Parallels Between Cytokinesis and Retroviral Budding: A Role for the ESCRT Machinery

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During cytokinesis, as dividing animal cells pull apart into two daughter cells, the final stage, termed abscission, requires breakage of the midbody, a thin membranous stalk connecting the daughter cells. This membrane fission event topologically resembles the budding of viruses, such as HIV-1, from infected cells. We found that two proteins involved in HIV-1 budding—tumor susceptibility gene 101 (Tsg101), a subunit of the endosomal sorting complex required for transport I (ESCRT-I), and Alix, an ESCRT-associated protein—were recruited to the midbody during cytokinesis by interaction with centrosome protein 55 (Cep55), a centrosome and midbody protein essential for abscission. Tsg101, Alix, and possibly other components of ESCRT-I were required for the completion of cytokinesis. Thus, HIV-1 budding and cytokinesis use a similar subset of cellular components to carry out topologically similar membrane fission events.

Completion of cytokinesis requires the scission of a thin bridge of membrane connecting the daughter cells. The site of abscission is the midbody, a complex structure that contains proteins required for cell cleavage (1). Cytokinesis also requires dramatic remodeling of plasma membranes (2), and a number of vesicle-trafficking components are thought to be involved in fusion events that precede abscission (3–5). The vesicle-tethering exocyst complex and two members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery, namely syntaxin-2 and endobrevin/vesicle-associated membrane protein 8 (VAMP-8) (6, 7), play an essential role in abscission, but it remains unclear whether SNARE-promoted fusion events are sufficient to complete the separation of the daughter cells. A topologically equivalent membrane scission event is needed to complete the last step of egress for enveloped viruses. Retroviral late budding domains (L domains) facilitate viral particle release from the infected cell by mediating a membrane fission event that separates the nascent virion from the plasma membrane (8). L domains in HIV-1, Ebola virus, and other enveloped viruses encode an essential Pro-Thr-Ala-Pro (PTAP) (9) motif that mediates its activity by recruiting Tsg101 (10–12). A second type of L domain is encoded by the LYPXL motif (where X is any amino acid), which facilitates retroviral egress by recruiting Alix (apoptosis-linked gene 2 interacting protein X), a class E vacuolar protein sorting (VPS) protein (13–15). Current models propose that ESCRT-III is the core machinery recruited by ESCRT-I and Alix to facilitate membrane fission (16), a function initially characterized in multivesicular body (MVB) formation.

Tsg101 preferentially localizes to late endosomal structures (17), although a cell cycle–dependent subcellular localization has been reported (18). To study Tsg101 localization in a physiological context, we replaced the endogenous protein with a monomeric Cherry
Fig. 2. Tsg101 and Alix are required for efficient completion of cytokinesis. (A) HeLa cells stably expressing both YFP-Cep55 and either mCh-Tsg101 or mCh-Alix were stained with α-tubulin and analyzed by confocal microscopy. (B) HeLa cells were transfected with siRNA targeting Cep55, Tsg101, or Alix, fixed, stained with α-tubulin, and scored for multinucleation (n = 5 ± SD). Representative micrographs are given, and arrows indicate cells at midbody stage. Cell lysates were normalized for Hsp90 levels and immunoblotted with antisera to Tsg101, Cep55, Alix, or Hsp90. Luc, luciferase. (C) 293T cells were transiently transfected with plasmids encoding YFP-tagged fusion proteins and pNL-HXB HIV-1 provirus. Cell lysates and virions were examined by immunoblotting with α-Gag antisera. β-Gal assay was performed on HeLa-TZM-bl cells infected with 293T supernatant (n = 4 ± SD). RLU, relative light units. (D) HeLa cells transfected with plasmids encoding YFP-tagged fusion proteins were fixed, stained with α-tubulin, and scored for multinucleated cells (n = 3 ± SD). Representative micrographs are given.
The most noticeable feature of mCh-Tsg101 was its localization to the midbody at late stages of cell division (Fig. 1B). Specifically, mCh-Tsg101 localized to the Flemming body, a phase-dense structure containing proteins involved in cell abscission (6). This localization led us to hypothesize that Tsg101 and perhaps other components of the ESCRT machinery might play a role in late stages of cytokinesis (Fig. 1C). A search for potential interactions with components of the cytokinesis machinery was performed by taking advantage of a proteome-scale map of human protein-protein interactions generated by yeast two-hybrid assay (20). Tsg101 was found to bind Cep55, a centrosomal protein that localizes to the midbody during late stages of cytokinesis and is required for abscission (21, 22). The Tsg101-Cep55 interaction and Cep55 homomultimerization activity were confirmed by yeast two-hybrid and coprecipitation assays (Fig. 1, D and E), suggesting that Tsg101 localization to the midbody might be mediated through interaction with Cep55. Indeed, depletion of Cep55 prevented Tsg101 recruitment to the midbody (Fig. 1B) and resulted in morphologically abnormal Flemming bodies (21), confirming Cep55 suppression in these cells.

