The midbody ring scaffolds the abscission machinery in the absence of midbody microtubules

Rebecca A. Green, 1 Jonathan R. Mayers, 2 Shaohe Wang, 1 Lindsay Lewellyn, 3 Arshad Desai, 1 Anjon Audhya, 2 and Karen Oegema 1

A
bscission completes cytokinesis to form the two
daughter cells. Although abscission could be or-
ganized from the inside out by the microtubule-
based midbody or from the outside in by the contractile
ring–derived midbody ring, it is assumed that midbody
microtubules scaffold the abscission machinery. In this
paper, we assess the contribution of midbody micro-
tubules versus the midbody ring in the Caenorhabditis
elegans embryo. We show that abscission occurs in two
stages. First, the cytoplasm in the daughter cells becomes
isolated, coincident with formation of the intercellular
bridge; proper progression through this stage required
the septins (a midbody ring component) but not the
membrane-remodeling endosomal sorting complex re-
quired for transport (ESCRT) machinery. Second, the midbody
and midbody ring are released into a specific daughter
cell during the subsequent cell division; this stage required
the septins and the ESCRT machinery. Surprisingly, mid-
body microtubules were dispensable for both stages.
These results delineate distinct steps during abscission and
highlight the central role of the midbody ring, rather than
midbody microtubules, in their execution.

Introduction

Cytokinesis can be partitioned into two phases: (1) contractile
ring constriction, which changes cell shape, and (2) abscission,
which isolates the cytoplasm in the daughter cells and alters
membrane topology to form two physically distinct cells. Con-
tractile ring constriction is orchestrated by an array of antiparal-
lel microtubule bundles called the central spindle and a cortical
contractile ring that forms around the cell equator. As constric-
tion nears completion, the central spindle and contractile ring
mature to form the midbody and the midbody ring, which direct
abscission (Fededa and Gerlich, 2012; Green et al., 2012;
Agromayor and Martin-Serrano, 2013).

The midbody is a densely packed antiparallel microtubule
array that sits in the center of the intercellular bridge (Glotzer,
2009; Green et al., 2012). The molecular composition of the
midbody includes three key components also required to form
the central spindle: (1) the microtubule cross-linking protein
PRC1 (Glotzer, 2009; Walczak and Shaw, 2010), (2) the cen-
tralspindlin complex, consisting of the Mklp1 kinesin and
the CYK4 Rho GAP 1 (White and Glotzer, 2012), and (3) the
chromosomal passenger complex containing the Aurora B
kinase (Carmena et al., 2012). Whereas PRC1 and the chromo-
somal passenger complex remain associated with midbody
microtubules (Hu et al., 2012), centralspindlin transitions in its
localization from the midbody to the midbody ring (Elia et al.,
2011; Hu et al., 2012).

The midbody ring, like the midbody, retains contractile
ring components, including actin filaments, Myosin II, and
septin filaments, as well as Anillin and Citron kinase (Madaule
et al., 1998; Gai et al., 2011; Hu et al., 2012; Kechad et al.,
2012). The septins bind directly to the plasma membrane and
are recruited to the contractile ring by Anillin (D’Avino, 2009;
Piekny and Maddox, 2010; Oh and Bi, 2011; Mostowy and
Cossart, 2012). In Drosophila melanogaster S2 cells, the Anillin
N terminus, which binds actin and Myosin II, is important for
midbody ring integrity, whereas the connection between the
Anillin C terminus and the septins links the midbody ring to the
plasma membrane (Kechad et al., 2012), a role similar to that
proposed for the CYK4 C1 domain (Lekomtsev et al., 2012).

© 2013 Green et al. This article is distributed under the terms of an Attribution–Noncommercial–
Share Alike-No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
Figure 1. **Abscission occurs in two stages: cytoplasmic isolation and release of the midbody/midbody ring.** (A) Furrow diameter was measured in projections of the central region of z stacks of embryos (n = 10) expressing a GFP-tagged plasma membrane probe. [right] Graph plots mean furrow diameter versus time after furrow initiation. Arrow indicates the last time point when a hole can be detected (apparent closure). Error bars are the SDs. [top] Schematics illustrate shape changes during the first division in the C. elegans embryo, highlighting intercellular bridge structure. MTs, microtubules. (B, left)
Abscission could be organized from the outside in by the midbody ring or from the inside out by the midbody. Although the relative contributions of the midbody and midbody ring in scaffolding abscission have not been directly tested, the midbody is thought to serve as the platform that brings together the abscission machinery, including membrane trafficking components that narrow the intercellular bridge (Schiel and Prekeris, 2013) and the endosomal sorting complex required for transport (ESCRT) machinery, which executes the final scission event (Agramayor and Martin-Serrano, 2013; McCullough et al., 2013). In human cells, the ESCRT machinery is recruited by CEP55, which binds to centralspindlin late in cytokinesis; CEP55 binds Alix and ESCRT-I, which in turn recruit ESCRT-III proteins thought to polymerize to drive membrane scission (Fabbro et al., 2005; Zhao et al., 2006; Carlton and Martin-Serrano, 2007; Morita et al., 2007; Carlton et al., 2008; Lee et al., 2008; Bastos and Barr, 2010; Elia et al., 2011; Guizetti et al., 2011).

