et al., 2017), abolished KATNB1 binding, demonstrating that the pulldown assay was sensitive to a mutation known to disrupt the interaction. Hence, our data imply that the KATNA1 V55D mutation discriminates between CHMP3 and KATNB1 binding, and therefore that CHMP3 is likely

1 responsible for localizing KATNA1 to Flemming bodies. Consistent with this model,
2 CHMP3 localizes to Flemming bodies(Mierzwa et al., 2017, Dukes et al., 2008), and its
3 depletion increases the propensity of cells to develop multiple nuclei and midbody
4 bridges(Morita et al., 2010), thereby phenocopying KATNA1 loss (Figure 5G).

5 Finally, our MIT screening data suggested that IST1 could be responsible for localizing
6 CAPN7 to midbodies (Figure 2). In support of this model, a point mutation in the second
7 CAPN7 MIT domain that decreased IST1 binding ~30-fold (F98D, see Figure 3) also
8 potently suppressed CAPN7 targeting to the Flemming body (Figure 6E and 6F). Thus,
9 in all three cases we examined in detail, our MIT screening data identified potential
10 ESCRT-III binding partners and point mutations that blocked binding. Furthermore, these
11 point mutations also abolished MIT protein localization to the midbody.

Discussion

We have comprehensively surveyed the MIT-ESCRT-III interactions between human ESCRT-III and MIT domain-containing proteins. We tested 228 pairwise interactions between MIT domains and ESCRT-III C-terminal tails, observed 58 positive interactions, and discovered 16 new interactions that have not been reported previously (Figures 2 and 3, see Supplemental discussion). We found that most human MIT domains can mediate ESCRT-III binding, further implicating MIT proteins as a major class of ESCRT pathway cofactors. Additionally, this comprehensive screen of potential interactions underscores the selectivity of partnerships in this network.

SPASTIN, KATNA1, and CAPN7 served as paradigms for using the binding data to discover new ESCRT cofactors that function in cytokinesis. Our screening assays identified ESCRT-III binding partners and interaction sites (Figure 3), enabled new structure determinations (Figure 4, Figure 4-figure supplement 1), and supported mutational analyses of MIT protein midbody localization (Figure 4, Figure 6). Together with previous studies, our work indicates that MIT domains are localization modules whose primary biological functions are to localize proteins to sites of ESCRT-driven membrane remodeling. MIT domains have also evolved to mediate protein-protein interactions in other pathways, most notably in autophagosome initiation (Fujioka et al., 2014, Young et al., 2019). Collectively, MIT-MIM interactions constitute a complex recognition network, and several principles that emerged from our studies are highlighted below. A more comprehensive discussion of the different interactions is provided in the Supplemental discussion.

MIT recruitment by ESCRT-III polymers

Although we studied the pairwise interactions of MIT domains and ESCRT-III tails, these interactions likely occur predominantly in the context of polymeric ESCRT-III filaments. Polymerization creates the potential for high avidity binding, and variations in ESCRT-III subunit compositions can alter binding modes and tune binding affinities. These properties are important because subunit compositions change as filaments mature and constrict membranes (Pfizner et al., 2021, Banjade et al., 2021, Pfizner et al., 2020). Current models for ESCRT-III assembly, derived from studies in simpler yeast systems, hold that CHMP6 initially nucleates the assembly of ESCRT-III strands composed of CHMP4 subunits. CHMP4 strands then recruit partner strands composed of CHMP2/CHMP3 subunits (Teis et al., 2008, Babst et al., 2002), which are subsequently exchanged for CHMP1/IST1 subunits (Pfizner et al., 2020). Hence, the cadre of recruited MIT protein binding partners may evolve as filaments mature.

These different principles are nicely illustrated by VPS4 enzymes and their associated activator LIP5 (Vta1 in yeast), which form “supercomplexes” that display 30 MIT domains; six from the VPS4 hexamer and 24 from the 12 associated LIP5(MIT)₂ elements (Skalicky et al., 2012, Yang et al., 2012). VPS4 MIT domains bind promiscuously to many ESCRT-III tails, and although binding is often weak, avidity effects can presumably create high

affinity binding. We measured strong to moderate, Type 1 VPS4 MIT binding to the late-acting CHMP1, CHMP2 and IST1 subunits (nM to mid- μ M K_{DS}), with 3-20-fold higher affinities consistently seen for VPS4A MIT vs. VPS4B MIT. CHMP3 binding was below our 200 μ M binding affinity cutoff for both VPS4 MIT domains, and the only case in which weaker, early-acting Type 2 interactors CHMP6 and CHMP4 made our binding cutoff was in the VPS4A MIT-CHMP6 complex. Nevertheless, there is good evidence that interactions between CHMP4 (Snf7 in yeast) and VPS4 are biologically important (Kieffer et al., 2008, Stuchell-Brereton et al., 2007, Shestakova et al., 2010, Buysse et al., 2020). The first of the tandem LIP5 MIT domains similarly binds ESCRT-III tails promiscuously, and again favors late-acting ESCRT-III subunits (Yang et al., 2012), whereas the second LIP5 MIT domain binds specifically and with high affinity to CHMP5 (Skalicky et al., 2012, Yang et al., 2012). Thus, these MIT interactions collectively explain how (VPS4)₆(LIP5)₁₂ supercomplexes can bind and remodel ESCRT-III filaments that contain essentially any combination of the 12 different human ESCRT-III subunits. Moreover, their different binding affinities can explain why VPS4 remodeling activity increases as ESCRT-III filaments mature and accumulate increasing fractions of the late-acting, and higher affinity CHMP2, CHMP1, and IST1 subunits (Pfizner et al., 2020).

In a similar fashion, ESCRT-III subunit binding specificities and affinities are also likely tuned to recruit other MIT cofactors as needed. It is therefore of interest that IST1, CHMP3 and CHMP1 were the most promiscuous ESCRT-III binders in our screen because each of these ESCRT-III subunits functions at transitional stages of ESCRT-III polymer maturation (Pfizner et al., 2020). Our binding data suggest that many MIT proteins will bind best to mature, IST1-containing filaments that form late, when membrane constriction is greatest and fission is most imminent. In contrast, MIT proteins like AMSH and MITD1 bind many different ESCRT-III subunits with similar affinities, perhaps because deubiquitination (AMSH) and midbody stabilization (MITD1) are required throughout abscission and other ESCRT-dependent processes.

IST1 as a versatile hub for cytokinetic cofactor recruitment

IST1 is the most promiscuous MIT binding ESCRT-III protein, and it binds most targets with high affinity (Figure 2, Figure 3C). IST1 promiscuity is achieved through combined use of two different MIM elements, and through multi-modal binding by the IST1 MIM1/3 element (Bajorek et al., 2009a, Guo and Xu, 2015) (Figure 3). Our screen revealed that both IST1 MIM elements can mediate MIT-binding, and that they often collaborate to enhance binding (Figure 3C). The SPASTIN MIT-IST1 structure shows that the IST1 MIM₃₄₄₋₃₆₆ element can make Type 3 MIT interactions, as seen previously in the SPARTIN-MIT-IST1 complex (Yang et al., 2008, Guo and Xu, 2015). However, the same hydrophobic surface of the IST1 MIM₃₄₄₋₃₆₆ helix can also form Type 1 interactions with the MIT domains of ULK3, VPS4B, and LIP5 (Guo and Xu, 2015, Skalicky et al., 2012, Caballe et al., 2015). IST1 depletion induces severe cytokinetic defects (Bajorek et al., 2009a, Agromayor et al., 2009) (Figure 5), reflecting its central role in abscission and as a versatile hub for MIT cofactor recruitment.

Comparison of the SPASTIN MIT complexes with IST1 and CHMP1B complexes allowed us to design a mutation that specifically disrupted only CHMP1B binding (Figure 4). This SPASTIN mutant still localized to intercellular bridges (Figure 6A), demonstrating that IST1 binding is sufficient (and CHMP1B dispensable) for midbody recruitment. Others have reported that SPASTIN midbody localization is impaired by CHMP1B depletion (Yang et al., 2008), but this observation can be reconciled because IST1 recruitment is also likely affected by the absence of CHMP1B (Bajorek et al., 2009a, Rue et al., 2008, Dimaano et al., 2008).

MIT binding modes of CHMP1 and CHMP3 proteins

Like IST1, CHMP1B and CHMP3 are also promiscuous MIT binders. CHMP1B binds two MIT domains (SPASTIN, USP8) that the closely related CHMP1A protein does not. This expanded binding range apparently reflects the ability of CHMP1B to access the Type 3 interaction mode. Type 3 interactions require 3 additional turns of the N-terminus of the CHMP1B MIM helix (vs. Type 1 interactions). These additional interactions allow SPASTIN to discriminate between CHMP1B and CHMP1A, whose sequences are highly similar at the C-terminus but diverge N-terminally (Yang et al., 2008). The USP8 MIT domain can also discriminate between CHMP1B and CHMP1A (Figure 2), and we therefore speculate that USP8 MIT may also form a Type 3 interaction with CHMP1B.

The CHMP3 MIM appears to be another example of a promiscuous helical MIM element that can bind in at least two separate grooves of different MIT domains. The CHMP3 MIM element binds the MIT domains of AMSH using a Type 4 mode (Figure 1C) (Solomons et al., 2011), and LIP5 (MIT1 using a Type 1/4 mode) (Skalicky et al., 2012). Remarkably, our study suggests that CHMP3 can also adopt yet another binding mode, as we mapped binding to the KATNA1 MIT domain between helices H1 and H3. The ability to adopt multiple different binding modes increases the number of possible partnerships with MIT domain proteins, and helps to explain why IST1, CHMP3 and CHMP1B are the most promiscuous MIT binders.

SPASTIN and KATNA1 midbody recruitment and microtubule severing

ESCRT-III proteins coordinate the recruitment of the microtubule severing enzymes KATNA1 and SPASTIN to complete abscission. Following anaphase, densely packed midbody microtubules must be cleared from the midbody to facilitate cytokinetic membrane abscission (Sharp and Ross, 2012). Both SPASTIN and KATNA1 form hexameric rings (Hartman and Vale, 1999, Eckert et al., 2012) that can engage the C-terminal tails of TUBULIN subunits within the central pore (Kuo and Howard, 2021). ATP hydrolysis then drives polypeptide translocation, thereby promoting subunit exchange and/or microtubule severing (Roll-Mecak and Vale, 2008, Zehr et al., 2017). We observed distinct midbody localization patterns for SPASTIN and KATNA1 (Figure 5), which may reflect their recruitment by distinct sets of ESCRT-III proteins (CHMP1B-IST1 vs CHMP3). In turn, this could allow the two enzymes to act on different pools of midbody microtubules.

Our discovery that SPASTIN, but not necessarily KATNA1, is required for abscission checkpoint maintenance further underscores that these enzymes likely mediate different aspects of microtubule dynamics during abscission. Furthermore, the surprising checkpoint requirement for SPASTIN suggests that microtubule severing may also be required to support abscission arrest (in addition to physically removing microtubule barriers to allow abscission). Possible roles for SPASTIN in maintaining abscission arrest include: 1) slowing microtubule catastrophe via an ATP-independent activity(Kuo et al., 2019) and thereby paradoxically stabilizing severed midbody microtubules, or 2) creating shorter microtubules that promote regrowth and dynamically increase microtubule networks(Kuo et al., 2019, Vemu et al., 2018), and could thereby stabilize the midbody and/or traffic abscission factors into or out of the abscission zone(Fremont and Echard, 2018). Our findings point to the need for more detailed studies of the mechanistic roles of microtubule remodeling in abscission and in checkpoint regulation.

The discovery that CHMP3 and KATNB1(Rezabkova et al., 2017) share overlapping binding sites on the KATNA1 MIT domain has important functional implications (Figure 4). KATNB1 targets KATNA1 to centrosomes during mitosis(Matsuo et al., 2013), whereas CHMP3 targets KATNA1 to midbody arms during cytokinesis (Figure 6). Thus, KATNA1 likely switches partnerships from KATNB1 to CHMP3 during this cell cycle transition. KATNB1 binding also regulates KATNA1 microtubule severing activities(Faltova et al., 2019), specificities(Faltova et al., 2019), and binding partners(Jiang et al., 2017, Jiang et al., 2018), and it will therefore be of interest to learn how CHMP3 replacement alters these KATNA1 activities.

CAPN7 functions in abscission and the abscission checkpoint

Finally, we have discovered that the cysteine protease, CAPN7, localizes to midbodies and supports abscission and the abscission checkpoint. Our screen identified IST1 as the sole ESCRT-III binding partner for the CAPN7 MIT domains (Figure 2, Figure 3), in good agreement with previous reports of IST1 binding in pulldown assays(Osako et al., 2010, Maemoto et al., 2011). The CAPN7 MIT domain also reportedly binds the second alpha-helical region of the CHMP1B core domain (but not the C-terminal MIM region)(Maemoto et al., 2011). We did not survey this interaction because our screen included only C-terminal ESCRT-III tails, but our mutational and localization analyses indicate that CAPN7 midbody recruitment is tightly dependent on IST1 binding (Figure 4, Figure 6).

In addition to recruiting CAPN7, IST1 binding may also enhance CAPN7 proteolytic activity within the midbody because IST1 binding was shown to activate CAPN7 proteolysis of an artificial substrate(Osako et al., 2010, Maemoto et al., 2013). Interestingly, CAPN7 orthologues in *Aspergillus* (PalB) and budding yeast (Rim13) function together with ESCRT-III binding partners to cleave the PEST peptide sequences of the transcription factors Rim101 and PacC, and thereby enhance gene expression(Rodriguez-Galan et al., 2009, Subramanian et al., 2012). Similarly, human CAPN7 can bind and cleave PEST elements of the HOXA10 transcription factor(Yan et al., 2018). Thus, IST1 recruitment of CAPN7 to the midbody could provide spatial and

temporal control of proteolysis, leading to downstream signaling activities required for checkpoint maintenance and/or abscission.

Cofactors for other ESCRT-dependent processes

Although we have focused on cytokinetic abscission, the same experimental framework can be used to characterize MIT cofactors for other cellular ESCRT functions, including intraluminal vesicle formation at the multi-vesicular body, nuclear envelope resealing, plasma membrane repair, and enveloped virus budding(Christ et al., 2017, Vietri et al., 2020, Zhen et al., 2021). Many of the newly discovered partnerships merit investigation in these other ESCRT functions. For example, CHMP7 is a specialized ESCRT-III protein that functions in post-mitotic closure and repair of the nuclear envelope(Vietri et al., 2015, Gu et al., 2017, Olmos et al., 2015, von Appen et al., 2020, Denais et al., 2016, Thaller et al., 2019). Our observation that CHMP7 interacts exclusively with the MIT domain of the AMSH deubiquitinase supports the possibility that these events may be dynamically regulated by ubiquitin-dependent processes, as has been recently reported(Wallis et al., 2021). More generally, our quantitative definition of the ESCRT-III-MIT interactome should provide a basis for probing how disruption of ESCRT-III and MIT cofactor activities can contribute to disease states such as hereditary spastic paraplegia(Ciccarelli et al., 2003), or can be used therapeutically, for example in anti-cancer strategies based on VPS4 synthetic lethality(Neggers et al., 2020, Szymanska et al., 2020).

Supplemental discussion

Meiotic clade AAA-ATPases

Single N-terminal MIT domains are prominent in most meiotic clade AAA+ ATPases including VPS4A, VPS4B, SPASTIN, KATNA1, and KATNAL1 (Monroe and Hill, 2016). These ATPases function as hexamers and avidity will therefore enhance their binding to polymeric ESCRT-III binding partners. KATNAL2 and FIDGETIN are the only meiotic clade ATPases that appear to lack MIT domains. In the place of a MIT domain, KATNAL2 contains an LisH domain (Iwaya et al., 2010), and secondary structure predictions do not reveal three characteristic MIT alpha helices at the N-terminus of FIDGETIN (data not shown). Of the AAA+ ATPases, the VPS4A and B MIT domains are the most promiscuous ESCRT-III interactors, and previous studies using biosensor binding and yeast two hybrid approaches had identified CHMP1A, 1B, 2A, 3, 4A-C, 6, and IST1 as VPS4 binding partners (von Schwedler et al., 2003, Scott et al., 2005, Stuchell-Brereton et al., 2007, Kieffer et al., 2008, Bajorek et al., 2009a). Our screen confirmed VPS4 binding to CHMP1A, 1B, 2A, 2B, 6, and IST1, with 1:1 K_D 's ranging from 0.5 – 54 μ M, with VPS4B consistently showing 3 – 20 fold weaker binding than VPS4A. CHMP3 and the CHMP4 proteins are also likely biologically relevant VPS4 MIT binding partners (Lin et al., 2005, Kieffer et al., 2008, Stuchell-Brereton et al., 2007, Adell et al., 2014), but their 1:1 binding affinities were below our cutoff. Thus, VPS4 enzymes bind all ESCRT-III paralogs, except the specialized CHMP5 and CHMP7 subunits, consistent with the central role that VPS4 enzymes play in remodeling ESCRT-III filaments.

SPASTIN MIT, in contrast, binds specifically to two ESCRT-III proteins: CHMP1B and IST1 (Yang et al., 2008, Renvoise et al., 2010). Our screen confirmed these interactions and showed weak or non-binding to the remaining ten ESCRT-III proteins ($K_D > 200 \mu$ M). Both CHMP1B and IST1 form an amphipathic helix that binds in the MIT groove formed by helices H1 and H3 (Yang et al., 2008) (Type 3 binding, Figure 4, and Figure 4-figure supplement 1). The CHMP1B and IST1 binding surfaces on the MIT are overlapped leading to mutually exclusive and competitive binding. Our screen revealed for the first time that KATNA1 and KATNAL1 MITs are also specific binders, in this case interacting with only CHMP3 (Figure 2). CHMP3 binding to the KATNA1 MIT domain was eliminated by the H1/3 groove mutant V55D, which is consistent with Type 3 binding (Figure 4G). Though KATNA1 and KATNAL1 binding constants are relatively weak, 32 and 120 μ M respectively, avidity is likely prevalent for these interactions *in cell* due to the hexameric association of KATNA1/KATNAL2 subunits and polymeric CHMP3 assemblies.