A subsequent screen against the human class E VPS pathway identified the interaction of Cep55 with Alix, a second protein required for retroviral egress (Fig. 1, D and E). The Cep55-Alix binding was confirmed by pull-down assays (Fig. 1E) and microscopy in cell lines stably expressing a combination of yellow fluorescent protein (YFP)-Cep55 and either mCh-Tsg101 or mCh-Alix at near endogenous levels (Fig. 2 and fig. S1A). YFP-Cep55 localized to the midbodies as described for the endogenous protein (21, 22), and both mCh-Tsg101 and mCh-Alix colocalized with YFP-Cep55 in the central region of the midbody (Fig. 2A). Localization of mCh-Alix to the midbody was also abolished in Cep55-depleted cells (fig. S1B).

We then used RNA interference to determine the roles of Tsg101 and Alix in cytokinesis. In this assay, defects in cytokinesis are manifested by the appearance of multinucleated cells. Supporting an essential role in cytokinesis, depletion of Alix resulted in a 14-fold increase in the percentage of multinucleated cells as compared with control cells (Fig. 2B). This phenotype was nearly identical to that observed upon depletion of Cep55 (21, 22). Depletion of Tsg101 also resulted in an increased proportion of multinucleated cells (Fig. 2B), supporting the essential role of Tsg101 in abscission. Additionally, a marked toxicity was observed in cells depleted of Tsg101 (fig. S2A) (23), suggesting that cytokinetic defects may contribute to the reduced proliferative capacity and embryonic lethality observed in tsg101 knockout embryos (24).

Thus, the cellular machineries involved in midbody abscission, MVB formation, and retroviral budding share some components and are functionally related. To extend this notion, we followed a dominant-negative approach taking advantage of VPS4, an AAA–adenosine triphosphatase that mediates disassembly and recycling of the ESCRT complexes from the endosomal membranes. Specifically, a catalytically inactive VPS4 (VPS4-DN) inhibits retroviral L-domain activity (10, 25). We also followed a strategy whereby components of the ESCRT machinery exhibit a dominant-negative effect when transiently overexpressed as fusions to heterologous proteins (15), and we used forms of Syntaxin-2 and Vamp-8 that lack transmembrane domains (STX2-8TM and Vamp8-8TM) and arrest cell division at late stages through inhibition of midbody abscission (7). Transfection of VPS4-DN induced an accumulation of multinucleated cells comparable to the effect of STX2-8TM and Vamp8-8TM (Fig. 2D). In contrast, these truncated SNAREs did not inhibit retroviral L-domain

Fig. 3. Residues P158-P-N-T-S162 in the proline-rich domain of Tsg101 coordinate Cep55. (A) Tsg101 or Tsg101(6158-162) fused to the Gal4 DNA binding domain were tested for interaction with a variety of Tsg101-interacting proteins fused to the VP16 activation domain through yeast 2-hybrid assay. Error bars indicate SD. (B) HeLa cells transiently transfected with plasmids encoding mCh-Cep55 and either YFP-Tsg101 or YFP-Tsg101(6158-162) were fixed and stained with α-tubulin.
activity as determined by measuring infectious virus release (Fig. 2C). Furthermore, the overexpression of a YFP-Tsg101 fusion inhibited L-domain activity, presumably by disrupting the ESCRT-I stoichiometry, and a similar inhibition was observed in cell division (Fig. 2D). Similar to HIV-1 budding (15), overexpression of charged multivesicular body protein 4 (CHMP4)–ESCRT-III, but not ESCRT-II subunits, inhibited cytokinesis (fig. S2B). An analogous correlation between viral budding and cytokinesis was observed by overexpressing a YFP-Alix fusion, resulting in the inhibition of L-domain activity observed by overexpressing a YFP-Alix fusion, as illustrated by the lack of inhibitory activity of the Syntaxin-2 and Vamp-8 deletions in retroviral budding.