The point when the cytoplasm in the daughter cells becomes isolated from each other (hereafter termed cytoplasmic isolation) has been monitored by following the diffusion of fluorescent probes (Lo and Gilula, 1979; Sanger et al., 1985; Steigemann et al., 2009; Guizetti et al., 2011). In HeLa cells, cytoplasmic exchange ceases ~60 min after the completion of furrowing, coincident with ESCRT-III–mediated scission (Steigemann et al., 2009; Guizetti et al., 2011).

Here, we analyze abscission in the early Caenorhabditis elegans embryo. We show that abscission occurs in two stages: cytoplasmic isolation and midbody/midbody ring release. Inhibition of the midbody ring–associated septins affects both stages, whereas the membrane-remodeling ESCRT machinery is only required for the second stage. In contrast to the idea that the midbody plays a central role in orchestrating abscission, both cytoplasmic isolation and midbody ring release occur normally in the absence of midbody microtubules. These results define distinct events during abscission and highlight the central role of the midbody ring, rather than midbody microtubules, in directing abscission.

## Results

### Cytoplasmic isolation occurs coincident with the completion of contractile ring constriction during the first division of the C. elegans embryo

To monitor contractile ring closure, we collected time-lapse 3D images of embryos expressing a GFP fusion with a pleckstrin homology (PH) domain that binds a phospholipid produced specifically on the plasma membrane (Audhya et al., 2005) and generated end-on views by rotating and projecting the data from the central portion of the embryo (Maddox et al., 2007). The last time when a hole can be detected is ~280 s after furrow initiation, a point we refer to as apparent closure (Fig. 1 A). To determine whether cytoplasmic isolation is coincident with apparent closure, we loaded caged carboxy-Q-rhodamine–labeled 10-kD dextran into embryos expressing the GFP-tagged plasma membrane probe by injection into the syncytial gonad of adult worms (Fig. S1 B). Diffusion was monitored by imaging at 5-s intervals after photoactivating the probe on one side of the embryo with a pulse of UV light. Before cytokinesis onset, the photoactivated probe equilibrated between the two halves of the embryo with a half-time of ~40 s; however, after apparent closure, there was no detectable equilibration (Fig. 1 B and Video 1). We conclude that the cytoplasm in the daughter cells becomes diffusionaly isolated coincident with the completion of contractile ring constriction.

The midbody/midbody ring from the first cytokinesis is released after anaphase of the subsequent cell division

In vertebrate cells, cytoplasmic isolation is thought to occur simultaneously with ESCRT-mediated scission (Steigemann et al., 2009; Guizetti et al., 2011). Scission releases the midbody into one of the daughter cells, if the bridge is severed on one side, or into the extracellular space, if it is cut on both sides (Chen et al., 2013). Midbodies are released into the extracellular space during the Q cell neuroblast divisions in C. elegans larva (Chai et al., 2012). However, older work suggested that the midbody/midbody ring from the first embryonic division remains after the completion of furrowing, in which it serves as a cortical site that guides centrosome rotation at the two-cell stage (Hyman, 1989; Waddle et al., 1994; Keating and White, 1998).