Proteases

The deubiquitinases AMSH, AMSHLP, USP8 and USP54, and the protease CAPN7 all contain MIT domains that can bind ESCRT-III subunits. CAPN7 is discussed in the main text, and therefore will not be extensively discussed here, and structures of the MIT domains of USP8 (Avvakumov et al., 2006) and AMSH (Solomons et al., 2011) have been determined by others. The MIT domain from the deubiquitinase AMSH is promiscuous,

binding 10/12 ESCRT-III proteins: CHMP1A, 1B, 2A, 2B, 3, 4A, 4B, 4C, 5 and 7. Seven of these interactions have been reported (Tsang et al., 2006, Ma et al., 2007) (Agromayor and Martin-Serrano, 2006, Row et al., 2007, Solomons et al., 2011), whereas the CHMP5 and CHMP7 interactions are new. The CHMP5(Colcher et al., 1977, Tsang et al., 2006) and CHMP4C(Tsang et al., 2006) interactions tested negative in previous screens, and the CHMP7 interaction had not been tested previously. CHMP3 makes a tight ($K_D = 0.011 \mu M$) Type 4 interaction with AMSH MIT(Solomons et al., 2011)(Figure 1C). The remaining ESCRT-III proteins are strong to moderate binders (K_D 's = 7 - 85 μM), and CHMP6 and IST1 do not bind ($K_D > 200 \mu M$). The AMSH paralogue, AMSHLP, also binds preferentially to CHMP3, albeit weakly. To our knowledge, this protein had not previously been tested for ESCRT-III interactions.

USP54 is a catalytically inactive deubiquitinase, and was predicted to contain an MIT domain in a bioinformatics study(Rigden et al., 2009). We found that the USP54 MIT domain bound weakly to all 12 ESCRT-III proteins ($K_D = 70-170 \mu M$, see Figure 2-figure supplement 4), suggesting nonspecific binding in our assay. CHMP1B, CHMP2A, CHMP2B, CHMP4C and CHMP6 were previously identified as USP54 MIT binding partners in *in vitro* pulldown binding assays(Rigden et al., 2009). USP54 is overexpressed in colorectal cancer(Fraile et al., 2016) and mutations outside of the predicted MIT domain are enriched in patients relapsing with acute lymphoblastic leukemia(Xiao et al., 2016). Mechanistic roles for WT and mutant USP54 proteins in tumorigenesis remain to be determined.

The USP8 MIT domain bound CHMP1B, CHMP2A, CHMP2B, CHMP3, CHMP4A, CHMP4B, and IST1 in our screen (Figure 2, Figure 2-figure supplement 1). We did not observe the CHMP1A or CHMP4C binding reported in other studies(Row et al., 2007, Rigden et al., 2009), but we uniquely detected CHMP4A and CHMP4B binding (Figure 2, Figure 2-figure supplement 1, Figure 2-figure supplement 5) . The tightest interactions were with IST1 ($K_D = 0.5 \mu M$) and CHMP3 ($K_D = 12 \mu M$) while the other interactions were weak ($K_D = 70-185 \mu M$). IST1 binding had been reported previously(Agromayor et al., 2009).

Kinases

Three MIT proteins contain kinase domains: ULK3, RPS6KC1, and RPS6KL1. Of these, only ULK3 has been shown to have catalytic activity(Caballe et al., 2015, Maloverjan et al., 2010a, Maloverjan et al., 2010b), and RPS6KC1 and RPS6KL1 are predicted to contain pseudo-kinase domains(Hayashi et al., 2002). Our screen confirmed ULK3 MIT interactions with CHMP1A, CHMP1B, CHMP2A and IST1, which were previously identified by pulldown and yeast two hybrid assays(Caballe et al., 2015), and we found that ULK3 MIT also binds CHMP3. The MIT domains of RPS6KC1 and RPS6KL1 had not previously been assayed for ESCRT-III binding, and our new findings that they interact with IST1 and CHMP3, respectively, now link these understudied proteins to the ESCRT pathway. Interestingly, RPS6KC1 binds sphingosine kinase 1 (SPHK1) and localizes to

endosomes(Hayashi et al., 2002, Liu et al., 2005), where ESCRT machinery creates cargo-carrying intraluminal(Hurley, 2015) and cytoplasmic vesicles(McCullough et al., 2015, Allison et al., 2013).

Other MIT proteins

MITD1 showed the broadest ESCRT-III promiscuity of the other MIT proteins tested, binding CHMP1A, 1B, 2A, 3, 4A, 4B, 6 and IST1. The CHMP1A, 1B, 2A, and IST1 interactions were detected previously using pulldowns and yeast two hybrid assays(Hadders et al., 2012, Lee et al., 2012, Agromayor et al., 2009), and a structure of the MITD1 MIT-CHMP1A complex has been reported(Hadders et al., 2012). Interestingly, Hadders and colleagues identified a PEG molecule bound to the H1/H3 groove of one of two inequivalent MITD1 MIT domains and noted that this might indicate a propensity for binding ESCRT-III molecules in this groove(Hadders et al., 2012). The new interactions that we identified with CHMP3, CHMP4A, CHMP4B, and CHMP6 could, in principle, all utilize the H1/H3 groove because CHMP4A, 4B, and 6 all make Type 2 interactions(Kieffer et al., 2008, Samson et al., 2008), and CHMP3 can apparently bind in this groove in a Type 3 mode (see main text for details). A key difference between our experiments and those reported previously, is that our screen employed isolated MIT domains whereas others have used full-length proteins. In the full-length protein, the H1/H3 groove may be autoinhibited by the C-terminal PLD domain (Figure 1B) and may therefore only be accessible in specific biological contexts, i.e., when the protein binds membranes or dimerizes. As noted in the main text, MITD1 stabilizes the intercellular bridge to promote proper abscission(Hadders et al., 2012, Lee et al., 2012), and this extended function may explain the need for promiscuous ESCRT-III binding.

LIP5 was also quite promiscuous, and bound CHMP1A, 1B, 2A, 2B, 3, 5, and IST1. We, and others had previously reported these interactions(Shim et al., 2008, Ward et al., 2005, Skalicky et al., 2012, Guo and Xu, 2015). In contrast to Guo et al(Guo and Xu, 2015), however, we found that the IST1 MIM₃₁₆₋₃₄₃ element contributed to binding, as an IST1 peptide that encompassed both MIM elements bound ~10-fold more tightly than a peptide spanning MIM₃₄₄₋₃₆₆ alone (Figure 3C). As mentioned in the main text, the LIP5(MIT)₂-CHMP5 complex was the highest affinity interaction seen in all of our studies (2.5 nM), which is explained by the Type 5 interaction mode, in which the extended CHMP5 tail and associated linkers wrap almost completely around the second MIT domain(Skalicky et al., 2012) (Yang et al., 2012) (see Figure 1C). In contrast, other ESCRT-III proteins bind in the H2/H3 groove of the first MIT domain in a Type 1 interaction(Guo and Xu, 2015, Skalicky et al., 2012).

The MIT domains of SNX15 and SPARTIN both bind exclusively to IST1. The SPARTIN-IST1 interaction had been reported previously(Renvoise et al., 2010, Guo and Xu, 2015), and we found that the IST1 MIM₃₄₄₋₃₆₆ element provided most of the binding energy (Figure 3C), in good agreement with previous studies. This Type 3 interaction was visualized in a structure of the SPARTIN MIT-IST1 complex(Guo and Xu, 2015). Ours is the first report of ESCRT-III binding by SNX15, and we found that both IST1 MIM

1 elements contribute significantly, indicating that both elements can simultaneously
 2 occupy both the H1/H3 and H2/H3 grooves. SNX15 has been reported to function in
 3 endosomal protein trafficking(Phillips et al., 2001), which is also a major site of ESCRT
 4 function (see above).

5 As discussed in the main text, the MIT domains ULK1, VPS9D1 and NRBF2 did not bind
 6 ESCRT-III proteins in our screen.

Materials and methods

Identification and cloning of human MIT domains

MIT domains were selected from the literature (Ciccarelli et al., 2003, Rigden et al., 2009, Row et al., 2007, Skalicky et al., 2012, Xiao et al., 2008, Fujioka et al., 2014). Bacterial expression constructs were designed using previous reports (when available)(Stuchell-Brereton et al., 2007, Caballe et al., 2015, Fujioka et al., 2014, Hadders et al., 2012, Solomons et al., 2011, Avvakumov et al., 2006, Yang et al., 2008, Guo and Xu, 2015, Iwaya et al., 2010, Yorikawa et al., 2008, Osako et al., 2010, Skalicky et al., 2012)(NRBF2; PDB 2CRB; unpublished) or were guided by secondary structure predictions using Phyre2(Kelley et al., 2015).

Bacterial expression constructs for ULK3, VPS4A, VPS4B, and LIP5 have been reported(Caballe et al., 2015, Scott et al., 2005, Stuchell-Brereton et al., 2007, Skalicky et al., 2012). Other DNAs were obtained from Addgene (RPS6KC1: 23460; ULK1: 31961), Dharmacon (SPARTIN: MHS6278-2028092; Clone ID: 5313379) and DNASU (NRBF2: HSCD00424935; KATNA1:HSCD00445516; KATNL1:HSCD00079435; KATNAL2: HSCD00733526; KATANA1B: HSCD00042784; RPS6KL1:HSCD00045064; AMSH: HSCD00078710; AMSHLP: HSCD00438568; USP8: HSCD004366965; SNX15: HSCD00404630; CAPN7: HSCD00404981; VPS9D1:HSCD00620221; MITD1: HSCD00356996; NRBF2:HSCD00434935). Bacterial and mammalian expression constructs for CAPN7 were further mutated to match the reference sequence NP055111.1 by making the following substitutions using quick change mutagenesis: G151S, E173V, E495K. SPASTIN was subcloned from EST ACCT 7491861(Han et al., 2020). USP54 MIT was made as a gene string (Thermofisher), and the Myc-ATG13 expression construct was a gift from Do-Hyung Kim (Addgene plasmid #31965; <http://n2t.net/addgene:31965>; RRID: Addgene_31965)(Jung et al., 2009).

Expression inserts for cell lines were generated by PCR amplification and ligated into the pLVX-tight-Puro vector (Clontech) using the NEB HiFi DNA Assembly Kit (New England Biolabs) according to the manufacturer's instructions. Human SPASTIN has four major isoforms(Mancuso and Rugarli, 2008, Claudiani et al., 2005), and we used the most abundant (M87) isoform, including residues encoded by exon 4.

Gene names, DNA sources, and amino acid sequences are given in Supplemental Tables 1 and 4. All plasmids generated by this study have been deposited to Addgene for distribution (See Supplemental Table 4 for Addgene accession numbers).

Bacterial expression of MIT domains

Proteins were expressed in BL21 RIPL cells grown in ZYP-5052 autoinduction media(Studier, 2005). Transformed cells were initially grown for 3-6 h at 37°C, and then switched to 19°C for an additional 20 h. Cells were harvested by centrifugation at 5,400 x g and cell pellets were stored at -80°C. MIT domains from VPS4A, VPS4B, ULK3, and

LIP5 were expressed and purified as described previously (Caballe et al., 2015, Skalicky et al., 2012, Scott et al., 2005, Stuchell-Brereton et al., 2007).

Purification of (His)₆-fusion proteins

All steps were carried out at 4°C except where noted. Frozen cell pellets were thawed and resuspended in lysis buffer: 50 mM Tris pH 8.0, 500 mM NaCl, 1 mM Dithiothreitol (DTT), 0.5 mM EDTA supplemented with 0.125% sodium deoxycholate, lysozyme (25 µg/mL) PMSF (35 µg/mL), pepstatin (1 µg/mL), leupeptin (0.5 µg/mL), aprotinin (0.1 µg/mL), DNase1 (25 µg/mL), and 1 mM MgSO₄. Cells were lysed by sonication and lysates were clarified by centrifugation at 37,000 x g for 60 min. The clarified supernatant was filtered through a 0.45 µm cartridge filter and incubated with 10 mL of cOmplete His-Tag purification beads (Roche, Germany) for 45 min. Beads were washed with 500 mL of wash buffer: 25 mM Tris (pH 8.0), 500 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and then with 500 mL wash buffer with 200 mM NaCl. Fusion proteins were eluted with 50 mL of 200 mM NaCl wash buffer supplemented with 200 mM imidazole, pH 8.0. Eluted protein solutions were treated with 100 µg of protease ((His)₆-ULP1 for (His)₆-SUMO fusions; GST-HRV3C preScission protease for (His)₆-GST fusions) in 3.5 kDa cutoff dialysis bags while dialyzing against 2 x 2 L of 200 mM NaCl wash buffer for 16-24 h. Uncut (His)₆-SUMO-MIT fusion, (His)₆-SUMO tag, and (His)₆-ULP1 proteases were removed with 5 mL of cOmplete His-Tag purification beads, and the MIT fusion proteins were concentrated and prepared for gel filtration chromatography.

For (His)₆ fusions of RPS6KL1, RPS6KC1, AMSH, AMSHLP, and USP8, NRBF2, MITD1, and SPARTIN, nickel column eluates were dialyzed against 2 x 2L of 25 mM Tris pH 8.0 (25 °C), 50 mM NaCl, 1 mM EDTA, 1 mM DTT in the presence of ~100 µg protease ((His)₆ULP1 for (His)₆SUMO fusions; GST-HRV3C preScission protease for (His)₆GST fusions). Dialyzed cleavage reactions were purified by chromatography on a 5 mL HiTrapQ Sepharose Column (GE Healthcare Life Sciences, USA) (or for SPARTIN, a HiTrap SP column) and eluted with gradient of 50 – 1000 mM NaCl to separate HIS-SUMO, or HIS-GST from pure MIT fractions. Pure MIT fractions were pooled, concentrated, and further purified by gel filtration chromatography.

Purification protocols for the CAPN7, SNX15 and VSP9D1 MIT domains were sufficiently different to merit separate descriptions (below).

(His)₆-GST-CAPN7(MIT)₂ was bound to GST-sepharose beads (10 mL, GE Healthcare Life Sciences, USA, 6 h), washed with 1 L wash buffer: 25 mM Tris (pH 8), 500 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and eluted with ~50 mL of wash buffer supplemented with 20 mM reduced L-glutathione (pH 8). The fusion tag was removed using ~100 µg GST-HRV3C (preScission) protease in a 3.5 kDa cutoff dialysis bag while dialyzing against 2 x 2 L wash buffer and 2 L of wash buffer with low salt (50 mM NaCl) over two days. The dialysate was purified by Q Sepharose chromatography (50 mL; GE Healthcare Life Sciences, USA) with a linear gradient of 50-500 mM NaCl. Fractions containing highly pure CAPN7(MIT)₂ were pooled and concentrated for gel filtration chromatography.

(His)₆-GST-SNX15 MIT was bound to cOmplete His-Tag purification beads (5 mL, Roche, Germany, 2h) and washed with 2 L wash buffer: 25 mM Tris (pH 8), 500 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and eluted with wash buffer supplemented with 250 mM imidazole (pH 8). The eluent was bound to glutathione sepharose beads (10 mL, GE Healthcare Life Sciences, USA, 6 h), washed with 1 L wash buffer: 25 mM Tris (pH 8), 500 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and eluted with ~50 mL of wash buffer supplemented with 20 mM reduced L-glutathione (pH 8). The fusion tag was removed using ~100 µg GST-HRV3C (preScission) protease in a 3.5 kDa cutoff dialysis bag while dialyzing against 2 x 2 L wash buffer and 2 x 2 L of wash buffer with low salt (25 mM NaCl) over two days. The dialysate was purified by Q Sepharose chromatography (50 mL, GE Healthcare Life Sciences, USA). SNX15 MIT eluted in the flow through and was concentrated for gel filtration chromatography.

(His)₆-GST-VPS9D1 MIT was lysed in 50 mM Tris pH 7.4 (25 °C), 500 mM NaCl, 0.5 mM EDTA, and 1 mM TCEP supplemented with 0.125% sodium deoxycholate, lysozyme, PMSF, pepstatin, leupeptin, aprotinin and DNase I (as described above). The clarified cell lysate was bound to 10 mL of cOmplete His-Tag purification resin (Roche, Germany, 30 min), washed with 1 L base buffer, and eluted with ~50 mL of base buffer supplemented with 250 mM imidazole (pH 8.0). The fusion tag was removed by incubation with ~100 µg GST-HRV3C protease in 3.5 kDa cutoff dialysis bag while dialyzing against 2 x 2 L of 25 mM Tris pH 7.4 (25 °C), 50 mM NaCl, 1 mM DTT, 0.5 mM EDTA. The dialysate was purified by Q Sepharose chromatography (50 mL, GE Healthcare Life Sciences, USA) and eluted with a 50-1000 mM NaCl linear gradient. VPS9D1 eluted in the flow through and was concentrated for gel filtration chromatography.

Finally, all MIT proteins were purified by Superdex 75 gel filtration chromatography (GE Healthcare Life Sciences, USA) in 25 mM Tris (pH 7.2 at 25 °C), 150 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. MIT protein fractions were pooled and concentrated. MIT domain masses were confirmed with ESI-MS or MALDI-TOF (University of Utah Mass Spectrometry Core Facility, see Supplemental Table 1). Yields ranged between 0.5-35 mg/L of bacterial culture.