The Cep55-binding site in Tsg101 was mapped to residues P158-P-N-T-S162 in Tsg101’s proline-rich region (PRR) (fig. S3A). Deletion of residues 158 to 162 in full-length Tsg101 [Tsg101(158-162)] resulted in the loss of binding to Cep55, whereas binding of Tsg101(158-162) to other ESCRT-I components (VPS28 and VPS37A-D), viral proteins (HIV-1 Gag and EbVP40), and endosomal proteins [hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs), Tsg101, Alix, and Tsg101-associated ligase (Tal)] was unaffected (Fig. 3A). These results were confirmed by colocalization experiments. In transiently transfected cells, both YFP-Tsg101 and mCh-Cep55 exhibited a punctate distribution at interphase and localized to the Flemming body at late stages of cytokinesis (Fig. 3B). In both situations, a nearly complete colocalization of Tsg101 and Cep55 was observed. In contrast, YFP-Tsg101(158-162) failed to colocalize with mCh-Cep55 despite displaying a punctate distribution in cells at interphase similar to that presented by wild-type Tsg101 (Fig. 3B). More importantly, YFP-Tsg101(158-162) was not recruited to the midbody (Fig. 3B), suggesting that residues 158 to 162 in Tsg101 mediate its recruitment to the midbody by interacting with Cep55.

Thus, Cep55 is functionally linked to the ESCRT machinery through binding to Tsg101

**Fig. 4.** The Tsg101-Cep55 interaction is required for cytokinesis but not for viral budding. (A) 293T cells were treated with siRNA targeting Luc or Tsg101. pNL/HXB HIV-1 provirus and YFP-encoding plasmids or siRNA-resistant YFP-Tsg101–encoding plasmids were included in the second transfection. 293T lysates were examined by immunoblotting with α-GFP antisera. Lysates and virions were examined with antisera to Gag. β-Gal assay was performed upon HeLa-TZM-bl reporter cells infected with 293T supernatant. WT, wild type. Error bars indicate SD. (B) Stably transduced HeLa cells expressing mCh or the indicated siRNA-resistant mCh-Tsg101 plasmids were treated with the indicated siRNA. Cells were fixed, stained with α-tubulin, and scored for multinucleation or arrest at midbody stage (n = 5 ± SD). Cell lysates were examined by immunoblotting with α-Tsg101 or α-Hsp90 antisera. (C) Representative micrographs describing midbody localization of mCh or mCh-Tsg101 mutants in dividing cells.
and Alix. The budding inhibition by Cep55 overexpression confirmed this functional link but did not necessarily imply that Cep55 was required for L-domain activity. To address this issue, we depleted endogenous Tsg101 from 293T cells by small interfering RNA (siRNA) and reintroduced it by transfecting siRNA-resistant plasmiads with mutations in either the PTAP-binding site [Met⁹⁵→Ala⁹⁵ (M95A)] (26) or in the Cep55-binding region (6158-162). Depletion of Tsg101 results in a dramatic reduction of HIV-1 infectious particle release (Fig. 4A) (10), and the L-domain activity was rescued by transfecting an siRNA-resistant Tsg101 but not with Tsg101(M95A). Similar experiments with Tsg101(Δ158-162) showed that the Cep55-binding region in Tsg101 was not required for HIV-1 L-domain activity (Fig. 4A). Additionally, depletion of Cep55 had no effect on either Tsg101- or Alix-dependent budding (fig. S4), indicating that Cep55 is not required for L-domain activity.