To determine whether the midbody/midbody ring are released, we used live-cell imaging to monitor fluorescently tagged fusions with two midbody components, mCherry-MKlp1\textsuperscript{ZEN-4} and GFP–Aurora B\textsuperscript{ARX-2}, and two components of the midbody ring, Myosin \textit{II}\textsuperscript{NMY-2}–GFP and GFP–CYK-7, a new contractile/midbody ring component that we recently identified (Green et al., 2011). Imaged embryos also expressed mCherry- or GFP-tagged fusions to label the plasma membrane. As contractile ring constriction completed, the two midbody markers were enveloped by the closing furrow and remained localized to a single tight focus embedded within the plasma membrane at the...
Figure 2. The ESCRT machinery is required for midbody/midbody ring release. (A) Deconvolved wide-field image of an embryo stained for tubulin (cyan), Mklp\(^{ZEN-4}\), and ESCRT-I\(^{TSG-101}\) (\(n = 5\) embryos). (B) Central plane confocal images of embryos expressing GFP-ESCRT-I\(^{MVB-12}\) (\(n = 6\) embryos). Times are relative to anaphase of the second division. Dashed yellow lines mark the cell boundaries. The white arrowhead and yellow arrow mark the focus.
cell–cell boundary, the intercellular bridge, for the next ~12 min (Fig. 1 C, yellow arrows; and Fig. S1 C; for a comparison of the intercellular bridge in the C. elegans embryo and vertebrate cells see Fig. S1 A). Coincident with anaphase of the second division (in the anterior AB cell; ~909 ± 102 s after furrow initiation), tiny pieces labeled with the plasma membrane marker were shed asymmetrically from the midbody region predominantly into the posterior cell (Fig. 1 C, white arrowheads). Approximately 200 s after the onset of membrane shedding (~1,100 s after initiation of the first division furrow), a focus containing both midbody markers was released into the posterior (P₁) cell (Fig. 1 C, green arrows; Fig. S1 C; and Videos 2 and 3).

Imaging the midbody ring components revealed a similar pattern. As the contractile ring completed its constriction, both Myosin II<sup>NMY-2</sup>-GFP and GFP–CYK-7 remained concentrated in a tight focus in the center of the cell–cell boundary (Fig. 1 C, yellow arrows; Fig. S1 C; and Video 4). Beginning at anaphase of the second division, tiny fragments of both markers were shed coincident with fragments containing the plasma membrane marker (Fig. 1 C, white arrowheads). When the midbody was released, a large mass of both midbody ring markers was also released from the cell–cell boundary (Figs. 1 C, green arrows; Fig. S1 C; and Video 4). Tracking of the released midbody through the four-cell stage revealed that it was always retained in the EMS cell (Fig. S1 D). Although fragments staining for the plasma membrane marker, which binds phosphatidylinositol 4,5-bisphosphate, were associated with the midbody, the released midbody was not encased in this marker. Thus, the released midbody is either not encased in plasma membrane or the phosphatidylinositol 4,5-bisphosphate associated with the membrane surrounding the midbody is lost during release.

These results suggest that abscission in the early C. elegans embryo occurs in two stages (Fig. 2 F). During the first stage, the cytoplasm in the two daughter cells becomes diffusely isolated, coincident with the completion of furrowing and formation of the intercellular bridge. During the second stage, which begins during anaphase of the subsequent cell division, the cortex surrounding the midbody is remodeled, releasing fragments containing plasma membrane and midbody ring markers. Remodeling culminates, ~200 s later, in the release of the midbody and midbody ring into the posterior cell.

The ESCRT machinery is required for midbody/midbody ring release

Midbody/midbody ring release could be mediated by ESCRT-dependent membrane scission (Henne et al., 2011; Agromayor and Martin-Serrano, 2013; McCullough et al., 2013). To investigate ESCRT function in the C. elegans embryo, we first localized the four-protein ESCRT-I complex by immunofluorescence against ESCRT-I<sup>TSG-101</sup> and time-lapse imaging of a GFP fusion with ESCRT-I<sup>MVB-12</sup>. ESCRT-I<sup>TSG-101</sup> colocalized with MKLP1<sup>ZEN-4</sup> to the midbody at the two-cell stage (Fig. 2 A). GFP–ESCRT-I<sup>MVB-12</sup> was first detected at the midbody ~400 s after furrow initiation; levels subsequently increased (not depicted), and GFP–ESCRT-I<sup>MVB-12</sup> was released with other midbody/midbody ring components after anaphase of the second division (Fig. 2 B and Video 5). To analyze ESCRT function, we depleted the ESCRT-I component TSG-101 because of its essential non-redundant role in membrane scission during cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007). ESCRT-I<sup>TSG-101</sup> depletion did not affect the timing of cytoplasmic isolation as assessed by monitoring photoactivated dextran diffusion (Fig. 2 C). During the second stage of abscission, fragments containing the plasma membrane marker and midbody ring markers were shed in ESCRT-I<sup>P<sup>101</sup>(RNAi)</sup> embryos (Fig. 2 E and Fig. S2) with timing similar to that in controls (onset = 9.5 ± 28 s in ESCRT-I<sup>P<sup>101</sup>(RNAi)</sup> embryos [n = 20] compared with 24 ± 41 s in control embryos [n = 66]). Strikingly, all four midbody/midbody ring components failed to release in the majority of ESCRT-I<sup>P<sup>101</sup>(RNAi)</sup> embryos (Fig. 2, D and E, yellow arrows; Fig. S2; and Video 6) and instead remained at the cell–cell interface even when monitored beyond the four-cell stage (Fig. 2, D and E). Consistent with previous work in C. elegans (Audhya et al., 2007; Michelelet et al., 2009), furrows were not observed to open back up in ESCRT-inhibited embryos. We conclude that the ESCRT machinery is required to release the midbody/midbody ring from the cell–cell boundary but not for cytoplasmic isolation or to maintain a closed connection between the daughter cells.