Expression and purification of ESCRT-III C-terminal peptides

ESCRT-III C-terminal peptides were prepared as described previously (Talledge et al., 2018). Briefly, most peptides were expressed as (His)₆-SUMO-fusions, except for IST1-MIM₃₁₆₋₃₄₃ and IST1 MIM₃₄₄₋₃₆₆ which were made synthetically by the University of Utah Peptide Synthesis Core. Peptides were expressed in BL21-Codon Plus (DE3) RIPL cells (Agilent, Santa Clara, CA, USA) in ZYP-5052 auto-induction media (Studier, 2005). Cells were lysed by sonication in lysis buffer (40 mL/L of culture) containing 50 mM Tris, pH 7.2, 150 mM NaCl, 5 mM imidazole, 2 mM DTT, 0.5 mM EDTA, and 0.125% sodium deoxycholate, supplemented with lysozyme, protease inhibitors, and DNase I (Roche, Germany) (as described above). Clarified cell lysates were incubated with 10 mL of cOmplete His-Tag purification resin (Roche, Germany) for 30 min, washed with 500 mL wash buffer: 50 mM Tris, pH 7.2, 500 mM NaCl, 5 mM Imidazole, 5 mM DTT, 0.5 mM

EDTA, and then with 500 mL wash buffer containing 150 mM NaCl. (His)₆-SUMO affinity tags were removed by on-column cleavage with (His)₆-ULP1 (0.7 mg, overnight, 4°C) in 40 mL of the 150 mM NaCl wash buffer. Cleaved peptides were collected from the column flow through and dialyzed against 25 mM sodium phosphate, pH 6.5, 50 mM NaCl, 2 mM DTT, 0.5 mM EDTA, and then purified by Q-sepharose ion exchange chromatography (GE Healthcare Life Sciences, USA) with a linear gradient from 50 mM to 1 M NaCl. Peptide fractions were pooled and dialyzed against 25 mM Tris, pH 7.2, 150 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, and further purified by Superdex-75 size exclusion chromatography (GE Healthcare Life Sciences, USA). Typical IST1 peptide yields were 4.5 mg/L culture. Purified ESCRT-III C-terminal fragments contained non-native 'GlyCys' or 'Cys' residues at their N-termini, and masses were confirmed by mass spectrometry (see Supplemental Table 1) either before labeling (CHMP4C, CHMP1A₁₄₀₋₁₉₆, CHMP1B₁₄₃₋₁₉₉) or after labeling (all other peptides; dye adds a mass shift of 463.4 Da). Competition experiments used peptides lacking N-terminal 'Cys' residues to avoid disulfide cross-linking.

Peptide fluorescent labeling

Fluorescent labeling was performed by the University of Utah DNA/Peptide Synthesis Core as described previously (Caballe et al., 2015, Talledge et al., 2018). Briefly, peptides were labeled in DMSO using ~1.3-fold molar excess of Oregon Green 488 maleimide (Life Technologies/Molecular Probes #O6034, USA) dissolved in a 1:1 solution of acetonitrile:DMSO. Reversed phase HPLC was used to monitor the reactions and separate labeled peptides from unreacted dye and unlabeled peptides using reversed phase HPLC. Labeled peptide fractions were dried under vacuum and dissolved in water. Peptide concentrations were quantified using the absorbance of Oregon Green 488 at 491 nm ($\epsilon = 83,000 \text{ cm}^{-1} \text{ M}^{-1}$ in 50 mM potassium phosphate, pH 9.0).

Fluorescence polarization anisotropy binding assays

Binding experiments were performed as described previously (Caballe et al., 2015) in 25 mM Tris, pH 7.2, 150 mM NaCl, 0.1 mg/mL Bovine Serum Albumin (BSA), 0.01% Tween-20, and 1 mM DTT, with 250-500 pM fluor-labeled ESCRT-III peptides and purified MIT domains at the indicated concentrations. A Biotek Synergy Neo Multi-Mode plate reader (Biotek, USA) was used to measure fluorescence polarization with excitation at 485 nm and emission (detection) at 535 nm. Binding isotherms were fit to 1:1 models using KaleidaGraph (Synergy Software) as described previously (Skalicky et al., 2012, Talledge et al., 2018). Reported K_D values are averages from at least three independent isotherms. Non-binding ($K_D > 200 \text{ } \mu\text{M}$) was confirmed at least twice independently. Competitive binding experiments were used to calculate the K_i for the interaction of USP8 with IST1, as direct binding experiments failed to generate the large changes in polarization needed to reliably fit dissociation constants (Figure 2-figure supplement 5C). Competition experiments were performed as described previously (Sadler et al., 2018). Briefly, complexes of MIT domains and fluorescently labeled peptides (26.6 μM MITD1 and 0.5 nM CHMP4B peptide; 0.75 μM USP8 and 0.5 nM IST1 peptide, 12.5 μM USP8 MIT and

0.5 nM CHMP4B peptide) were titrated with the indicated concentrations of unlabeled peptides. IC₅₀s were calculated using KaleidaGraph (Synergy Software) and then converted to K_i values (Cer et al., 2009). Competitive binding curves were measured independently three or more times for each peptide, but only once for each positive control peptide.

Co-immunoprecipitation experiments

KATNA1 pulldowns. HEK293T cells were seeded at 0.5 x 10⁶ cells per well in 6-well plates and transfected 24 h later with 1 µg of plasmid encoding Myc-KATNB1 and one of the following: 2 µg empty vector control, 1.5 µg OSF-KATNA1-WT, 2 µg of OSF-KATNA1-R14A, or 1.5 µg of OSF-KATNA1-V55D P60. Empty vector was added as necessary to bring the total transfected DNA to 3 µg/well. Cells were harvested 48 h post-transfection and lysed in 400 µl of 50 mM Tris, pH 7.2, 150 mM NaCl, 0.5% Triton-X100, 1 mM DTT, protease inhibitors (1:100, Sigma). Lysates were clarified by centrifugation at 16,100 x g for 10 min at 4°C, and clarified lysates were incubated with 20 µl of a 50% slurry of Strep-Tactin resin (IBA Biosciences) for 30 min. Beads were washed 4x with 500 µl lysis buffer. After the final wash, Strep-Tactin beads were aspirated to near dryness and bound proteins were eluted by boiling in 40 µl of 2x Laemmli sample buffer, resolved by SDS-PAGE, and detected by Western blotting.

ULK(MIT)₂ pulldowns. HEK293T cells were seeded in 6 well plates as above and DNA levels were optimized to normalize expression levels: 3 µg pCAG-OSF-ULK3(MIT)₂ (residues 277-449) and 250 ng of pCAG-CHMP1A-myc, pCAG-CHMP1B-myc, or pCAG-Myc-IST1; 1.5 µg pCAG-OSF-PP-ULK1(MIT)₂ (residues 833-1050) and 500 ng of pCAG-CHMP1A-myc, pCAG-CHMP1B-myc, or pCAG-Myc-IST1; 500 ng pCAG-OSF-PP-ULK1(MIT)₂ and 2 µg Myc-ATG13; 2.5 µg pCAG-OSF empty vector and 500 ng of pCAG-CHMP1A-myc, pCAG-CHMP1B-myc, or pCAG-Myc-IST1; 1 µg pCAG-OSF empty vector and 2 µg Myc-ATG13. Cells were harvested 24 h post transfection and lysed in 50 mM Tris, pH 7.2, 1% Triton, 150 mM NaCl, 1mM DTT, 1:100 mammalian protease inhibitors (Sigma). Lysates were clarified by centrifugation at 16,100 x g for 10 min at 4°C, and incubated with 20 µl of a 50% slurry of Strep-Tactin resin (IBA Biosciences) for 30 min at 4°C. Beads were washed 4x with 500 µl lysis buffer. After the final wash, Strep-Tactin beads were aspirated and bound proteins were eluted and detected by Western blotting as described above.

For ULK(MIT)₂ pulldowns with CHMP2A (Figure 1-figure supplement 1B); 10 cm dishes of 60-80% confluent HEK293T cells were individually transfected with PEI and 12 µg of pCAG-OSF-ULK3(MIT)₂, pCAG-OSF-PP-ULK1(MIT)₂, pCAG-CHMP2A-myc, pCAG-CHMP2A-L216D/L219D, Myc-ATG13, or pCAG-OSF-empty vector control. Cells were harvested 24 h post-transfection, lysed in 25 mM Tris, pH 7.2, 150 mM NaCl, 1 mM DTT, 1% Triton-X100 supplemented with 1:100 mammalian protease inhibitors (Sigma), and lysates were clarified by centrifugation at 16,100 x g for 10 min at 4°C. Clarified lysates expressing CHMP2A-Myc were mixed with lysates expressing OSF-ULK(MIT)₂ or empty vector controls and incubated overnight at 4°C. Lysate mixes were then incubated with

20 μ l of a 50% slurry of Strep-Tactin resin (IBA Biosciences) for 1 h at 4°C. Beads were washed 5x with 500 μ l of 25 mM Tris, pH 7.2, 150 mM NaCl, 1mM DTT, 0.5% Triton-X100 buffer. After the final wash, Strep-Tactin beads were aspirated, and bound proteins were eluted and detected by Western blotting.

X-ray crystallography

SPASTIN MIT and IST1 peptide (residues 344-366) were prepared as described above, mixed at a 1:1.2 molar ratio (14.25 mg/ml SPASTIN MIT and 4.8 mg/ml IST1 peptide), and filtered using a 0.2 μ m cartridge filter. This complex was mixed in a 2:1, 1:1, and 1:2 (v/v) ratio with 100 mM sodium cacodylate/ hydrochloric acid pH 6.5, 40% (v/v) PEG 300, 200 mM calcium acetate (0.6 μ L final volume; Wizard Cryo 1/2 screen (Rigaku, USA), condition D1). Crystals formed by sitting drop vapor diffusion after ten days at 4°C in 1:1 and 1:2 (v/v) ratios. A crystal from 1:1 or 1:2 ratio was suspended in a small nylon loop and cryocooled by plunging in liquid nitrogen. Supplemental cryoprotection was not used for the crystals grown in these conditions.

X-ray diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) using beamline 9.1. The crystal was maintained at 100 °K with the aid of a cold nitrogen gas stream during data collection. Data were integrated and scaled using XDS(Kabsch, 2010a, Kabsch, 2010b) and AIMLESS(Evans and Murshudov, 2013, Evans, 2011). Initial phases were obtained from phenix-phaser(Bunkoczi et al., 2013) using SPASTIN MIT (PDB 3EAB)(Yang et al., 2008) as a search model. The resulting electron density was readily interpretable and further built using Coot(Emsley and Cowtan, 2004, Emsley et al., 2010) and phenix-refine(Liebschner et al., 2019).

The model was evaluated using Molprobity(Davis et al., 2007, Williams et al., 2018) and judged to be of good quality. Two regions of unexplained density were present in the Fo-Fc electron density map. PEG 300 is present at 40% (v/v) in the crystallization condition and the density resembled smaller PEG molecules. Several molecules were tested by model building and refinement and the best fit to the density was obtained with one molecule of triethylene glycol (PGE) and one of tetraethylene glycol (PG4). The PEG molecules improved the model statistics and maintained good geometry. Two initially assigned water molecules also produced unexplained density in the Fo-Fc electron density map. Several ions were tested by model building and refinement until the best fit was obtained with one chloride ion and one calcium ion. Both ions are present in the crystallization condition (hydrochloric acid and calcium acetate), and their inclusion improved the model statistics and maintained good geometry. The final model refined to $R_{work}=0.149$ and $R_{free}=0.159$. Full statistics and data collection details are provided in Supplemental Table 7.

An omit map of the IST1 portion of the complex was generated for figure presentation. This map was calculated by removing IST1 from the final model followed with phenix refinement and simulated annealing (Cartesian) to minimize phase bias (Figure 4 - supplemental figure 4A).

Cell culture

HEK293T and HeLa cells were cultured and maintained at 37 °C and 5% CO₂ in DMEM supplemented with 10% FBS. TetOn-HeLa cells were supplemented with 500 µg/mL G418 (Invitrogen) to maintain expression of the Tet-On Advanced protein. DOX-inducible cell lines generated in the parental TetOn-HeLa cell line were supplemented with 500 µg/mL G418 + 0.5 µg/mL puromycin (Invivogen).

Cell lines

Our parental HeLa cell line was authenticated by genomic sequencing of 24 loci (University of Utah Sequencing Core) and confirmed to be mycoplasma-free by routine PCR testing (ABM) following the manufacturer's protocols. HeLa cells were transfected with the pLVX-TetOn-Advanced plasmid (Clontech) and selected with 500 µg/mL G418 for 14 days. Single colonies were isolated, expanded, and screened for TetOn-Advanced expression by Western blot using a TetR monoclonal antibody. The optimal clone, which was selected based on a combination of TetOn Advanced expression and tight control of DOX-inducible expression, was used as the parental HeLa TetOn line. To generate stable cell lines with doxycycline-inducible expression, the parental TetOn cell line was transfected with pLVX-tight puro plasmids containing the MIT genes of interest (see Supplemental Table 4) and selected for 14 days in 500 µg/mL G418 + 0.5 µg/mL puromycin. Single colonies were expanded and screened for expression by immunofluorescence and western blotting. Selected clones were further validated by sequencing the PCR amplified MIT gene of interest from genomic DNA. Protein expression was induced by addition of 1 µg/mL doxycycline.

siRNA transfections

For experiments in Figure 5, transfection protocols were as follows: Day 1 – 350,000 cells were reverse transfected with 20 nM siRNA targeting MIT protein (as indicated) in a 35 mm dish using Lipofectamine RNAiMAX and following manufacturer's instructions; Day 2 – cells from Day 1 were trypsinized, resuspended in a total volume of 6 mL DMEM, and divided as follows: 0.5 mL into each of four wells of a 24-well dish containing 12 mm circle glass coverslips, and 2 mL into each of two 35 mm dishes. These samples were again reverse transfected with 20 nM siRNA (as indicated); for active abscission checkpoint samples, cells in two wells of a 24 well dish (for IF) and one 35 mm dish (for WB) were also reverse transfected with 10 nM siNUP153 and 10 nM siNUP50 at this time, incubated for 8 h, then treated with 2 mM thymidine for 24 h; Day 3 – thymidine was removed, cells were washed 2x with warm PBS, and fresh medium added to all cells; Day 4 – 16 h after thymidine release cells were harvested for analysis. For experiments in Figure 6, transfection protocols were as follows: Day 1 – either 70,000 cells (in one well of a 24 well dish containing a 12mm glass coverslip for IF) or 350,000 cells (in a 35mm dish for WB) were reverse transfected with 20 nM siRNA targeting the indicated MIT protein plus 10 nM siNUP153 and 10 nM siNUP50, incubated for 8 h, then treated with 2 mM thymidine for 24 h; Day 2 – thymidine was removed and fresh medium added to all cells (as

described above); Day 3 – 16 h after thymidine release cells were harvested for analysis. siRNA sequences are reported in Supplemental Table 5.

Immunoblotting

Cells were lysed in RIPA buffer (Thermo Fisher) supplemented with mammalian protease inhibitor cocktail (Sigma; used at 1:100 dilution) for 15 min on ice with brief vortexing every 5 min. Lysates were cleared by centrifugation at 17,000 x g for 10 min at 4°C. Lysate protein concentrations were determined using the BCA Assay (Thermo Fisher) and normalized prior to SDS-PAGE. 12 µg lysate per sample were prepared with SDS loading buffer, resolved by SDS-PAGE, and transferred to either PVDF or nitrocellulose. Membranes were blocked for 1 h at room temperature in 5% milk in TBS, then incubated overnight at 4°C with primary antibodies (see Supplemental Table 6 for dilutions). Following 3 x 10 min washes in TBS-T, membranes were incubated with the corresponding secondary antibodies for 1 h at 23°C, washed again with TBS-T, and imaged using a LiCor Odyssey infrared scanner.

Immunofluorescence imaging and phenotype quantification

Cells were seeded on fibronectin-coated glass coverslips and treated with the indicated siRNAs, according to the individual experimental protocol as described in “siRNA transfections”. For analysis, cells were briefly washed with 1X PHEM buffer (60 mM PIPES, 25 mM HEPES, pH 6.9, 1 mM EGTA, 2 mM MgCl₂) and then fixed for 20 min at 23°C in 4% formaldehyde + 0.5% Triton X-100 (in 1X PHEM buffer). Following fixation, cells were washed with PBS and incubated 30 min in blocking buffer (3% FBS + 0.1% Triton X-100 in PBS). Primary antibodies were incubated for least 1 h at 23°C (see Supplemental Table 6 for dilutions). Coverslips were then washed with PBS and incubated with the secondary antibodies (Thermo Fisher) for 1 h at 23°C. Following a final wash in PBS, coverslips were mounted onto glass microscope slides using Prolong Gold Antifade Reagent with or without DAPI (Thermo Fisher).

Images were acquired using a Nikon Ti-E inverted microscope equipped with a 60X PlanApo oil immersion objective, an Andor Zyla CMOS camera, and an automated Prior II motorized stage controlled with the Nikon Elements software. For phenotype quantification in Figure 5, the software was used to acquire 49 images using a randomized 7x7 grid pattern. The images were then blinded and scored independently by four individuals. For Figure 6, 25 images for each treatment were acquired, blinded, and scored independently by three individuals. Quantification and statistical analyses were performed using GraphPad Prism.