We next determined the role of the Cep55-Tsg101 interaction in cytokinesis by following a similar depletion-replacement approach in HeLa cells. The percentage of multinucleated cells in Tsg101-depleted cells was restored to normal levels when the siRNA-resistant Tsg101 was reintroduced (Fig. 4B), showing that the cytokinesis defect observed with the siRNA against Tsg101 was specific. An accumulation of cells arrested at the midbody stage was also observed in Tsg101-depleted cells (Fig. 4B), and midbody morphology was nearly identical to that in control cells, with formation of apparently normal Fanning bodies. Overall, the cytokinesis arrest observed in Tsg101-depleted cells is therefore consistent with defects in abscission. The replacement of Tsg101 mutants showed a partial cytokinesis defect in cells expressing Tsg101(Δ158-162), and a similar partial phenotype was observed in Tsg101(M95A)-expressing cells (Fig. 4B), whereas a Tsg101 double mutant (M95A, Δ158-162) recapitulated the phenotype of Tsg101-depleted cells (Fig. 4B). The effect of Tsg101(Δ158-162) could be explained by the lack of binding to Cep55 and recruitment to the midbody, but Tsg101(M95A) was recruited to the Fanning body (Fig. 4C), suggesting that a downstream defect might explain its phenotype. Alternatively, efficient Tsg101 recruitment to the midbody might occur in a complex with Alix and Cep55. Alix binds to the ubiquitin E2 variant domain of Tsg101 through a PSAP motif in the PRR, and Tsg101(M95A) cannot bind Alix (13), although its binding to other components of the ESCRT machinery remained unchanged (fig. S3). Thus, the partial phenotype observed with Tsg101(M95A) may indicate a requirement for the Tsg101-Alix interaction to complete abscission, although more work is needed to prove this point unequivocally. An additional requirement for other components of ESCRT-I, specifically VPS28, was strongly suggested by the phenotype observed in cells expressing Tsg101(A3), which does not bind VPS28 (25). Tsg101(A3) was recruited to the central region of the midbody (Fig. 4C), and the percentage of multinucleated cells induced by the A3 mutation fully accounted for the phenotype of Tsg101-depleted cells (Fig. 4B), suggesting that the Tsg101-VPS28 interaction is required to complete abscission.

We found that Cep55, a key component of the cellular machinery that mediates abscission, interacts with two endosomal proteins that facilitate retroviral budding, namely Tsg101 and Alix. The cellular pathways that mediate retroviral L-domain activity and abscission are closely interconnected, which are consistent with a model whereby the ESCRT machinery mediates membrane fission events essential for efficient separation of the daughter cells in the last step of cell division. The role of ESCRT complexes in yeast cytokinesis is unclear, but mutations in the Arabidopsis homolog of Tsg101 induce cytokinesis defects (27), suggesting that the role of the ESCRT machinery in abscission in might be conserved in multicellular organisms.

References and Notes
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9. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

HIV-1 Proviral DNA Excision Using an Evolved Recombinase
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†HIV-1 integrates into the host chromosome and persists as a provirus flanked by long terminal repeats (LTRs). To date, treatment regimens primarily target the virus enzymes or virus-cell fusion, but not the integrated provirus. We report here the substrate-linked protein evolution of a tailored recombinase that recognizes an asymmetric sequence within an HIV-1 LTR. This evolved recombinase efficiently excised integrated HIV proviral DNA from the genome of infected cells. Although a long way from use in the clinic, we speculate that this type of technology might be adapted in future antiretroviral therapies, among other possible uses.

Current highly active antiretroviral therapy (HAART) targeting the viral reverse transcriptase, protease, and virus-host fusion (1, 2) has transformed HIV-1 infection into a chronic illness and curtailed the morbidity of infected individuals. Furthermore, new viral targets and novel inhibition strategies are being tested for improved control of HIV-1 (3–7). However, the current treatment strategies only suppress the viral life cycle without eradicating the infection, and new strains of HIV-1 are emerging that are resistant to suppressive treatments (8). An attractive alternative would be the specific eradication of the HIV-1 provirus.

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