**PRC1<sup>SPD-1</sup> depletion prevents the formation of midbody microtubule bundles and Aurora B<sup>AIR-2</sup> targeting to the intercellular bridge**

To examine the role of midbody microtubules in abscission, we depleted the microtubule-bundling protein PRC1<sup>SPD-1</sup>. As in other systems (Mollinari et al., 2002, 2005; Vermi et al., 2004; D’Avino et al., 2007), PRC1<sup>SPD-1</sup> inhibition in the C. elegans embryo prevents the formation of the microtubule bundles that make up the central spindle and blocks midbody assembly (Verbrugge and White, 2004). We confirmed loss of midbody of GFP–ESCRT-I<sup>MVB-12</sup> before and after release from the cell–cell boundary, respectively. (C) Example of an ESCRT-I<sup>P<sup>101</sup>(RNAi)</sup> embryo in which a 10-kD dextran probe was photoactivated after apparent closure ([n = 4 embryos]). Central plane images show the embryo before activation (~5 s), immediately after activation (~5 s), and 140 s after activation (140 s). A kymograph was constructed by aligning strips (narrow rectangle) from images collected at 5-s intervals. Red arrow denotes the point of photoactivation. (D and E, top) Central plane confocal images of ESCRT-I<sup>P<sup>101</sup>(RNAi)</sup> embryos expressing a fluorescently tagged plasma membrane probe along with the midbody marker mCherry–MKlp1<sup>ZEN-4</sup> ([D; n = 10 embryos] or the midbody ring marker GFP–CYK-7 ([E; n = 6 embryos]). Times are relative to anaphase of the second division. Released fragments marked with the plasma membrane probe (white arrowheads) and the mCherry–MKlp1<sup>ZEN-4</sup>-marked or GFP–CYK-7–marked midbody remnants are indicated (yellow arrows). Asterisks mark the new midbody/midbody rings arising from the second embryonic division. (bottom) Graphs plotting the times when the mCherry–MKlp1<sup>ZEN-4</sup>-marked midbodies or GFP–CYK-7–marked midbody rings were released in control and ESCRT-P<sup>101</sup>(RNAi) embryos. In cases in which the midbody/midbody ring was not released, the data point reflects the endpoint of the time-lapse sequence. (F) Timeline summarizes the key events during contractile ring constriction (light gray) and abscission (dark gray). MTs, microtubules. White boxes on the low magnification images in A, B, D, and E mark the location of the region shown at higher magnification in the adjacent images. Bars, 5 μm.
Figure 3. PRC1<sup>Spd-1</sup> depletion prevents the formation of midbody microtubule bundles and Aurora B<sup>AIR-2</sup> targeting to the intercellular bridge. (A) Central plane confocal images of control (top; n = 8 embryos) and PRC1<sup>Spd-1</sup> (RNAi) (bottom; n = 9) embryos expressing GFP-β-tubulin and mCherry-histone. Kymographs of the GFP-β-tubulin signal in the midbody region are also shown. Times are seconds after furrow initiation. (B) Central plane confocal images
microtubules by imaging control and $PRC_{1}^{nd-1}(RNAi)$ embryos expressing GFP–β-tubulin and mCherry::histone. In control embryos, bundled microtubules in the central spindle compacted to form the midbody, which could be monitored for >400 s after furrow initiation. After this point, which corresponds to the onset of mitosis of the second cell division, midbody microtubules appeared to dissipate, suggesting that relatively few microtubules span the intracellular bridge at the time of midbody release in control C. elegans embryos. In contrast, no central spindle or midbody microtubules were detected at any stage in $PRC_{1}^{nd-1}(RNAi)$ embryos (Fig. 3 A and Video 7). Consistent with the absence of midbody microtubules, as the furrow closed during the first division, we could not detect any focus of GFP–Aurora B$^{BR-2}$ or mCherry-Mklp1$^{ZEN-4}$ embedded within the cell–cell boundary in $PRC_{1}^{nd-1}(RNAi)$ embryos (Fig. 3, B and C). Although mCherry-Mklp1$^{ZEN-4}$ did not localize to the cell–cell boundary in $PRC_{1}^{nd-1}(RNAi)$ embryos, a population of mCherry-Mklp1$^{ZEN-4}$ was subsequently recruited to the midbody ring, becoming detectable ~500–600 s after furrow initiation (Fig. 3 C). In control embryos, the amount of mCherry-Mklp1$^{ZEN-4}$ in the focus at the cell–cell boundary also increased over time (Fig. 3 C). In fixed abscission stage embryos, the freeze-crack fixation procedure occasionally causes the midbody ring to release from the midbody; in such embryos, Mklp1$^{ZEN-4}$ localized to both the midbody and with Myosin II$^{NMY-2}$ to the midbody ring (Fig. 3 D). These findings are consistent with work in vertebrate cells, suggesting that Mklp1 transitions from the midbody to the midbody ring as the intercellular bridge matures (Elia et al., 2011; Hu et al., 2012). To further confirm the loss of midbody microtubules in $PRC_{1}^{nd-1}(RNAi)$ embryos, we performed immunofluorescence in fixed embryos using Mklp1$^{ZEN-4}$ as a marker for the location of the midbody ring. Whereas an intense microtubule bundle passed through the Mklp1$^{ZEN-4}$-marked midbody ring in interphase two-cell stage control embryos, no tubulin fluorescence above background was detected passing through the Mklp1$^{ZEN-4}$, marked midbody ring in $PRC_{1}^{nd-1}(RNAi)$ embryos (Fig. 3 E). We conclude that there are no detectable microtubule bundles passing through the intercellular bridge in $PRC_{1}^{nd-1}(RNAi)$ embryos. Our results further suggest that Mklp1$^{ZEN-4}$ is a component of the midbody ring as well as the midbody and can be directly recruited to the midbody ring independent of midbody microtubules.