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References

- ADDI, C., BAI, J. & ECHARD, A. 2018. Actin, microtubule, septin and ESCRT filament remodeling during late steps of cytokinesis. *Curr Opin Cell Biol*, 50, 27-34.
- ADDI, C., PRESLE, A., FREMONT, S., CUVELIER, F., ROCANCOURT, M., MILIN, F., SCHMUTZ, S., CHAMOT-ROOKE, J., DOUCHE, T., DUCHATEAU, M., GIAI GIANETTO, Q., SALLES, A., MENAGER, H., MATONDO, M., ZIMMERMANN, P., GUPTA-ROSSI, N. & ECHARD, A. 2020. The Flemmingsome reveals an ESCRT-to-membrane coupling via ALIX/syntenin/syndecan-4 required for completion of cytokinesis. *Nat Commun*, 11, 1941.
- ADELL, M. A., VOGEL, G. F., PAKDEL, M., MULLER, M., LINDNER, H., HESS, M. W. & TEIS, D. 2014. Coordinated binding of Vps4 to ESCRT-III drives membrane neck constriction during MVB vesicle formation. *J Cell Biol*, 205, 33-49.
- AGROMAYOR, M., CARLTON, J. G., PHELAN, J. P., MATTHEWS, D. R., CARLIN, L. M., AMEER-BEG, S., BOWERS, K. & MARTIN-SERRANO, J. 2009. Essential role of HIST1 in cytokinesis. *Mol Biol Cell*, 20, 1374-87.
- AGROMAYOR, M. & MARTIN-SERRANO, J. 2006. Interaction of AMSH with ESCRT-III and deubiquitination of endosomal cargo. *J Biol Chem*, 281, 23083-91.
- ALLISON, R., LUMB, J. H., FASSIER, C., CONNELL, J. W., TEN MARTIN, D., SEAMAN, M. N., HAZAN, J. & REID, E. 2013. An ESCRT-spastin interaction promotes fission of recycling tubules from the endosome. *J Cell Biol*, 202, 527-43.
- AMARAL, N., VENDRELL, A., FUNAYA, C., IDRISSE, F. Z., MAIER, M., KUMAR, A., NEUROHR, G., COLOMINA, N., TORRES-ROSELL, J., GELI, M. I. & MENDOZA, M. 2016. The Aurora-B-dependent NoCut checkpoint prevents damage of anaphase bridges after DNA replication stress. *Nat Cell Biol*, 18, 516-26.
- AVVAKUMOV, G. V., WALKER, J. R., XUE, S., FINERTY, P. J., JR., MACKENZIE, F., NEWMAN, E. M. & DHE-PAGANON, S. 2006. Amino-terminal dimerization, NRDP1-rhodanese interaction, and inhibited catalytic domain conformation of the ubiquitin-specific protease 8 (USP8). *J Biol Chem*, 281, 38061-70.
- AZMI, I. F., DAVIES, B. A., XIAO, J., BABST, M., XU, Z. & KATZMANN, D. J. 2008. ESCRT-III family members stimulate Vps4 ATPase activity directly or via Vta1. *Dev Cell*, 14, 50-61.
- BABST, M., KATZMANN, D. J., ESTEPA-SABAL, E. J., MEERLOO, T. & EMR, S. D. 2002. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev Cell*, 3, 271-82.
- BAJOREK, M., MORITA, E., SKALICKY, J. J., MORHAM, S. G., BABST, M. & SUNDQUIST, W. I. 2009a. Biochemical analyses of human IST1 and its function in cytokinesis. *Mol Biol Cell*, 20, 1360-73.
- BAJOREK, M., SCHUBERT, H. L., MCCULLOUGH, J., LANGELIER, C., ECKERT, D. M., STUBBLEFIELD, W. M., UTER, N. T., MYSZKA, D. G., HILL, C. P. & SUNDQUIST, W. I. 2009b. Structural basis for ESCRT-III protein autoinhibition. *Nat Struct Mol Biol*, 16, 754-62.
- BANJADE, S., SHAH, Y. H., TANG, S. & EMR, S. D. 2021. Design principles of the ESCRT-III Vps24-Vps2 module. *Elife*, 10.
- BENZ, C., CLUCAS, C., MOTTRAM, J. C. & HAMMARTON, T. C. 2012. Cytokinesis in bloodstream stage Trypanosoma brucei requires a family of katanins and spastin. *PLoS One*, 7, e30367.
- BUNKOCZI, G., ECHOLS, N., MCCOY, A. J., OEFFNER, R. D., ADAMS, P. D. & READ, R. J. 2013. Phaser.MRage: automated molecular replacement. *Acta Crystallogr D Biol Crystallogr*, 69, 2276-86.
- BUYSSE, D., PFITZNER, A. K., WEST, M., ROUX, A. & ODORIZZI, G. 2020. The ubiquitin hydrolase Doa4 directly binds Snf7 to inhibit recruitment of ESCRT-III remodeling factors in S. cerevisiae. *J Cell Sci*, 133.

- 1 CABALLE, A., WENZEL, D. M., AGROMAYOR, M., ALAM, S. L., SKALICKY, J. J., KLOC, M., CARLTON, J. G.,
2 LABRADOR, L., SUNDQUIST, W. I. & MARTIN-SERRANO, J. 2015. ULK3 regulates cytokinetic
3 abscission by phosphorylating ESCRT-III proteins. *Elife*, 4, e06547.
- 4 CAPALBO, L., MONTEBAULT, E., TAKEDA, T., BASSI, Z. I., GLOVER, D. M. & D'AVINO, P. P. 2012. The
5 chromosomal passenger complex controls the function of endosomal sorting complex required
6 for transport-III Snf7 proteins during cytokinesis. *Open Biol*, 2, 120070.
- 7 CARLTON, J. G., CABALLE, A., AGROMAYOR, M., KLOC, M. & MARTIN-SERRANO, J. 2012. ESCRT-III governs
8 the Aurora B-mediated abscission checkpoint through CHMP4C. *Science*, 336, 220-5.
- 9 CARLTON, J. G. & MARTIN-SERRANO, J. 2007. Parallels between cytokinesis and retroviral budding: a role
10 for the ESCRT machinery. *Science*, 316, 1908-12.
- 11 CER, R. Z., MUDUNURI, U., STEPHENS, R. & LEBEDA, F. J. 2009. IC50-to-Ki: a web-based tool for converting
12 IC50 to Ki values for inhibitors of enzyme activity and ligand binding. *Nucleic Acids Res*, 37, W441-
13 5.
- 14 CHAN, E. Y., LONGATTI, A., MCKNIGHT, N. C. & TOOZE, S. A. 2009. Kinase-inactivated ULK proteins inhibit
15 autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *Mol*
16 *Cell Biol*, 29, 157-71.
- 17 CHRIST, L., RAIBORG, C., WENZEL, E. M., CAMPSTEIJN, C. & STENMARK, H. 2017. Cellular Functions and
18 Molecular Mechanisms of the ESCRT Membrane-Scission Machinery. *Trends Biochem Sci*, 42, 42-
19 56.
- 20 CHRIST, L., WENZEL, E. M., LIESTOL, K., RAIBORG, C., CAMPSTEIJN, C. & STENMARK, H. 2016. ALIX and
21 ESCRT-I/II function as parallel ESCRT-III recruiters in cytokinetic abscission. *J Cell Biol*, 212, 499-
22 513.
- 23 CICCARELLI, F. D., PROUKAKIS, C., PATEL, H., CROSS, H., AZAM, S., PATTON, M. A., BORK, P. & CROSBY, A.
24 H. 2003. The identification of a conserved domain in both spartin and spastin, mutated in
25 hereditary spastic paraplegia. *Genomics*, 81, 437-41.
- 26 CLAUDIANI, P., RIANO, E., ERRICO, A., ANDOLFI, G. & RUGARLI, E. I. 2005. Spastin subcellular localization
27 is regulated through usage of different translation start sites and active export from the nucleus.
28 *Exp Cell Res*, 309, 358-69.
- 29 COLCHER, D., HEBERLING, R. L., KALTER, S. S. & SCHLOM, J. 1977. Squirrel monkey retrovirus: an
30 endogenous virus of a new world primate. *J Virol*, 23, 294-301.
- 31 CONNELL, J. W., LINDON, C., LUZIO, J. P. & REID, E. 2009. Spastin couples microtubule severing to
32 membrane traffic in completion of cytokinesis and secretion. *Traffic*, 10, 42-56.
- 33 CRASTA, K., GANEM, N. J., DAGHER, R., LANTERMANN, A. B., IVANOVA, E. V., PAN, Y., NEZI, L.,
34 PROTOPOPOV, A., CHOWDHURY, D. & PELLMAN, D. 2012. DNA breaks and chromosome
35 pulverization from errors in mitosis. *Nature*, 482, 53-8.
- 36 DAVIS, I. W., LEAVER-FAY, A., CHEN, V. B., BLOCK, J. N., KAPRAL, G. J., WANG, X., MURRAY, L. W.,
37 ARENDALL, W. B., SNOEYINK, J., RICHARDSON, J. S. & RICHARDSON, D. C. 2007. MolProbity: all-
38 atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Research*, 35,
39 W375-W383.
- 40 DENAIS, C. M., GILBERT, R. M., ISERMANN, P., MCGREGOR, A. L., TE LINDERT, M., WEIGELIN, B.,
41 DAVIDSON, P. M., FRIEDL, P., WOLF, K. & LAMMERDING, J. 2016. Nuclear envelope rupture and
42 repair during cancer cell migration. *Science*, 352, 353-8.
- 43 DIMAANO, C., JONES, C. B., HANONO, A., CURTISS, M. & BABST, M. 2008. Ist1 regulates Vps4 localization
44 and assembly. *Mol Biol Cell*, 19, 465-74.
- 45 DUKES, J. D., RICHARDSON, J. D., SIMMONS, R. & WHITLEY, P. 2008. A dominant-negative ESCRT-III protein
46 perturbs cytokinesis and trafficking to lysosomes. *Biochem J*, 411, 233-9.

- 1 ECKERT, T., LINK, S., LE, D. T., SOBCZAK, J. P., GIESEKE, A., RICHTER, K. & WOHLKE, G. 2012. Subunit
2 Interactions and cooperativity in the microtubule-severing AAA ATPase spastin. *J Biol Chem*, 287,
3 26278-90.
- 4 ELIA, N., FABRIKANT, G., KOZLOV, M. M. & LIPPINCOTT-SCHWARTZ, J. 2012. Computational model of
5 cytokinetic abscission driven by ESCRT-III polymerization and remodeling. *Biophys J*, 102, 2309-
6 20.
- 7 ELIA, N., SOUGRAT, R., SPURLIN, T. A., HURLEY, J. H. & LIPPINCOTT-SCHWARTZ, J. 2011. Dynamics of
8 endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its
9 role in abscission. *Proc Natl Acad Sci U S A*, 108, 4846-51.
- 10 EMSLEY, P. & COWTAN, K. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallographica*
11 *Section D-Structural Biology*, 60, 2126-2132.
- 12 EMSLEY, P., LOHKAMP, B., SCOTT, W. G. & COWTAN, K. 2010. Features and development of Coot. *Acta*
13 *Crystallographica Section D-Biological Crystallography*, 66, 486-501.
- 14 EVANS, P. R. 2011. An introduction to data reduction: space-group determination, scaling and intensity
15 statistics. *Acta Crystallographica Section D-Biological Crystallography*, 67, 282-292.
- 16 EVANS, P. R. & MURSHUDOV, G. N. 2013. How good are my data and what is the resolution? *Acta*
17 *Crystallographica Section D-Biological Crystallography*, 69, 1204-1214.
- 18 FABBRO, M., ZHOU, B. B., TAKAHASHI, M., SARCEVIC, B., LAL, P., GRAHAM, M. E., GABRIELLI, B. G.,
19 ROBINSON, P. J., NIGG, E. A., ONO, Y. & KHANNA, K. K. 2005. Cdk1/Erk2- and Plk1-dependent
20 phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and
21 cytokinesis. *Dev Cell*, 9, 477-88.
- 22 FALTOVA, L., JIANG, K., FREY, D., WU, Y., CAPITANI, G., PROTA, A. E., AKHMANOVA, A., STEINMETZ, M. O.
23 & KAMMERER, R. A. 2019. Crystal Structure of a Heterotetrameric Katanin p60:p80 Complex.
24 *Structure*, 27, 1375-1383 e3.
- 25 FRAILE, J. M., CAMPOS-IGLESIAS, D., RODRIGUEZ, F., ESPANOL, Y. & FREIJE, J. M. 2016. The deubiquitinase
26 USP54 is overexpressed in colorectal cancer stem cells and promotes intestinal tumorigenesis.
27 *Oncotarget*, 7, 74427-74434.
- 28 FREMONT, S. & ECHARD, A. 2018. Membrane Traffic in the Late Steps of Cytokinesis. *Curr Biol*, 28, R458-
29 R470.
- 30 FUJIOKA, Y., SUZUKI, S. W., YAMAMOTO, H., KONDO-KAKUTA, C., KIMURA, Y., HIRANO, H., AKADA, R.,
31 INAGAKI, F., OHSUMI, Y. & NODA, N. N. 2014. Structural basis of starvation-induced assembly of
32 the autophagy initiation complex. *Nat Struct Mol Biol*, 21, 513-21.
- 33 GU, M., LAJOIE, D., CHEN, O. S., VON APPEN, A., LADINSKY, M. S., REDD, M. J., NIKOLOVA, L., BJORKMAN,
34 P. J., SUNDQUIST, W. I., ULLMAN, K. S. & FROST, A. 2017. LEM2 recruits CHMP7 for ESCRT-
35 mediated nuclear envelope closure in fission yeast and human cells. *Proc Natl Acad Sci U S A*, 114,
36 E2166-E2175.
- 37 GUIZETTI, J., SCHERMELLEH, L., MANTLER, J., MAAR, S., POSER, I., LEONHARDT, H., MULLER-REICHERT, T.
38 & GERLICH, D. W. 2011. Cortical constriction during abscission involves helices of ESCRT-III-
39 dependent filaments. *Science*, 331, 1616-20.
- 40 GUO, E. Z. & XU, Z. 2015. Distinct mechanisms of recognizing endosomal sorting complex required for
41 transport III (ESCRT-III) protein IST1 by different microtubule interacting and trafficking (MIT)
42 domains. *J Biol Chem*, 290, 8396-408.
- 43 HADDERS, M. A., AGROMAYOR, M., OBITA, T., PERISIC, O., CABALLE, A., KLOC, M., LAMERS, M. H.,
44 WILLIAMS, R. L. & MARTIN-SERRANO, J. 2012. ESCRT-III binding protein MITD1 is involved in
45 cytokinesis and has an unanticipated PLD fold that binds membranes. *Proc Natl Acad Sci U S A*,
46 109, 17424-9.
- 47 HAN, H., MONROE, N., VOTTELER, J., SHAKYA, B., SUNDQUIST, W. I. & HILL, C. P. 2015. Binding of
48 Substrates to the Central Pore of the Vps4 ATPase Is Autoinhibited by the Microtubule Interacting

and Trafficking (MIT) Domain and Activated by MIT Interacting Motifs (MIMs). *J Biol Chem*, 290, 13490-9.

HAN, H., SCHUBERT, H. L., MCCULLOUGH, J., MONROE, N., PURDY, M. D., YEAGER, M., SUNDQUIST, W. I. & HILL, C. P. 2020. Structure of spastin bound to a glutamate-rich peptide implies a hand-over-hand mechanism of substrate translocation. *J Biol Chem*, 295, 435-443.

HARTMAN, J. J. & VALE, R. D. 1999. Microtubule disassembly by ATP-dependent oligomerization of the AAA enzyme katanin. *Science*, 286, 782-5.

HAYASHI, S., OKADA, T., IGARASHI, N., FUJITA, T., JAHANGEER, S. & NAKAMURA, S. 2002. Identification and characterization of RPK118, a novel sphingosine kinase-1-binding protein. *J Biol Chem*, 277, 33319-24.

HURLEY, J. H. 2015. ESCRTs are everywhere. *EMBO J*, 34, 2398-407.

HURLEY, J. H. & YANG, D. 2008. MIT domainia. *Dev Cell*, 14, 6-8.

IWAYA, N., KUWAHARA, Y., FUJIWARA, Y., GODA, N., TENNO, T., AKIYAMA, K., MASE, S., TOCHIO, H., IKEGAMI, T., SHIRAKAWA, M. & HIROAKI, H. 2010. A common substrate recognition mode conserved between katanin p60 and VPS4 governs microtubule severing and membrane skeleton reorganization. *J Biol Chem*, 285, 16822-9.

JANSSEN, A., VAN DER BURG, M., SZUHAI, K., KOPS, G. J. & MEDEMA, R. H. 2011. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. *Science*, 333, 1895-8.

JIANG, K., FALTOVA, L., HUA, S., CAPITANI, G., PROTA, A. E., LANDGRAF, C., VOLKMER, R., KAMMERER, R. A., STEINMETZ, M. O. & AKHMANOVA, A. 2018. Structural Basis of Formation of the Microtubule Minus-End-Regulating CAMSAP-Katanin Complex. *Structure*, 26, 375-382 e4.

JIANG, K., REZABKOVA, L., HUA, S., LIU, Q., CAPITANI, G., ALTELAAR, A. F. M., HECK, A. J. R., KAMMERER, R. A., STEINMETZ, M. O. & AKHMANOVA, A. 2017. Microtubule minus-end regulation at spindle poles by an ASPM-katanin complex. *Nat Cell Biol*, 19, 480-492.

JUNG, C. H., JUN, C. B., RO, S. H., KIM, Y. M., OTTO, N. M., CAO, J., KUNDU, M. & KIM, D. H. 2009. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell*, 20, 1992-2003.

KABSCH, W. 2010a. Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr D Biol Crystallogr*, 66, 133-44.

KABSCH, W. 2010b. Xds. *Acta Crystallogr D Biol Crystallogr*, 66, 125-32.