**Furrow ingression and cytoplasmic isolation occur with normal timing in the absence of midbody microtubules**

Next, we monitored abscission in $PRC_{1}^{nd-1}(RNAi)$ embryos. The kinetics of contractile ring closure in $PRC_{1}^{nd-1}(RNAi)$ embryos were similar to those in controls, and apparent closure of the hole between the daughter cells occurred at a similar time point (Fig. 4 A). Monitoring of the contractile/midbody ring components Myosin II$^{NMY-2}$–GFP, GFP-Septin$^{UNC-59}$, and GFP–CYK-7 revealed that despite the absence of the midzone/midbody, the contractile ring closed and was converted into a midbody ring embedded in the cell–cell boundary with normal kinetics in $PRC_{1}^{nd-1}(RNAi)$ embryos (Fig. 4, B and C; and Fig. 5 B).

To determine whether midbody microtubules affect cytoplasmic isolation, we compared the diffusion of photoactivated 10-kD dextran probe at different time points after furrow initiation in control and $PRC_{1}^{nd-1}(RNAi)$ embryos. To quantitatively compare diffusion across the division plane at different times during furrow ingression, we calculated the normalized intensity difference (NID) between the activated and unactivated halves of the embryo for each time point after photoactivation. Plotting the initial slope of the NID, which reflects the rate of probe diffusion across the division plane (Fig. 3 D), versus time revealed that the rate of diffusion across the division plane decreased with similar kinetics in control and $PRC_{1}^{nd-1}(RNAi)$ embryos (Fig. 3 E). In both cases, the rate progressively dropped until it reached 0 ~250 s after furrow initiation; the point at which diffusion stopped was reached slightly sooner (~30 s) in $PRC_{1}^{nd-1}(RNAi)$ embryos than in controls. We conclude that midbody microtubules are not required for cytoplasmic isolation.

**Midbody microtubules are not required for membrane shedding, ESCRT recruitment, or midbody ring release**

Next, we determined whether the absence of midbody microtubules affected membrane shedding or the fate of the midbody ring embedded in the cell–cell boundary. Imaging embryos coexpressing a fluorescent membrane probe with the midbody and midbody ring components revealed that $PRC_{1}^{nd-1}(RNAi)$ embryos shed fragments containing the plasma membrane marker and midbody ring markers at a time equivalent to that in controls (Fig. 5, A and C; and Video 8). The midbody ring markers Myosin II$^{NMY-2}$–GFP, GFP-Septin$^{UNC-59}$, and GFP–CYK-7 were embedded in the cell–cell boundary in the $PRC_{1}^{nd-1}(RNAi)$ embryos, and this focus was released into the posterior cell with normal timing (Fig. 5, B and C; and Video 8); Mklp1$^{ZEN-4}$ recruited to the midbody ring was also released into the posterior cell (Fig. S3).
The septins are required for timely cytoplasmic isolation and for release of the midbody and midbody ring.

The septins are midbody ring components across metazoans and have been shown to be important for abscission in vertebrate and Drosophila S2 cells (Estey et al., 2010; Kechad et al., 2012). In contrast to the combinatorial complexity of heterooligomeric septin complexes in humans, which have 13 different septins (Hall and Russell, 2012), C. elegans has only two septins (UNC-59 and UNC-61), and depletion of either is sufficient to disrupt septin recruitment to the contractile ring (Nguyen et al., 2000; John et al., 2007). To examine the effects of septin inhibition, we depleted the septin UNC-59. Consistent with the fact that the midbody ring is released normally in PRC1 spd-1 (RNAi) embryos suggests that ESCRT can be directly recruited to the midbody ring. Consistent with this expectation, time-lapse imaging revealed recruitment and release of ESCRT-I (MVB-12) from the cell–cell boundary (Fig. 4 D).

In summary, we conclude that despite the central role proposed for the midbody in orchestrating abscission, all the events we can monitor, including cytoplasmic isolation, ESCRT recruitment, membrane shedding, and release of the midbody ring into the posterior cell, succeed in the absence of midbody microtubules. These findings suggest that the midbody ring may play a greater role in scaffolding abscission than previously appreciated.
Figure 5. **Midbody microtubules are not required for membrane shedding, ESCRT recruitment, or midbody ring release.** (A) Central plane confocal images showing membrane shedding (white arrowheads) at the cell–cell boundary in a \( PRC_1^{spd-1}(RNAi) \) embryo (\( n = 9 \) embryos) expressing an mCherry-tagged plasma membrane probe and GFP–Aurora B\(^{AIR-2} \). Times are relative to anaphase of the second division. (B) Central plane confocal images showing midbody ring release in a \( PRC_1^{spd-1}(RNAi) \) embryo expressing the midbody ring markers Myosin II \(^{NMY-2} \)–GFP (\( n = 8 \) embryos), GFP–CYK-7 (\( n = 10 \) embryos), or GFP–septin \(^{UNC-59} \) (\( n = 5 \) embryos) along with the mCherry-tagged plasma membrane probe and mCherry-histone. Times are relative to anaphase of the second division. Midbody rings are highlighted before (yellow arrows) and after (green arrows) release from the cell–cell junction. (C) Graphs plotting the mean onset of membrane shedding (top) and midbody ring release (bottom) for control and \( PRC_1^{spd-1}(RNAi) \) embryos. Error bars are the SDs. (D) Central plane confocal images of control (\( n = 6 \) embryos) and \( PRC_1^{spd-1}(RNAi) \) (\( n = 7 \) embryos) embryos expressing GFP–ESCRT-I\(^{MVB-12} \). Times are relative to anaphase of the second division. Dashed yellow lines mark the cell boundaries. Images are scaled equivalently. White boxes on the low magnification images in A and B mark the location of the region shown at higher magnification in the adjacent images. Bars, 5 μm.
Figure 6. The septins are required for timely cytoplasmic isolation and for midbody release. (A) Graph plotting the mean initial slope of the NID versus time in seconds after furrow initiation for control and septin<sup>unc-59(RNAi)</sup> embryos. Error bars are the 90% confidence interval; mean n = 10 slope measurements per time point. (B and C, top) Central plane confocal images of control and septin<sup>unc-59(RNAi)</sup> embryos expressing a fluorescently tagged plasma membrane probe and the midbody markers mCherry-Mklp1<sup>ZEN-4</sup> (B; n = 11 embryos) or GFP-CYK-7 (C; n = 11 embryos). Times are relative to anaphase of the second division. Released fragments marked with the plasma membrane probe are indicated (white arrowheads). Arrows point to the midbody/midbody ring from the first division, which is released in control embryos (green arrows) and fails to be released in septin<sup>unc-59(RNAi)</sup> embryos (yellow arrows). Asterisks mark the tip of the ingressing furrow from the second embryonic division. (bottom) Graphs plotting the times when the mCherry-Mklp1<sup>ZEN-4</sup>-marked midbodies or GFP-CYK-7–marked midbody rings were released. In cases in which the midbody/midbody ring was not released, the data point refers to the endpoint of the time-lapse sequence. (D) The central region of confocal images of control (n = 11) and septin<sup>unc-59(RNAi)</sup> (n = 10) embryos expressing the mCherry-tagged plasma membrane probe and GFP–Aurora B<sup>AIR-2</sup>. (E) Confocal images of septin<sup>unc-59(RNAi)</sup> (n = 6 embryos) embryos expressing GFP–ESCRT-I<sup>MVB-12</sup>. Times in D and E are relative to anaphase of the second division. Dashed yellow lines mark the cell boundaries. Bars, 5 μm.
previous work (Maddox et al., 2007), the furrow closed with similar kinetics to controls until the very end, when septin\textsuperscript{unc-59} (RNAi) embryos persisted longer (~40 s) with a small hole between the daughter cells (Fig. S4 A). To determine how septin depletion affects the timing of cytoplasmic isolation, we used the approach described in Fig. 4 D to monitor the diffusion of photoactivated 10-kD dextran across the division plane. Plotting the mean initial slope of the NID versus time revealed that the rate of diffusion across the division plane decreases with similar kinetics for the first 250 s after furrow initiation. However, at this point, the curves diverge, and the septin\textsuperscript{unc-59} (RNAi) embryos remain diffusionally connected, with a small open channel between the daughter cells, for ~140 s longer than controls (Fig. 6 A). We conclude that cytoplasmic isolation is substantially delayed by septin depletion.