KARASMANIS, E. P., HWANG, D., NAKOS, K., BOWEN, J. R., ANGELIS, D. & SPILIOTIS, E. T. 2019. A Septin Double Ring Controls the Spatiotemporal Organization of the ESCRT Machinery in Cytokinetic Abscission. *Curr Biol*, 29, 2174-2182 e7.

KATOH, K., SHIBATA, H., HATTA, K. & MAKI, M. 2004. CHMP4b is a major binding partner of the ALG-2-interacting protein Alix among the three CHMP4 isoforms. *Arch Biochem Biophys*, 421, 159-65.

KATOH, K., SHIBATA, H., SUZUKI, H., NARA, A., ISHIDOH, K., KOMINAMI, E., YOSHIMORI, T. & MAKI, M. 2003. The ALG-2-interacting protein Alix associates with CHMP4b, a human homologue of yeast Snf7 that is involved in multivesicular body sorting. *J Biol Chem*, 278, 39104-13.

KELLEY, L. A., MEZULIS, S., YATES, C. M., WASS, M. N. & STERNBERG, M. J. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc*, 10, 845-58.

KIEFFER, C., SKALICKY, J. J., MORITA, E., DE DOMENICO, I., WARD, D. M., KAPLAN, J. & SUNDQUIST, W. I. 2008. Two distinct modes of ESCRT-III recognition are required for VPS4 functions in lysosomal protein targeting and HIV-1 budding. *Dev Cell*, 15, 62-73.

KIM, J., SITARAMAN, S., HIERRO, A., BEACH, B. M., ODORIZZI, G. & HURLEY, J. H. 2005. Structural basis for endosomal targeting by the Bro1 domain. *Dev Cell*, 8, 937-47.

KUO, Y. W. & HOWARD, J. 2021. Cutting, Amplifying, and Aligning Microtubules with Severing Enzymes. *Trends Cell Biol*, 31, 50-61.

- 1 KUO, Y. W., TROTTIER, O., MAHAMDEH, M. & HOWARD, J. 2019. Spastin is a dual-function enzyme that
2 severs microtubules and promotes their regrowth to increase the number and mass of
3 microtubules. *Proc Natl Acad Sci U S A*, 116, 5533-5541.
- 4 LAFAURIE-JANVORE, J., MAIURI, P., WANG, I., PINOT, M., MANNEVILLE, J. B., BETZ, T., BALLAND, M. & PIEL,
5 M. 2013. ESCRT-III assembly and cytokinetic abscission are induced by tension release in the
6 intercellular bridge. *Science*, 339, 1625-9.
- 7 LEE, H. H., ELIA, N., GHIRLANDO, R., LIPPINCOTT-SCHWARTZ, J. & HURLEY, J. H. 2008. Midbody targeting
8 of the ESCRT machinery by a noncanonical coiled coil in CEP55. *Science*, 322, 576-80.
- 9 LEE, S., CHANG, J., RENVOISE, B., TIPIRNENI, A., YANG, S. & BLACKSTONE, C. 2012. MITD1 is recruited to
10 midbodies by ESCRT-III and participates in cytokinesis. *Mol Biol Cell*, 23, 4347-61.
- 11 LIEBSCHNER, D., AFONINE, P. V., BAKER, M. L., BUNKOCZI, G., CHEN, V. B., CROLL, T. I., HINTZE, B., HUNG,
12 L. W., JAIN, S., MCCOY, A. J., MORIARTY, N. W., OEFFNER, R. D., POON, B. K., PRISANT, M. G., READ,
13 R. J., RICHARDSON, J. S., RICHARDSON, D. C., SAMMITO, M. D., SOBOLEV, O. V., STOCKWELL, D.
14 H., TERWILLIGER, T. C., URZHUMTSEV, A. G., VIDEAU, L. L., WILLIAMS, C. J. & ADAMS, P. D. 2019.
15 Macromolecular structure determination using X-rays, neutrons and electrons: recent
16 developments in Phenix. *Acta Crystallographica Section D-Structural Biology*, 75, 861-877.
- 17 LIN, Y., KIMPLER, L. A., NAISMITH, T. V., LAUER, J. M. & HANSON, P. I. 2005. Interaction of the mammalian
18 endosomal sorting complex required for transport (ESCRT) III protein hSnf7-1 with itself,
19 membranes, and the AAA+ ATPase SKD1. *J Biol Chem*, 280, 12799-809.
- 20 LITTLE, J. N., MCNEELY, K. C., MICHEL, N., BOTT, C. J., LETTIERI, K. S., HECHT, M. R., MARTIN, S. A. & DWYER,
21 N. D. 2021. Loss of Coiled-Coil Protein Cep55 Impairs Neural Stem Cell Abscission and Results in
22 p53-Dependent Apoptosis in Developing Cortex. *J Neurosci*, 41, 3344-3365.
- 23 LIU, L., YANG, C., YUAN, J., CHEN, X., XU, J., WEI, Y., YANG, J., LIN, G. & YU, L. 2005. RPK118, a PX domain-
24 containing protein, interacts with peroxiredoxin-3 through pseudo-kinase domains. *Mol Cells*, 19,
25 39-45.
- 26 MA, Y. M., BOUCROT, E., VILLEN, J., AFFAR, E. B., GYGI, S. P., GOTTLINGER, H. G. & KIRCHHAUSEN, T. 2007.
27 Targeting of AMSH to endosomes is required for epidermal growth factor receptor degradation. *J*
28 *Biol Chem*, 282, 9805-9812.
- 29 MACKAY, D. R., MAKISE, M. & ULLMAN, K. S. 2010. Defects in nuclear pore assembly lead to activation of
30 an Aurora B-mediated abscission checkpoint. *J Cell Biol*, 191, 923-31.
- 31 MACKAY, D. R. & ULLMAN, K. S. 2015. ATR and a Chk1-Aurora B pathway coordinate postmitotic genome
32 surveillance with cytokinetic abscission. *Mol Biol Cell*, 26, 2217-26.
- 33 MAEMOTO, Y., KISO, S., SHIBATA, H. & MAKI, M. 2013. Analysis of limited proteolytic activity of calpain-7
34 using non-physiological substrates in mammalian cells. *FEBS J*, 280, 2594-607.
- 35 MAEMOTO, Y., OSAKO, Y., GOTO, E., NOZAWA, E., SHIBATA, H. & MAKI, M. 2011. Calpain-7 binds to
36 CHMP1B at its second alpha-helical region and forms a ternary complex with IST1. *J Biochem*, 150,
37 411-21.
- 38 MALOVERJAN, A., PIIRSOO, M., KASAK, L., PEIL, L., OSTERLUND, T. & KOGERMAN, P. 2010a. Dual function
39 of UNC-51-like kinase 3 (Ulk3) in the Sonic hedgehog signaling pathway. *J Biol Chem*, 285, 30079-
40 90.
- 41 MALOVERJAN, A., PIIRSOO, M., MICHELSON, P., KOGERMAN, P. & OSTERLUND, T. 2010b. Identification of
42 a novel serine/threonine kinase ULK3 as a positive regulator of Hedgehog pathway. *Exp Cell Res*,
43 316, 627-37.
- 44 MANCUSO, G. & RUGARLI, E. I. 2008. A cryptic promoter in the first exon of the SPG4 gene directs the
45 synthesis of the 60-kDa spastin isoform. *BMC Biol*, 6, 31.
- 46 MATSUO, M., SHIMODAIRA, T., KASAMA, T., HATA, Y., ECHIGO, A., OKABE, M., ARAI, K., MAKINO, Y., NIWA,
47 S., SAYA, H. & KISHIMOTO, T. 2013. Katanin p60 contributes to microtubule instability around the
48 midbody and facilitates cytokinesis in rat cells. *PLoS One*, 8, e80392.

- 1 MCCULLOUGH, J., CLIPPINGER, A. K., TALLEGE, N., SKOWYRA, M. L., SAUNDERS, M. G., NAISMITH, T. V.,
2 COLF, L. A., AFONINE, P., ARTHUR, C., SUNDQUIST, W. I., HANSON, P. I. & FROST, A. 2015. Structure
3 and membrane remodeling activity of ESCRT-III helical polymers. *Science*, 350, 1548-51.
- 4 MCCULLOUGH, J., FISHER, R. D., WHITBY, F. G., SUNDQUIST, W. I. & HILL, C. P. 2008. ALIX-CHMP4
5 interactions in the human ESCRT pathway. *Proc Natl Acad Sci U S A*, 105, 7687-91.
- 6 MCCULLOUGH, J., FROST, A. & SUNDQUIST, W. I. 2018. Structures, Functions, and Dynamics of ESCRT-
7 III/Vps4 Membrane Remodeling and Fission Complexes. *Annu Rev Cell Dev Biol*, 34, 85-109.
- 8 MENDOZA, M. & BARRAL, Y. 2008. Co-ordination of cytokinesis with chromosome segregation. *Biochem*
9 *Soc Trans*, 36, 387-90.
- 10 MERIGLIANO, C., BURLA, R., LA TORRE, M., DEL GIUDICE, S., TEO, H., LIEW, C. W., CHOJNOWSKI, A., GOH,
11 W. I., OLMOS, Y., MACCARONI, K., GIUBETTINI, M., CHIOLO, I., CARLTON, J. G., RAIMONDO, D.,
12 VERNI, F., STEWART, C. L., RHODES, D., WRIGHT, G. D., BURKE, B. E. & SAGGIO, I. 2021. AKTIP
13 interacts with ESCRT I and is needed for the recruitment of ESCRT III subunits to the midbody.
14 *PLoS Genet*, 17, e1009757.
- 15 MIERZWA, B. E., CHIARUTTINI, N., REDONDO-MORATA, L., VON FILSECK, J. M., KONIG, J., LARIOS, J.,
16 POSER, I., MULLER-REICHERT, T., SCHEURING, S., ROUX, A. & GERLICH, D. W. 2017. Dynamic
17 subunit turnover in ESCRT-III assemblies is regulated by Vps4 to mediate membrane remodelling
18 during cytokinesis. *Nat Cell Biol*, 19, 787-798.
- 19 MONROE, N. & HILL, C. P. 2016. Meiotic Clade AAA ATPases: Protein Polymer Disassembly Machines. *J*
20 *Mol Biol*, 428, 1897-911.
- 21 MORITA, E., COLF, L. A., KARREN, M. A., SANDRIN, V., RODESCH, C. K. & SUNDQUIST, W. I. 2010. Human
22 ESCRT-III and VPS4 proteins are required for centrosome and spindle maintenance. *Proc Natl Acad*
23 *Sci U S A*, 107, 12889-94.
- 24 MORITA, E., SANDRIN, V., CHUNG, H. Y., MORHAM, S. G., GYGI, S. P., RODESCH, C. K. & SUNDQUIST, W. I.
25 2007. Human ESCRT and ALIX proteins interact with proteins of the midbody and function in
26 cytokinesis. *EMBO J*, 26, 4215-27.
- 27 NEGGERS, J. E., PAOLELLA, B. R., ASFAW, A., ROTHBERG, M. V., SKIPPER, T. A., YANG, A., KALEKAR, R. L.,
28 KRILL-BURGER, J. M., DHARIA, N. V., KUGENER, G., KALFON, J., YUAN, C., DUMONT, N., GONZALEZ,
29 A., ABDUSAMAD, M., LI, Y. Y., SPURR, L. F., WU, W. W., DURBIN, A. D., WOLPIN, B. M., PICCIONI,
30 F., ROOT, D. E., BOEHM, J. S., CHERNIACK, A. D., TSHERNIAK, A., HONG, A. L., HAHN, W. C.,
31 STEGMAIER, K., GOLUB, T. R., VAZQUEZ, F. & AGUIRRE, A. J. 2020. Synthetic Lethal Interaction
32 between the ESCRT Paralog Enzymes VPS4A and VPS4B in Cancers Harboring Loss of Chromosome
33 18q or 16q. *Cell Rep*, 33, 108493.
- 34 NGUYEN, H. C., TALLEGE, N., MCCULLOUGH, J., SHARMA, A., MOSS, F. R., 3RD, IWASA, J. H., VERSHININ,
35 M. D., SUNDQUIST, W. I. & FROST, A. 2020. Membrane constriction and thinning by sequential
36 ESCRT-III polymerization. *Nat Struct Mol Biol*, 27, 392-399.
- 37 NORDEN, C., MENDOZA, M., DOBBELAERE, J., KOTWALIWALE, C. V., BIGGINS, S. & BARRAL, Y. 2006. The
38 NoCut pathway links completion of cytokinesis to spindle midzone function to prevent
39 chromosome breakage. *Cell*, 125, 85-98.
- 40 NORGAN, A. P., DAVIES, B. A., AZMI, I. F., SCHROEDER, A. S., PAYNE, J. A., LYNCH, G. M., XU, Z. &
41 KATZMANN, D. J. 2013. Relief of autoinhibition enhances Vta1 activation of Vps4 via the Vps4
42 stimulatory element. *J Biol Chem*, 288, 26147-56.
- 43 OBITA, T., SAKSENA, S., GHAZI-TABATABAI, S., GILL, D. J., PERISIC, O., EMR, S. D. & WILLIAMS, R. L. 2007.
44 Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. *Nature*, 449, 735-
45 9.
- 46 OLMOS, Y., HODGSON, L., MANTELL, J., VERKADE, P. & CARLTON, J. G. 2015. ESCRT-III controls nuclear
47 envelope reformation. *Nature*, 522, 236-9.

- 1 OSAKO, Y., MAEMOTO, Y., TANAKA, R., SUZUKI, H., SHIBATA, H. & MAKI, M. 2010. Autolytic activity of
2 human calpain 7 is enhanced by ESCRT-III-related protein IST1 through MIT-MIM interaction. *FEBS*
3 *J*, 277, 4412-26.
- 4 PFITZNER, A. K., MERCIER, V., JIANG, X., MOSER VON FILSECK, J., BAUM, B., SARIC, A. & ROUX, A. 2020. An
5 ESCRT-III Polymerization Sequence Drives Membrane Deformation and Fission. *Cell*, 182, 1140-
6 1155 e18.
- 7 PFITZNER, A. K., MOSER VON FILSECK, J. & ROUX, A. 2021. Principles of membrane remodeling by dynamic
8 ESCRT-III polymers. *Trends Cell Biol.*
- 9 PHAROAH, P. D., TSAI, Y. Y., RAMUS, S. J., PHELAN, C. M., GOODE, E. L., LAWRENSEN, K., BUCKLEY, M.,
10 FRIDLEY, B. L., TYRER, J. P., SHEN, H., WEBER, R., KAREVAN, R., LARSON, M. C., SONG, H., TESSIER,
11 D. C., BACOT, F., VINCENT, D., CUNNINGHAM, J. M., DENNIS, J., DICKS, E., AUSTRALIAN CANCER,
12 S., AUSTRALIAN OVARIAN CANCER STUDY, G., ABEN, K. K., ANTON-CULVER, H., ANTONENKOVA,
13 N., ARMASU, S. M., BAGLIETTO, L., BANDERA, E. V., BECKMANN, M. W., BIRRER, M. J., BLOOM, G.,
14 BOGDANOVA, N., BRENTON, J. D., BRINTON, L. A., BROOKS-WILSON, A., BROWN, R., BUTZOW, R.,
15 CAMPBELL, I., CARNEY, M. E., CARVALHO, R. S., CHANG-CLAUDE, J., CHEN, Y. A., CHEN, Z., CHOW,
16 W. H., CICEK, M. S., COETZEE, G., COOK, L. S., CRAMER, D. W., CYBULSKI, C., DANSONKA-
17 MIESZKOWSKA, A., DESPIERRE, E., DOHERTY, J. A., DORK, T., DU BOIS, A., DURST, M., ECCLES, D.,
18 EDWARDS, R., EKICI, A. B., FASCHING, P. A., FENSTERMACHER, D., FLANAGAN, J., GAO, Y. T.,
19 GARCIA-CLOSAS, M., GENTRY-MAHARAJ, A., GILES, G., GJYSHI, A., GORE, M., GRONWALD, J., GUO,
20 Q., HALLE, M. K., HARTER, P., HEIN, A., HEITZ, F., HILLEMANN, P., HOATLIN, M., HOGDALL, E.,
21 HOGDALL, C. K., HOSONO, S., JAKUBOWSKA, A., JENSEN, A., KALLI, K. R., KARLAN, B. Y., KELEMEN,
22 L. E., KIEMENEY, L. A., KJAER, S. K., KONECNY, G. E., KRAKSTAD, C., KUPRYJANCZYK, J.,
23 LAMBRECHTS, D., LAMBRECHTS, S., LE, N. D., LEE, N., LEE, J., LEMINEN, A., LIM, B. K., LISSOWSKA,
24 J., LUBINSKI, J., LUNDVALL, L., LURIE, G., MASSUGER, L. F., et al. 2013. GWAS meta-analysis and
25 replication identifies three new susceptibility loci for ovarian cancer. *Nat Genet*, 45, 362-70,
26 370e1-2.
- 27 PHILLIPS, S. A., BARR, V. A., HAFT, D. H., TAYLOR, S. I. & HAFT, C. R. 2001. Identification and
28 characterization of SNX15, a novel sorting nexin involved in protein trafficking. *J Biol Chem*, 276,
29 5074-84.
- 30 REID, E., CONNELL, J., EDWARDS, T. L., DULEY, S., BROWN, S. E. & SANDERSON, C. M. 2005. The hereditary
31 spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal
32 protein CHMP1B. *Hum Mol Genet*, 14, 19-38.
- 33 RENVOISE, B., PARKER, R. L., YANG, D., BAKOWSKA, J. C., HURLEY, J. H. & BLACKSTONE, C. 2010. SPG20
34 protein spartin is recruited to midbodies by ESCRT-III protein Ist1 and participates in cytokinesis.
35 *Mol Biol Cell*, 21, 3293-303.
- 36 REZABKOVA, L., JIANG, K., CAPITANI, G., PROTA, A. E., AKHMANOVA, A., STEINMETZ, M. O. & KAMMERER,
37 R. A. 2017. Structural basis of katanin p60:p80 complex formation. *Sci Rep*, 7, 14893.
- 38 RIGDEN, D. J., LIU, H., HAYES, S. D., URBE, S. & CLAGUE, M. J. 2009. Ab initio protein modelling reveals
39 novel human MIT domains. *FEBS Lett*, 583, 872-8.
- 40 RODRIGUEZ-GALAN, O., GALINDO, A., HERVAS-AGUILAR, A., ARST, H. N., JR. & PENALVA, M. A. 2009.
41 Physiological involvement in pH signaling of Vps24-mediated recruitment of Aspergillus PalB
42 cysteine protease to ESCRT-III. *J Biol Chem*, 284, 4404-12.
- 43 ROLL-MECAK, A. & VALE, R. D. 2008. Structural basis of microtubule severing by the hereditary spastic
44 paraplegia protein spastin. *Nature*, 451, 363-7.
- 45 ROW, P. E., LIU, H., HAYES, S., WELCHMAN, R., CHARALABOUS, P., HOFMANN, K., CLAGUE, M. J.,
46 SANDERSON, C. M. & URBE, S. 2007. The MIT domain of UBPY constitutes a CHMP binding and
47 endosomal localization signal required for efficient epidermal growth factor receptor
48 degradation. *J Biol Chem*, 282, 30929-37.

- 1 RUE, S. M., MATTEI, S., SAKSENA, S. & EMR, S. D. 2008. Novel Ist1-Did2 complex functions at a late step in
2 multivesicular body sorting. *Mol Biol Cell*, 19, 475-84.
- 3 SADLER, J. B. A., WENZEL, D. M., WILLIAMS, L. K., GUINDO-MARTINEZ, M., ALAM, S. L., MERCADER, J. M.,
4 TORRENTS, D., ULLMAN, K. S., SUNDQUIST, W. I. & MARTIN-SERRANO, J. 2018. A cancer-
5 associated polymorphism in ESCRT-III disrupts the abscission checkpoint and promotes genome
6 instability. *Proc Natl Acad Sci U S A*, 115, E8900-E8908.
- 7 SAMSON, R. Y., OBITA, T., FREUND, S. M., WILLIAMS, R. L. & BELL, S. D. 2008. A role for the ESCRT system
8 in cell division in archaea. *Science*, 322, 1710-3.
- 9 SCOTT, A., GASPAR, J., STUCHELL-BRERETON, M. D., ALAM, S. L., SKALICKY, J. J. & SUNDQUIST, W. I. 2005.
10 Structure and ESCRT-III protein interactions of the MIT domain of human VPS4A. *Proc Natl Acad*
11 *Sci U S A*, 102, 13813-8.
- 12 SHARP, D. J. & ROSS, J. L. 2012. Microtubule-severing enzymes at the cutting edge. *J Cell Sci*, 125, 2561-9.
- 13 SHESTAKOVA, A., HANONO, A., DROSNER, S., CURTISS, M., DAVIES, B. A., KATZMANN, D. J. & BABST, M.
14 2010. Assembly of the AAA ATPase Vps4 on ESCRT-III. *Mol Biol Cell*, 21, 1059-71.
- 15 SHIM, S., MERRILL, S. A. & HANSON, P. I. 2008. Novel interactions of ESCRT-III with LIP5 and VPS4 and their
16 implications for ESCRT-III disassembly. *Mol Biol Cell*, 19, 2661-72.
- 17 SKALICKY, J. J., ARII, J., WENZEL, D. M., STUBBLEFIELD, W. M., KATSUYAMA, A., UTER, N. T., BAJOREK, M.,
18 MYSZKA, D. G. & SUNDQUIST, W. I. 2012. Interactions of the human LIP5 regulatory protein with
19 endosomal sorting complexes required for transport. *J Biol Chem*, 287, 43910-26.
- 20 SOLOMONS, J., SABIN, C., POUDÉVIGNE, E., USAMI, Y., HULSIK, D. L., MACHEBOEUF, P., HARTLIEB, B.,
21 GOTTLINGER, H. & WEISSENHORN, W. 2011. Structural basis for ESCRT-III CHMP3 recruitment of
22 AMSH. *Structure*, 19, 1149-59.
- 23 STEIGEMANN, P., WURZENBERGER, C., SCHMITZ, M. H., HELD, M., GUIZETTI, J., MAAR, S. & GERLICH, D.
24 W. 2009. Aurora B-mediated abscission checkpoint protects against tetraploidization. *Cell*, 136,
25 473-84.
- 26 STROHACKER, L. K., MACKAY, D. R., WHITNEY, M. A., COULDWELL, G. C., SUNDQUIST, W. I. & ULLMAN, K.
27 S. 2021. Identification of abscission checkpoint bodies as structures that regulate ESCRT factors
28 to control abscission timing. *Elife*, 10.
- 29 STUCHELL-BRERETON, M. D., SKALICKY, J. J., KIEFFER, C., KARREN, M. A., GHAFARIAN, S. & SUNDQUIST,
30 W. I. 2007. ESCRT-III recognition by VPS4 ATPases. *Nature*, 449, 740-4.
- 31 STUDIER, F. W. 2005. Protein production by auto-induction in high density shaking cultures. *Protein Expr*
32 *Purif*, 41, 207-34.
- 33 SUBRAMANIAN, S., WOOLFORD, C. A., DESAI, J. V., LANNI, F. & MITCHELL, A. P. 2012. cis- and trans-acting
34 localization determinants of pH response regulator Rim13 in *Saccharomyces cerevisiae*. *Eukaryot*
35 *Cell*, 11, 1201-9.
- 36 SZYMANSKA, E., NOWAK, P., KOLMUS, K., CYBULSKA, M., GORYCA, K., DEREZINSKA-WOLEK, E., SZUMERA-
37 CIECKIEWICZ, A., BREWINSKA-OLCHOWIK, M., GROCHOWSKA, A., PIWOCKA, K., PROCHOREC-
38 SOBIESZEK, M., MIKULA, M. & MIACZYNSKA, M. 2020. Synthetic lethality between VPS4A and
39 VPS4B triggers an inflammatory response in colorectal cancer. *EMBO Mol Med*, 12, e10812.
- 40 TALLEGE, N., MCCULLOUGH, J., WENZEL, D., NGUYEN, H. C., LALONDE, M. S., BAJOREK, M., SKALICKY, J.,
41 FROST, A. & SUNDQUST, W. I. 2018. The ESCRT-III proteins IST1 and CHMP1B assemble around
42 nucleic acids. *bioRxiv*, 386532.
- 43 TEDESCHI, A., ALMAGRO, J., RENSHAW, M. J., MESSAL, H. A., BEHRENS, A. & PETRONCZKI, M. 2020. Cep55
44 promotes cytokinesis of neural progenitors but is dispensable for most mammalian cell divisions.
45 *Nat Commun*, 11, 1746.
- 46 TEIS, D., SAKSENA, S. & EMR, S. D. 2008. Ordered assembly of the ESCRT-III complex on endosomes is
47 required to sequester cargo during MVB formation. *Dev Cell*, 15, 578-89.

- 1 THALLER, D. J., ALLEGRETTI, M., BORAH, S., RONCHI, P., BECK, M. & LUSK, C. P. 2019. An ESCRT-LEM
2 protein surveillance system is poised to directly monitor the nuclear envelope and nuclear
3 transport system. *Elife*, 8.
- 4 THORESEN, S. B., CAMPSTEIJN, C., VIETRI, M., SCHINK, K. O., LIESTOL, K., ANDERSEN, J. S., RAIBORG, C. &
5 STENMARK, H. 2014. ANCHR mediates Aurora-B-dependent abscission checkpoint control
6 through retention of VPS4. *Nat Cell Biol*, 16, 550-60.
- 7 TSANG, H. T., CONNELL, J. W., BROWN, S. E., THOMPSON, A., REID, E. & SANDERSON, C. M. 2006. A
8 systematic analysis of human CHMP protein interactions: additional MIT domain-containing
9 proteins bind to multiple components of the human ESCRT III complex. *Genomics*, 88, 333-46.
- 10 UMBREIT, N. T., ZHANG, C. Z., LYNCH, L. D., BLAINE, L. J., CHENG, A. M., TOURDOT, R., SUN, L.,
11 ALMUBARAK, H. F., JUDGE, K., MITCHELL, T. J., SPEKTOR, A. & PELLMAN, D. 2020. Mechanisms
12 generating cancer genome complexity from a single cell division error. *Science*, 368.
- 13 VEMU, A., SZCZESNA, E., ZEHR, E. A., SPECTOR, J. O., GRIGORIEFF, N., DEACONESCU, A. M. & ROLL-MECAK,
14 A. 2018. Severing enzymes amplify microtubule arrays through lattice GTP-tubulin incorporation.
15 *Science*, 361.
- 16 VIETRI, M., RADULOVIC, M. & STENMARK, H. 2020. The many functions of ESCRTs. *Nat Rev Mol Cell Biol*,
17 21, 25-42.
- 18 VIETRI, M., SCHINK, K. O., CAMPSTEIJN, C., WEGNER, C. S., SCHULTZ, S. W., CHRIST, L., THORESEN, S. B.,
19 BRECH, A., RAIBORG, C. & STENMARK, H. 2015. Spastin and ESCRT-III coordinate mitotic spindle
20 disassembly and nuclear envelope sealing. *Nature*, 522, 231-5.
- 21 VILD, C. J., LI, Y., GUO, E. Z., LIU, Y. & XU, Z. 2015. A novel mechanism of regulating the ATPase VPS4 by its
22 cofactor LIP5 and the endosomal sorting complex required for transport (ESCRT)-III protein
23 CHMP5. *J Biol Chem*, 290, 7291-303.
- 24 VON APPEN, A., LAJOIE, D., JOHNSON, I. E., TRNKA, M. J., PICK, S. M., BURLINGAME, A. L., ULLMAN, K. S.
25 & FROST, A. 2020. LEM2 phase separation promotes ESCRT-mediated nuclear envelope
26 reformation. *Nature*, 582, 115-118.
- 27 VON SCHWEDLER, U. K., STUCHELL, M., MULLER, B., WARD, D. M., CHUNG, H. Y., MORITA, E., WANG, H.
28 E., DAVIS, T., HE, G. P., CIMBORA, D. M., SCOTT, A., KRAUSSLICH, H. G., KAPLAN, J., MORHAM, S.
29 G. & SUNDQUIST, W. I. 2003. The protein network of HIV budding. *Cell*, 114, 701-13.
- 30 WALLIS, S. S., VENTIMIGLIA, L. N., OTIGBAH, E., INFANTE, E., CUESTA-GEIJO, M. A., KIDIYOOR, G. R.,
31 CARBAJAL, M. A., FLECK, R. A., FOIANI, M., GARCIA-MANYES, S., MARTIN-SERRANO, J. &
32 AGROMAYOR, M. 2021. The ESCRT machinery counteracts Nesprin-2G-mediated mechanical
33 forces during nuclear envelope repair. *Dev Cell*, 56, 3192-3202 e8.
- 34 WARD, D. M., VAUGHN, M. B., SHIFLETT, S. L., WHITE, P. L., POLLOCK, A. L., HILL, J., SCHNEGELBERGER, R.,
35 SUNDQUIST, W. I. & KAPLAN, J. 2005. The role of LIP5 and CHMP5 in multivesicular body
36 formation and HIV-1 budding in mammalian cells. *J Biol Chem*, 280, 10548-55.
- 37 WILLIAMS, C. J., HEADD, J. J., MORIARTY, N. W., PRISANT, M. G., VIDEAU, L. L., DEIS, L. N., VERMA, V.,
38 KEEDY, D. A., HINTZE, B. J., CHEN, V. B., JAIN, S., LEWIS, S. M., ARENDALL, W. B., SNOEYINK, J.,
39 ADAMS, P. D., LOVELL, S. C., RICHARDSON, J. S. & RICHARDSON, D. C. 2018. MolProbity: More and
40 better reference data for improved all-atom structure validation. *Protein Science*, 27, 293-315.
- 41 XIAO, H., WANG, L. M., LUO, Y., LAI, X., LI, C., SHI, J., TAN, Y., FU, S., WANG, Y., ZHU, N., HE, J., ZHENG, W.,
42 YU, X., CAI, Z. & HUANG, H. 2016. Mutations in epigenetic regulators are involved in acute
43 lymphoblastic leukemia relapse following allogeneic hematopoietic stem cell transplantation.
44 *Oncotarget*, 7, 2696-708.
- 45 XIAO, J., XIA, H., ZHOU, J., AZMI, I. F., DAVIES, B. A., KATZMANN, D. J. & XU, Z. 2008. Structural basis of
46 Vta1 function in the multivesicular body sorting pathway. *Dev Cell*, 14, 37-49.

1 YAN, Q., HUANG, C., JIANG, Y., SHAN, H., JIANG, R., WANG, J., LIU, J., DING, L., YAN, G. & SUN, H. 2018.
2 Calpain7 impairs embryo implantation by downregulating beta3-integrin expression via
3 degradation of HOXA10. *Cell Death Dis*, 9, 291.
4 YANG, D., RISMANCHI, N., RENVOISE, B., LIPPINCOTT-SCHWARTZ, J., BLACKSTONE, C. & HURLEY, J. H.
5 2008. Structural basis for midbody targeting of spastin by the ESCRT-III protein CHMP1B. *Nat*
6 *Struct Mol Biol*, 15, 1278-86.
7 YANG, Z., VILD, C., JU, J., ZHANG, X., LIU, J., SHEN, J., ZHAO, B., LAN, W., GONG, F., LIU, M., CAO, C. & XU,
8 Z. 2012. Structural basis of molecular recognition between ESCRT-III-like protein Vps60 and AAA-
9 ATPase regulator Vta1 in the multivesicular body pathway. *J Biol Chem*, 287, 43899-908.
10 YORIKAWA, C., TAKAYA, E., OSAKO, Y., TANAKA, R., TERASAWA, Y., HAMAKUBO, T., MOCHIZUKI, Y.,
11 IWANARI, H., KODAMA, T., MAEDA, T., HITOMI, K., SHIBATA, H. & MAKI, M. 2008. Human calpain
12 7/PalBH associates with a subset of ESCRT-III-related proteins in its N-terminal region and partly
13 localizes to endocytic membrane compartments. *J Biochem*, 143, 731-45.
14 YOUNG, L. N., GOERDELER, F. & HURLEY, J. H. 2019. Structural pathway for allosteric activation of the
15 autophagic PI 3-kinase complex I. *Proc Natl Acad Sci U S A*, 116, 21508-21513.
16 ZEHR, E., SZYK, A., PISZCZEK, G., SZCZESNA, E., ZUO, X. & ROLL-MECAK, A. 2017. Katanin spiral and ring
17 structures shed light on power stroke for microtubule severing. *Nat Struct Mol Biol*, 24, 717-725.
18 ZHANG, C. Z., SPEKTOR, A., CORNILS, H., FRANCIS, J. M., JACKSON, E. K., LIU, S., MEYERSON, M. &
19 PELLMAN, D. 2015. Chromothripsis from DNA damage in micronuclei. *Nature*, 522, 179-84.
20 ZHEN, Y., RADULOVIC, M., VIETRI, M. & STENMARK, H. 2021. Sealing holes in cellular membranes. *EMBO*
21 *J*, 40, e106922.

22

23

A ESCRT-III

B

ATPases

Kinases

Proteases

Other

100 aa

Figure 1. Domain organization of ESCRT-III and MIT domain-containing proteins.

(A) Generalized ESCRT-III schematic, depicting the conserved helical core domain and the variable C-terminal tail that contains MIM (MIT-interacting Motif) elements. **(B)** Human proteins with MIT domains, grouped by enzymatic categories. Three dimensional structures (PDB IDs in parenthesis) are available for the MIT domains from human VPS4A (1YXR, 2JQ9, 2K3W), VPS4B (2JQH, 2JQK, 4U7Y, 1WRO, 2CPT), SPASTIN (3EAB), USP8 (2A9U), AMSH (2XZE), NRBF2 (4ZEY), LIP5 (2LXL, 2LXM, 4TXP, 4TXQ, 4TXR, 4U7E), SPARTIN (4U7I), MITD1 (4A5X), and ULK3 MIT2 (4WZX). Abbreviations: MIT, Microtubule Interacting and Trafficking; MTB, Microtubule Binding Domain; S/T Kinase, Serine/Threonine Kinase domain; PSK, Pseudokinase domain; PX, Phosphoinositide binding domain; CysPc, Calpain protease domain; USP, Ubiquitin Specific Protease Domain; PLD, Phospholipase D-like domain; VSL, Vta1-SBP1-LIP5 domain; JAMM, JAB1/MPN/Mov34 metalloenzyme domain; CBSW, calpain-type beta-sandwich domain. **(C)** Gallery showing the different types of binding interactions formed between MIT domains (blue) and MIM elements (green). For clarity, the three helix bundles of the different MIT domains are shown in approximately equivalent orientations. *Type 1 interaction:* a helical MIM binds in the groove between MIT helices 2 and 3 (H2/H3 groove), and is oriented parallel to MIT helix 3. *Type 2 interaction:* the MIM forms an extended strand that binds in the MIT H1/H3 groove, parallel to MIT helix 3. *Type 3 interaction:* a helical MIM binds in the MIT H1/3 groove, parallel to MIT helix 3. *Type 4 interaction:* Similar to Type 1, except that the longer MIM helix binds lower in the H2/H3 groove and interacts with the H2/H3 loop. *Type 5 interaction:* Two MIM helices and adjacent linkers wrap nearly completely around the MIT domain. *Type 6 interaction:* a mixed helix/strand MIM element binds in the MIT H1/3 groove, antiparallel to MIT helix 3. *Type 7 interaction:* a helical MIM element binds in the MIT 2/3 groove, antiparallel to helix 3. The specific complexes shown in the figure are labeled below, together with their PDB accession codes.