Cytoplasmic isolation normally occurs as the contractile ring envelops the midbody to form the intercellular bridge. The substantial delay in cytoplasmic isolation in the septin\textsuperscript{unc-59} (RNAi) embryos suggested that this process was not occurring normally. Consistent with this idea, examination of mCherry-Mkp1\textsuperscript{ZEN-4} and GFP–CYK-7 revealed that the midbody and midbody ring in septin\textsuperscript{unc-59} (RNAi) embryos protruded out toward the cytoplasm in the posterior cell at the two-cell stage (Fig. 6, B and C, top). In contrast to control embryos, in which both mCherry-Mkp1\textsuperscript{ZEN-4} and GFP–Aurora B\textsuperscript{AIR-2} remained at the cell–cell junction until midbody release, GFP–Aurora B\textsuperscript{AIR-2} gradually disappeared from the cell–cell junction in septin\textsuperscript{unc-59} (RNAi) two-cell embryos coincident with entry of the daughter cells into mitosis (~500 s before anaphase of the second division; Fig. 6 D). One possibility, suggested by the fact that midbody microtubules become difficult to detect during this time frame in control embryos, is that Aurora B\textsuperscript{AIR-2} transitions from midbody microtubules to a septin-dependent localization on the midbody ring coincident with mitotic entry. Shedding of plasma membrane and midbody ring markers was observed in septin\textsuperscript{unc-59} (RNAi) embryos coincident with anaphase of the second division (onset = −19 ± 39 s in septin\textsuperscript{unc-59} (RNAi) embryos [n = 20] compared with 24 ± 41 s in control embryos [n = 66]). At the time when the midbody and midbody ring were released into the posterior cell in control embryos, a focus containing mCherry–MKLP-1\textsuperscript{ZEN-4}, Myosin II\textsuperscript{NMV-2}, GFP, and GFP–CYK-7 protruded into the posterior cell of septin\textsuperscript{unc-59} (RNAi) embryos but did not release (Figs. 6, B and C, yellow arrows; Fig. S4 B; and Video 9). After septin\textsuperscript{unc-59} (RNAi), GFP–ESCRT-I\textsuperscript{FEB-12} was recruited at levels comparable to controls (Fig. 6 E), but also failed to release, suggesting that the failure to release the midbody/midbody ring in septin\textsuperscript{unc-59} (RNAi) embryos was not caused by failure to recruit the ESCRT machinery. We conclude that inhibition of the septins delays cytoplasmic isolation and results in the formation of a defective intercellular bridge; the intercellular bridge permits ESCRT machinery recruitment but cannot support ESCRT-mediated midbody/midbody ring release into the posterior cell.