Figure 2. ESCRT-III-MIT domain interaction network. Equilibrium dissociation constants (μ M) for pairwise binding interactions are displayed for each ESCRT-III-MIT pair and are reported as averages of at least three independent measurements. Values in parenthesis denote $\pm$ standard deviation. Interactions are color-coded based on strength of measured binding affinity (see key bar). Asterisks denote K_i values that were determined from competition experiments (see Materials and methods and Figure 2-figure supplement 5B-E). Binding constants for ULK3(MIT)₂ and IST1 are from Caballe et al., 2015, but were measured in the same fashion as the other interactions and are reproduced here for comparison.

1 **Figure 2-figure supplement 1. Binding isotherms for MIT domains that bind**
2 **promiscuously to ESCRT-III protein tails.** Representative binding curves for pairwise
3 interactions between MIT domains and ESCRT-III C-terminal tails. Binding isotherms with
4 $K_D < 200 \mu\text{M}$ are fit with colored curves. Data points for weak and non-binders ($K_D > 200$
5 μM) are plotted in black and white but not fit. Data for ULK3(MIT)₂ and IST1, IST1344-366,
6 IST1-MIM₃₁₆₋₃₄₃ are from Caballe et al., 2015, but are reproduced here for comparison.
7

Figure 2-figure supplement 2. Binding isotherms for MIT domains that bind specifically to ≤ 2 different ESCRT-III tails. Representative binding curves for pairwise interactions between MIT domains and ESCRT-III C-terminal tails. Binding isotherms with $K_D < 200 \mu\text{M}$ are fit with colored curves. Data points for weak and non-binders ($K_D > 200 \mu\text{M}$) are plotted in black and white but not fit. Binding curves for SPASTIN, KATNAL1, and CALPAIN-7 are shown in Figure 3A.

Figure 2-figure supplement 3. USP54 MIT binds weakly to all ESCRT-III C-terminal tails. Representative binding curves for pairwise interactions between USP54 MIT and ESCRT-III C-terminal tails. Binding isotherms with $K_D < 200 \mu$ M are fit with colored curves, whereas non and weak binders ($K_D > 200 \mu$ M) are plotted as black and white points but not fit. Dissociation constants shown are averages calculated from three independent experiments $\pm$ standard deviation.

Figure 2-figure supplement 4. Raw binding data for MIT domains that do not bind any ESCRT-III tails. Representative raw FP values for pairwise interactions between ESCRT-III C-terminal tails and MIT domains are shown for KATNAL2 (A), NRBF2 (B), and VPS9D1 (C).

Figure 2-figure supplement 5. Competitive binding analyses of CHMP4C tails binding to MIT domains. (A) Comparison of the C-terminal peptides from different CHMP4 paralogs. Serines are colored red to highlight the unique CHMP4C insert residing between the MIM2 sequence and the ALIX binding site, suggesting that this tail might be unusually flexible and reduce fluorescence polarization anisotropy in CHMP4C-bound complexes, leading to false negative results. The cysteine attachment for the Oregon Green 488 maleimide is highlighted yellow. (B) Fluorescence polarization competition assay. Fluorescently labeled CHMP4B peptide in complex with the MIT domain from AMSH was titrated with unlabeled CHMP4B or CHMP4C peptides. Representative curves are shown here and throughout. (C) Same experiment as in B, except with the MITD1 MIT domain. (D) Same experiment as in B, except with the USP8 MIT domain. (E) Same experiment as in B except that the labeled peptide is IST1-MIM₃₄₄₋₃₆₆ and the unlabeled peptides are IST1 (both MIM elements) and IST1-MIM₃₄₄₋₃₆₆ (positive control).

Figure 2-figure supplement 6. ESCRT-III binding is not conserved across ULK family members. (A) HEK 293T Cells were co-transfected with constructs encoding One-Strep-Flag (OSF) ULK3(MIT)₂ or ULK1(MIT)₂ and the indicated Myc-tagged proteins. OSF-tagged proteins were bound to Strep-Tactin resin, and co-precipitating Myc-tagged proteins were detected by probing with α -Myc antibody. **(B)** Lysates from 293T cells overexpressing Myc-CHMP2A constructs were mixed with lysates from cells overexpressing OSF-ULK3(MIT)₂ or OSF-ULK1(MIT)₂. OSF-ULK proteins were bound to Strep-Tactin resin, and co-precipitating Myc-tagged proteins were detected with α -Myc antibody.

Figure 2-figure supplement 7. Uncropped Western blots for Figure 2-figure supplement 6. (A) Uncropped blots for Figure 2-figure supplement 6A. Asterisk in middle panel denotes cross-reactivity with the band corresponding to IST1 and/or its secondary antibody. **(B)** Uncropped blots for Figure 2-figure supplement 6B.

Figure 3. MIT-MIM Binding Interactions of SPASTIN, KATNA1 and CAPN7 and IST1. (A) Binding isotherms from human ESCRT-III C-terminal tails and the MIT domains from SPASTIN (left), KATNA1 (middle), and CAPN7 (right). Representative binding data are shown for each ESCRT-III-MIT pairwise binding interaction. Binding isotherms with $K_D < 200 \mu M$ are fit with colored curves. Weak and non-binders ($K_D > 200 \mu M$) are plotted as black and white points. (B) Domain schematic of the ESCRT-III protein IST1 showing the position of the Type 2, MIM₃₁₆₋₃₄₃ and Type 1/3, MIM₃₄₄₋₃₆₆ elements within the full-length protein. Data for 'IST1' peptide encompasses residues 316-366. (C) Dissociation constants for MIM₃₄₄₋₃₆₆ and MIM₃₁₆₋₃₄₃ peptides with IST1-binding MIT domains. Dissociation constants shown are averages calculated from at least three independent experiments with standard deviation reported in parentheses. Interactions are color-coded based on strength of measured binding affinity (see key bar). Asterisk denotes K_i values determined from competition experiments (see Materials and methods and Figure 2-figure supplement 5E). Binding data for ULK3(MIT)₂ and IST1, IST1-MIM₃₄₄₋₃₆₆ and IST1-MIM₃₁₆₋₃₆₆ are from Caballe et al., 2015, but are reproduced here for comparison.

1 **Figure 4. Identification of MIT-MIM binding mutants. (A and B)** Two orientations of
2 the SPASTIN MIT-IST1 Type 3 structure (PDB 7S7J) displaying the locations of binding
3 mutants (stick representation). See Figure 4-figure supplement 1A, B for a detailed view
4 of the interface and Supplemental Table 7 for data collection and structure refinement
5 statistics. **(C)** Cartoon depicting the positions of mutation sites in the SPASTIN MIT H1/H3
6 groove. **(D)** SPASTIN-CHMP1B structure (PDB 3EAB) showing the location of binding
7 mutants (stick representation). See Figure 2-figure supplement 1C for an overlay of both
8 SPASTIN structures. **(E-G)** Binding isotherms for WT and mutant MIT domains with the
9 designated MIM elements. **(E)** SPASTIN-IST1. **(F)** SPASTIN-CHMP1B. **(G)** KATNA1-
10 CHMP3, and **(H)** CAPN7-IST1. For E and H, binding was measured using IST1 peptides
11 composed of both MIM elements (residues 316-366). MIT groove binding cartoons within
12 the figures show locations of mutations in the relevant MIT domain.

Figure 4-figure supplement 1. Analysis of SPASTIN-IST1 complex and comparisons with SPASTIN-CHMP1B and SPASTIN-IST1. (A) Fo-Fc IST1 omit map contoured at 3σ overlaid with the SPASTIN-IST1 structure (left) and expanded view (right) highlighting the omit map quality. SPASTIN and IST1 are shown in blue and green respectively. Four hydrophobic IST1 residues, F352, L355, F359, and L362 are shown binding in the H1/H3 groove of the SPASTIN MIT domain. (B) SPASTIN-IST1 interface details. The interface buries $1400 \text{ \AA}^2$ of surface area over 32 residues from both faces and is further stabilized with four hydrogen bonds and seven salt bridges. SPASTIN side chains with significant

1 buried surface area are colored olive for contrast. Left shows full structure and right shows
2 an expanded view. Salt bridges and hydrogen bonds are indicated with dotted lines. The
3 SPASTIN intramolecular salt bridge D134-R191 is shown with olive sticks. **(C)** Overlay of
4 SPASTIN-CHMP1B (PDB 3EAB, SPASTIN cartoon shown gray and CHMP1B yellow)
5 with SPASTIN-IST1 (PDB 7S7J, SPASTIN cartoon shown blue and IST1 green),
6 highlighting similar overlay of four prominent ESCRT-III hydrophobic side chains buried
7 in the H1/H3 binding groove, a small shift in helical register between IST1 and CHMP1B,
8 and an expanded H1/H3 groove in CHMP1B complex (compare the N-terminal region of
9 SPASTIN H3 demarked by residue E169). **(D)** Overlay of SPASTIN-IST1 complex (PDB
10 7S7J, SPASTIN cartoon shown dark blue and IST1 shown green) and SPASTIN-IST1
11 complex (PDB 4UI, SPASTIN shown gray cartoon and IST1 shown dark green) show
12 nearly identical structures.
13

Figure 5. ESCRT-III-interacting proteins SPASTIN, KATNA1, and CAPN7 are recruited to the midbody and are differentially required for abscission and maintenance of the abscission checkpoint. Immunofluorescence images showing the localization of SPASTIN (**A, D**), KATNA1 (**B, E**), and CAPN7 (**C, F**) after treatment with the indicated siRNAs. Here and throughout ‘NT’ refers to non-targeting control and ‘siNups’ refers to co-depletion of NUP153/NUP50. Checkpoint Active samples (**D-F**) were generated by additional treatment with siNups, combined with a thymidine synchronization/release. Antibodies specific for α -TUBULIN and CEP55 were used to identify midbody-stage cells and the Flemming body, respectively. Enlargements of selected regions are shown at the right of each image, with the target protein shown alone (**top**) or together with CEP55 (**bottom**). (**G, H**) Quantification of abscission defects (midbody-stage cells and multinucleate cells) under asynchronous conditions (**G**) or with a sustained abscission checkpoint (**H**). Bars represent the average and standard deviation from n=5 independent experiments where N>500 cells were counted per experiment. Statistical analysis was performed using an Unpaired t-test comparing the combined abscission defects (multinucleate and midbody-stage) for each individual treatment compared to siNT; ****p<0.0001, ***p<0.0005, ns=not significant.

Figure 5 - figure supplement 1. Confirmation of the efficiency and specificity of protein depletion by siRNA treatments. (A) Western blots from experiments in Figure 5. (B) Western blots using a second, independent siRNA for SPASTIN (SPAS-b), KATNA1 (KATNA1-b), and CAPN7 (CAPN7-b). (C, D) Quantification of abscission defects (midbody-stage cells and multinucleate cells) under asynchronous conditions (C) or with a sustained active abscission checkpoint (D), following treatment with the indicated siRNAs. Bars represent the average and standard deviation from 5 independent experiments where >500 cells were counted per experiment. Statistical analysis was performed using an Unpaired t-test comparing the combined abscission defects (multinucleate and midbody-stage) for each individual treatment to siNT. ****p<0.0001, ***p<0.0005, and **p<0.005.

Figure 5-figure supplement 2. Uncropped Western blots for Figure 5-figure supplement 1A.

Figure 5-figure supplement 3. Uncropped Western blots for Figure 5-figure supplement 1B. The CAPN7 blot shown at the bottom left was reprobbed with the NUP153 antibody (shown middle right). * Indicates the residual CAPN7 signal.

Figure 6. ESCRT-III binding by SPASTIN, KATNA1, and CAPN7 is required for their midbody localization. (A) Immunofluorescence of DOX-inducible cell lines expressing siRNA-resistant mCherry-SPASTIN-WT, mCherry-SPASTIN-F124D, and mCherry-SPASTIN-L177D constructs under sustained abscission checkpoint conditions (see Materials and methods). Endogenous SPASTIN was depleted by siRNA treatment of the DOX-inducible cell lines, and induced protein expression/localization was detected using an anti-mCherry antibody. Antibodies to α -TUBULIN and CEP55 were used to identify midbody-stage cells and the Flemming Body, respectively. Enlargements of selected regions are shown at the right of each image, with the target protein shown alone (top)

or together with CEP55 (**bottom**). **(B)** Quantification of midbody localization for endogenous SPASTIN from Figure 5 (Endo) and the indicated DOX-inducible cell lines. Bars are the average and standard deviation from 3 independent experiments in which >100 midbody-stage cells were counted. Statistical analysis was performed using an Unpaired t-test comparing the combined midbody localization for each individual cell line to WT. **(C, D)** Immunofluorescence and quantification of midbody localization for endogenous KATNA1 (Figure 5) and DOX-inducible cell lines expressing siRNA-resistant mCherry-KATNA1-WT and mCherry-KATNA1-V55D constructs as in A and B, except that endogenous KATNA1 was depleted by siRNA in the DOX-inducible cell lines. **(E, F)** Immunofluorescence and quantification of midbody localization for endogenous CAPN7 (Figure 5) and DOX-inducible cell lines expressing siRNA-resistant CAPN7-mCherry and CAPN7-F98D-mCherry constructs as in A and B, except that endogenous CAPN7 was depleted by siRNA in the DOX-inducible cell lines. *** $p < 0.0005$, ** $p < 0.01$, ns=not significant.

Figure 6-figure supplement 1. Confirmation of the efficiency and specificity of protein depletion by siRNA and DOX-inducible protein expression. (A-C) Western blots from experiments in Figure 6.

Figure 6-figure supplement 2. The KATNA1 V55D mutation does not disrupt KATNB1 binding. (A) Structure of KATNA1 MIT domain showing locations of V55 (cyan, stick) and R14 (red, stick). Left image shows the MIT domain alone and right image shows the MIT domain in complex with KATNB1 (PDB 5NBT). (B) Co-precipitations from HEK293T cells co-transfected with Myc-KATNB1 and WT or mutant OSF-KATNA1 proteins. An empty vector expressing OSF alone was used as a control (-).

Figure 6- figure supplement 3. Uncropped Western blots for Figure 6-figure supplement 1A.

Figure 6-figure supplement 4. Uncropped Western blots for Figure 6-figure supplement 1B.

Figure 6-figure supplement 5. Uncropped Western blots for Figure 6-figure supplement 1C.

Figure 6-figure supplement 6. Uncropped Western blots for Figure 6-figure supplement 2.

1 Supplemental Table 1. MIT Domains

MIT DOMAINS	Expected Mass (Da)	Measured Mass (Da)	Uniprot #	Notes
pCA528 AMSH (1-178)	21172.0	21171.0	O95630	
pCA528 AMSHLP (16-218)	23973.2	23973.2	Q96FJ0	
pGEX-THTAP CALPAIN-7 (1-165)	18871.0	18870.5	Q9Y6W3	GPH tag
pCA528 CALPAIN-7 (1-165)	18579.80	18581.34	Q9Y6W3	
pCA528 CALPAIN-7 V18D (1-165)	18595.75	18595.35	Q9Y6W3	
pCA528 CALPAIN-7 F98D (1-165)	18547.71	18547.35	Q9Y6W3	
pCA528 KATNA1 (1-79)	9429.9	9428.8	A8K7S5	
pCA528 KATNA1-V55D (1-79)	9445.8	9444.7	A8K7S5	
pCA528 KATNAL1 (1-87)	10098.5	10098.0	Q9BW62	
pCA528 KATNAL2 (1-98)	11681.35	11680.83	Q8IYT4	
pGEX2T LIP5 (1-168)			Q9NP79	Skalicky, 2012
pGEX-THTAP MITD1 (1-85)	9897.1	9897.1	Q8WV92	GPH tag
pGEX-THTAP NRBF2 (1-111)	12785.0	12784.0	Q96F24	GPH tag
pCA528 RPS6KC1 (235-316)	9313.9	9312.8	Q96S38	
pCA528 RPS6KL1 (20-140)	9512.8	9512.0	Q9Y6S9	
pGEX-THTAP SNX15 (263-342)	8675.9	8676.2	Q9NRS6	GP tag
pCA528 SNX15 (263-342)	8521.5	8520.6	Q9NRS6	
pGEX-THTAP SPARTIN (1-101)	11697.0	11698.0	Q8N0X7	GPH tag
pCA528 SPASTIN WT (112-196)	9763.2	9762.1	Q9UBP0	
pCA528 SPASTIN WT (108-200)	10687.3	10686.1	Q9UBP0	
pCA528 SPASTIN F124D (108-200)	10652.3	10653.0	Q9UBP0	
pCA528 SPASTIN L177D (108-200)	10689.3	10688.5	Q9UBP0	
ULK3 (MIT) ₂ (277-449)			Q6PHR2	Caballe, 2015
pCA528 USP8 (1-147)	17260.0	17260.0	P40818	
pCA528 USP54 (731-878)	16728.5	16727.4	Q70EL1	

MIT DOMAINS	Expected Mass (Da)	Measured Mass (Da)	Uniprot #	Notes
pET16b VPS4A (1-84)			Q9UN37	Stuchell-Brereton,2007
pGEX VPS4B (1-86)			O75351	Stuchell-Brereton,2007
pGEX-THTAP VPS9D1 (1-97)	10657.2	10656.6	Q9Y2B5	GPH tag

1

1 Supplemental Table 2. Fluor-labeled ESCRT-III Peptides¹

ESCRT-III	Sequence ¹	Calculated Mass (Da)	Measured Mass (Da)
IST1 316-366	G C P A D N Y D N F V L P E L P S V P D T L P T A S A G A S T S A S E D I D F D D L S R R F E E L K K K T	6196	6192
IST1 C-Cys 316-366	G P A D N Y D N F V L P E L P S V P D T L P T A S A G A S T S A S E D I D F D D L S R R F E E L K K K T G C	6271	6270
IST1 MIM ₃₁₆₋₃₄₃	G C P A D N Y D N F V L P E L P S V P D T L P T A S A G A S	3480	3480
IST1 MIM ₃₄₄₋₃₆₆	C T S A S E D I D F D D L S R R F E E L K K K T	3296	3296
CHMP1A 140-196	G C T T P Q E Q V D S L I M Q I A E E N G L E V D Q L S Q L P E G A S A V G E S S V R S Q E D Q L S R R L A A L R N	6369	6365
CHMP1B 143-199	G C T T P Q N Q V D M L L Q E M A D E A G L D L N M E L P Q G Q T G S V G T S V A S A E Q D E L S Q R L A R L R D Q V	6347	6343
CHMP2A 152-222	G C G D E E D E E E S D A V S Q V L D E L G L S L T D E L S N L P S T G G S L S V A A G G K K A E A A A S A L A D A D A D L E E R L K N L R R D	7939	7939
CHMP2B 141-213	G C M I N D T L D D I F D G S D E E E S Q D I V N Q V L D E I G I E I S G K M A K A P S A A R S L P S A S T S K A T I S D E E I E R Q L K A L G V D	8448	8446
CHMP3 159-222	G C E E A E M E I D R I L F E I T A G A L G K A P S K V T D A L P E P E P P G A M A A S E D E E E E E E E A L E A M Q S R L A T L R S	7525	7525
CHMP4A 153-222	G C G D D V D E D E L L E E E L E Q E L A Q E L L N V G D K E E E P S V K L P S V P S T H L P A G P A P K V D E D E E A L K Q L A E W V S	8375	8372
CHMP4B 156-224	G C G E E F D E D E L M A E L E E L E Q E L D K N L L E I S G P E T V P L P N V P S I A L P S K P A K K E E E D D D M K E L E N W A G S M	8424	8423
CHMP4C 156-233	G C G D D F D E D E L M A E L E E L E Q E L N K K M T N I R L P N V P S S S L P A Q P N R K P G M S S T A R R S R A A S S Q R A E E E D D I K Q L A A W A T	8907	8904
CHMP5 148-219	G C A L S R S Y G T P E L D E D D L E A E L D A L G D E L L A D E D S S Y L D E A A S A P A I E G V P T D T K N K D G V L V D E F G L P Q I P A S	8187	8186

CHMP6 145-201	GC AGSFTQEDEDAILLELSAITQEIQIELPEVPSEPLPEKI PENVPVKARPRQAELVAAS	6835	6835
CHMP7 366-453	GC AGGVVTNGLDFDSEELEKELDILLQDTTKEPLDLPDN PRNRHFTNSVPNPRISDAELEAELEKLSLSEGGLVPSSK SPKRQLEPTLKPL	10315	10314

1 ¹Cysteines of non-native **GC** or **C** residues were used to couple Alexa Green 488 maleimide fluorescent
2 dyes to the peptides.

3

1 **Supplemental Table 3. Unlabeled ESCRT-III Peptides for Crystallography and**
2 **Competition Binding Assays**

ESCRT-III	Sequence	Calculated Mass (Da)	Measured Mass (Da)
IST1 MIM ₃₄₄₋₃₆₆	TSASEDIDFDDLSRRFEELKKKT	2730.9 Da	2730.4 Da
CHMP4B	GEEFDEDELMAELEELEQEELDKNLLEISGPETVPL PNVPSIALPSKPAKKKEEEDDDMKELN WAGSM	7800.6 Da	7799.7 Da
CHMP4C	GDDFDEDELMAELEELEQEELNKKMTNIRLPNVPS SSLPAQPNRKPGMSSTARRSRAASSQRAEEEDDD IKQLAAWAT	8746.5 Da	8746.2 Da
IST1 316-366	GPADNYDNFVLPELPSVPTLPTASAGASTSASEDI DFDDLSRRFEELKKKT	5629.3 Da	5628.7 Da

3

1 Supplemental Table 4. Plasmids

Plasmid	Internal ID	DNASU# Addgene #	or Uniprot #	Source/ Reference
MIT DOMAINS				
pCA528-AMSH (1-178)	WISP18-22	160050 (Addgene)	O95630	This Study
pCA528 AMSHLP (16-218)	WISP20-83	180609 (Addgene)	Q96FJ0	This Study
pGEX-THTAP-CALPAIN-7 (1-165)	WISP21-129	180610 (Addgene)	Q9Y6W3	This Study
pCA528 CALPAIN-7 WT (1-165)	WISP21-25	180611 (Addgene)	Q9Y6W3	This Study
pCA528 CALPAIN-7 V18D (1-165)	WISP21-26	180612 (Addgene)	Q9Y6W3	This Study
pCA528 CALPAIN-7 F98D (1-165)	WISP21-27	180613 (Addgene)	Q9Y6W3	This Study
pCA528 KATNA1 (1-79)	WISP19-20	180614 (Addgene)	A8K7S5	This Study
pCA528 KATNA1-V55D (1-79)	WISP20-23	180615 (Addgene)	A8K7S5	This Study
pCA528 KATNAL1 (1-87)	WISP20-54	180618 (Addgene)	Q9BW62	This Study
pCA528 KATNAL2 (1-98)	WISP21-28	180619 (Addgene)	Q8IYT4	This Study
pGEX2T-LIP5 (1-168)	WISP10-486	HSCD00587773	Q9NP79	Skalicky, 2012
pGEX-THTAP-MITD1 (1-85)	WISP20-35	180620 (Addgene)	Q8WV92	This Study
pGEX-THTAP-NRBF2 (1-111)	WISP20-88	180621 (Addgene)	Q96F24	This Study
pCA528 RPS6KC1 (235-316)	WISP20-87	180622 (Addgene)	Q96S38	This Study
pCA528 RPS6KL1 (20-140)	WISP20-32	180623 (Addgene)	Q9Y6S9	This Study
pGEX-THTAP-SNX15 (263-342)	WISP21-130	180624 (Addgene)	Q9NRS6	This Study
pCA528-SNX15 (263-342)	WISP21-14	180625 (Addgene)	Q9NRS6	This Study
pGEX-THTAP-SPARTIN (1-101)	WISP20-57	180626 (Addgene)	Q8N0X7	This Study
pCA528 SPASTIN WT (112-196)	WISP21-11	180627 (Addgene)	Q9UBP0	This Study; xray
pCA528 SPASTIN WT (108-200)	WISP21-10	180628 (Addgene)	Q9UBP0	This Study; FP
pCA528 SPASTIN F124D (108-200)	WISP21-12	180629 (Addgene)	Q9UBP0	This Study
pCA528 SPASTIN L177D (108-200)	WISP21-13	180634 (Addgene)	Q9UBP0	This Study
pGEX-PP-ULK3 (MIT) ₂ (277-449)	WISP14-112	HSCD00696230	Q6PHR2	Caballe, 2015
pCA528 USP8 (1-147)	WSIP20-86	180636 (Addgene)	P40818	This Study

Plasmid	Internal ID	DNASU# Addgene #	or Uniprot #	Source/ Reference
pCA528 USP54 (731-878)	WISP20-85	180637 (Addgene)	Q70EL1	This Study
pET16B VPS4A (1-84)	WISP05-43	180638 (Addgene)	Q9UN37	Stuchell- Brereton, 2007
pGEXET16B VPS4B (1-86)	WISP04-155	180639 (Addgene)	O75351	Stuchell- Brereton, 2007
pGEX-THTAP VPS9D1 (1-97)	WISP20-89	180640 (Addgene)	Q9Y2B5	This Study
ESCRT-III PEPTIDES				
pCA528 CHMP1A (140-196)	WISP18-1	104593 (Addgene)	Q9HD42	This Study
pCA528 CHMP1B (143-199)	WISP18-2	108255 (Addgene)	Q7LBR1	This Study
pCA528 CHMP2A (152-222)	WISP18-3	104594 (Addgene)	O43633	This Study
pCA528 CHMP2B (141-213)	WISP18-4	104595 (Addgene)	Q9UQN3	This Study
pCA538 CHMP3 (159-222)	WISP18-5	104599 (Addgene)	Q9Y3E7	This Study
pCA528 CHMP4A (153-222)	WISP18-6	108256 (Addgene)	Q9BY43	This Study
pCA528 CHMP4B (156-224)	WISP18-7	104596 (Addgene)	Q9H444	This Study
pCA528 CHMP4B (156-224; no Cys)	WISP20-140	180641 (Addgene)	Q9H444	This Study
pCA528 CHMP4C (156-233)	WISP18-9	108257 (Addgene)	Q96CF2	This Study
pCA528 CHMP4C (156-233; no Cys)	WISP20-14	180642 (Addgene)	Q96CF2	This Study
pCA528 CHMP5 (148-219)	WISP18-10	108259 (Addgene)	Q9NZZ3	This Study
pCA528 CHMP6 (145-201)	WISP18-11	104597 (Addgene)	Q96FZ7	This Study
pCA528 CHMP7 (366-453)	WISP18-12	104598 (Addgene)	Q8WVX9	This Study
pCA528 IST1 (316-366)	WISP14-121	HSCD00696258	P53390	Caballe, 2015
pCA528 IST1 (316-366; no Cys)	WISP20-99	180643 (Addgene)	P53390	This Study
Mammalian Expression Vectors				
pCAG-OSF-PP-ULK1(MIT) ₂ (833-1050)	WISP20-37	180644 (Addgene)	O75385	This Study

Plasmid	Internal ID	DNASU# Addgene #	or Uniprot #	Source/ Reference
pCAG-OSF-ULK3(MIT) ₂ (277-449)	WISP14-112	HSCD00696230	Q6PHR2	Caballe, 2015
pCAG-CHMP1A-myc	WISP08-77	HSCD00520978	Q9HD42	Caballe, 2015
pCAG-CHMP1B-myc	WISP08-78	HSCD00696225	Q7LBR1	Caballe, 2015
pCAG-CHMP2A-myc	WISP08-97	HSCD00696226	O43633	Caballe, 2015
pCAG-CHMP2A-myc L216D/L219D	WISP20-13	180645 (Addgene)	O43633	This Study
pCAG-Myc-IST1	WISP07-77	HSCD00751713	P53390	Caballe, 2015
pCAG-OSF empty	WISP06-65			
pRK5-Myc-ATG13	WISP20-30	31965 (Addgene)	O75143	Jung, 2009
pCAG-Myc-KATNB1	WISP20-16	160054 (Addgene)	Q9BVA0	This Study
pCAG-OSF-KATNA1 WT	WISP20-18	160051 (Addgene)	A8K7S5	This Study
pCAG-OSF-KATNA1 V55D	WISP20-17	160053 (Addgene)	A8K7S5	This Study
pCAG-OSF-KATNA1 R14A	WISP20-19	160052 (Addgene)	A8K7S5	This Study
pLVX-TetOn-Advanced				Clontech/ Don Ayer gift
pLVX-tight-puro				Clontech/ Don Ayer gift
pLVX-mCherry	WISP20-61	180646 (Addgene)		This Study
pLVX-mCherry-SPASTIN	WISP20-43	180647 (Addgene)	Q9UBP0	This Study
pLVX-mCherry-SPASTIN-F124D	WISP20-44	180648 (Addgene)	Q9UBP0	This Study
pLVX-mCherry-SPASTIN-L177D	WISP20-45	180649 (Addgene)	Q9UBP0	This Study
pLVX-mCherry-KATNA1	WISP20-47	180650 (Addgene)	Q9BVA0	This Study
pLVX-mCherry-KATNA1-V55D	WISP20-48	180651 (Addgene)	Q9BVA0	This Study
pLVX-CAPN7-mCherry	WISP20-50	180652 (Addgene)	Q9Y6W3	This Study
pLVX-CAPN7-F98D-mCherry	WISP20-53	180653 (Addgene)	Q9Y6W3	This Study

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1 Supplemental Table 5. siRNA Sequences

siRNA	Protein Target	Sense Sequence	Source	Reference
siCHMP4C (siC4C)	CHMP4C	CACUCAGAUUGAUGGCACA	U of U Cores	Strohacker, 2021
siCAPN7-2213 (siCAPN7)	CALPAIN7	GCACCCAUACCUUUACAUU	U of U Cores	This Study
siCAPN7-2444 (siCAPN7-b)	CALPAIN7	GGCCGUUACUGAUUGAGCU	Thermo Fisher	Cat # s23893
siIST1-490	IST1	AGAUACCUGAUUGAAAUUG	U of U Cores	Bajorek, 2009
siKATNA1-583 (siKATNA1)	KATNA1	GGACAGCACUCCCUUGAAA	U of U Cores	This Study
siKATNA1-pool (siKATNA1-b)	KATNA1	ON-TARGET-Plus siRNA SMARTPool	Horizon Discovery	Cat # L-005157-02-005
siNT	Non-targeting	GCAAAUCUCCGAUCGUAGA	U of U Cores	Mackay, 2010
siNUP50	Nup50	GGAGGACGCUUUUCUGGAU	U of U Cores	Mackay, 2010
siNUP153	Nup153	GGACUUGUUAGAUCUAGUU	U of U Cores	Mackay, 2010
siSPAS-726 (siSPAS)	SPASTIN	GAACAGUGUGAAAGAGCUA	U of U Cores	This study
siSPAS-4146 (siSPAS-b)	SPASTIN	CGUUAUUGAUACUUGGAUA	U of U Cores	This study

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1 Supplemental Table 6. Antibodies

Target	Host Organism	Source	Product Number	Application ¹	Dilution Factor
CAPN-7	Rabbit	Proteintech	26985-1-AP	IF WB	1:1000 1:5000
CEP55	Sheep	Bastos and Barr, 2010	N/A	IF	1:3500
CHMP4C	Rabbit	Sadler, M.-Serrano, 2018	N/A	WB	1:500
GAPDH	Mouse	Millipore	MAB374	WB	1:20,000
IST1	Rabbit	Sundquist Lab/ Covance	UT560	WB	1:1000
KATNA1	Rabbit	Proteintech	17560-1-AP	IF	1:1000
KATNA1	Rabbit	Abcam	ab111881	WB	1:1000
mCherry	Rabbit	Abcam	ab167453	IF	1:2000
mCherry	Rabbit	Novus Biologicals	NBP2-25157	WB	1:1000
NUP153 (SA1)	Mouse	Brian Burke, Singapore	N/A	WB	1:50
NUP50	Rabbit	Mackay, et al., 2010	N/A	WB	1:2500
SPASTIN	Mouse	Sigma	S7074	IF WB	1:1000 1:1000
α -TUBULIN (DM1A)	Mouse	Cell Signaling Technology	3873S	IF	1:2500
Myc	Mouse	EMD Millipore	Clone 4A6	WB	1:2500
Flag	Mouse	Sigma	M2	WB	1:10,000
IRDye 800 CW, Mouse	Donkey	Licor	926-32212	WB	1:10,000
IRDye 680, Mouse	Donkey	Licor	926-68072	WB	1:10,000
IRDye 800 CW,	Donkey	LiCor	926-32213	WB	1:10,000

Rabbit					
IRDye 680, Rabbit	Donkey	LiCor	926-68073	WB	1:10,000
Alexa Fluor Plus 647, Mouse IgG	Donkey	Thermo Fisher	A-32787	IF	1:1000
Alexa Fluor Plus 405, Rabbit IgG	Donkey	Thermo Fisher	A-48258	IF	1:1000
Alexa Fluor Plus 594, Rabbit IgG	Donkey	Thermo Fisher	A-32754	IF	1:1000
Alexa Fluor Plus 647, Rabbit IgG	Donkey	Thermo Fisher	A-32795	IF	1:1000
Alexa Fluor 488, Sheep IgG	Donkey	Thermo Fisher	A-11015	IF	1:1000

¹IF = Immunofluorescence; WB = Western Blot

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1 **Supplemental Table 7. SPASTIN MIT-IST1 Complex Data Collection and**
2 **Refinement Statistics**

Data	
Crystal	SPASTIN MIT (112-195):IST1 (344-366)
Source, Wavelength (Å), Collection Date	SSRL 9-1, 0.97946, 03-13-2016
Space group, unit cell constants (a b c α β γ)	I4 ₁ , (79.614 79.614 36.331 90 90 90)
Resolution range	28.15-1.15 (1.191-1.15)
Total reflections	519,763
Unique reflections	38,262 (3250)
Multiplicity	13.6 (12.3)
Completeness (%)	94.19 (80.39)
Mean I/sigma (I)	16.4 (4.0)
Wilson B-factor	10.40
R-merge	0.103 (1.647)
R-meas	0.107 (1.718)
R-pim	0.029 (0.480)
CC1/2	0.988 (0.865)
Refinement	
Program	Phenix.refine, version 1.17.1
Reflections used in refinement	38,243 (3239)
Reflections used for R-free	2001 (170)
R-work	0.1494 (0.1969)
R-free	0.1586 (0.1886)
Number of non-hydrogen atoms	998
macromolecules	861
ligands	25
solvent	112
Protein residues	102
RMS (bonds), (angles)	0.007, 0.90

Ramachandran favored, allowed, outliers (%)	100.00, 0.00, 0.00
Rotamer outliers (%)	1.10
Clashscore	2.22
Average B-factor (Å²)	22.12
macromolecules	20.60
ligands	28.80
solvent	32.36
Number of TLS groups	1

1 Statistics for the highest-resolution shell are shown in parentheses.

2