Septins and ESCRT function at different steps during abscission

Because both ESCRT and septin inhibitions result in failure of midbody/midbody ring release (Fig. 2, D and E; and Fig. 6, B and C), we performed a more careful comparison of these two conditions. In control embryos, expressing a GFP-tagged plasma membrane probe, the ingressing furrow enveloped the midbody, generating a smooth cell–cell boundary. In septin\textsuperscript{unc-59} (RNAi) embryos, envelopment of the midbody by the plasma membrane was delayed, but the boundary remained smooth (Fig. 6 A). In ESCRT-I\textsuperscript{FEB-12} (RNAi) embryos, the furrow enveloped the midbody with normal timing, consistent with our analysis indicating that cytoplasmic isolation occurs coincident with the completion of furrowing (Fig. 2 C); however, the intercellular bridge was often distended, suggesting the presence of an obstruction enveloped along with the midbody (Fig. 7 A and Video 10). An occlusion was also visible in the cell–cell boundary in differential interference contrast images of ESCRT-I\textsuperscript{FEB-12} (RNAi) embryos (Fig. 7 A). Given that we do not observe ESCRT-I on the midbody/midbody ring until after cytoplasmic isolation, we suspect that the obstruction is a consequence of the effect of ESCRT inhibition on the formation of multivesicular bodies (Henne et al., 2011; McCullough et al., 2013), rather than caused by its role in midbody/midbody release. The midbody release defect in septin\textsuperscript{unc-59} (RNAi) embryos also differed from that in ESCRT-I\textsuperscript{FEB-12} (RNAi) depleted embryos. In septin\textsuperscript{unc-59} (RNAi) depleted embryos, the midbody/midbody ring protruded into the posterior cell and did not appear to encase in plasma membrane marker (Fig. 7 B and Fig. 6, B and C). In contrast, in ESCRT-I\textsuperscript{FEB-12} (RNAi) depleted embryos, the midbody/midbody ring was encased in a ring of plasma membrane embedded in the cell–cell boundary (Fig. 7 B). These distinct defects suggest that the septins and the ESCRT machinery function at different points during abscission (Fig. 8 and accompanying text in the Discussion).

Discussion

Here, we use assays for cytoplasmic diffusion across the division plane and the fate of midbody and midbody ring components to analyze abscission in the early C. elegans embryo. Our results partition abscission into two distinct stages and define molecular components required for each stage (Fig. 8). Contrary to the proposal that abscission is orchestrated by the midbody, we show that all events during abscission that we can assay proceed normally in the absence of midbody microtubules. These results suggest that the midbody ring, rather than the midbody, orchestrates abscission in C. elegans.

Abscission occurs in two stages: cytoplasmic isolation and midbody/midbody ring release

Previous work in HeLa cells suggested that the cessation of diffusion between the daughter cells and ESCRT-mediated scission are coupled events (Steigemann et al., 2009; Guizetti et al., 2011). In contrast, our characterization partitions abscission into two temporally distinct stages with different molecular requirements. During the first stage (Fig. 8, early abscission), the cytoplasm in the daughter cells becomes diffusionally isolated, coincident with the formation of an intercellular bridge containing the midbody and midbody ring. This step requires the septins but is ESCRT independent. During the second stage...
abscission (Schiel and Prekeris, 2013) will be needed to determine how membrane shedding occurs and to assess its importance to abscission.

Our results show that the septins are required for release of the midbody/midbody ring as well as for timely cytoplasmic isolation. In septin-inhibited embryos, cytoplasmic isolation eventually occurs, but the midbody/midbody ring ends up in an aberrant configuration protruding into the posterior cell (Fig. 8). Thus, when the septins are absent, a septin-independent process is able to bring about cytoplasmic isolation, albeit in an aberrant way that does not result in the midbody/midbody ring being properly enveloped within the cell–cell boundary. In septin-depleted embryos, the ESCRT machinery is recruited, but the midbody/midbody ring fails to release. An appealing possibility is that shedding is the result of a septin- and ESCRT-independent membrane remodeling event that acts on the intercellular bridge in conjunction with the ESCRT machinery to release the midbody/midbody ring. Additional work targeting some of the membrane trafficking components implicated in (Fig. 8, late abscission), which occurs concurrent with the subsequent division of the daughter cells, the intercellular bridge is remodeled, as indicated by plasma membrane shedding, followed by the ESCRT-dependent release of the midbody/midbody ring. Remodeling of the intercellular bridge releases fragments containing plasma membrane and midbody ring markers (Fig. 8, late abscission–membrane shedding). Membrane shedding is observed after septin\(^{unc-59}\), PRC1\(^{p-1}\), and ESCRT-\(^{Isg-101}\) depletion. An attractive possibility based on these results is that shedding is the result of a septin- and ESCRT-independent membrane remodeling event that acts on the intercellular bridge in conjunction with the ESCRT machinery to release the midbody/midbody ring. Additional work targeting some of the membrane trafficking components implicated in abscission (Schiel and Prekeris, 2013) will be needed to determine how membrane shedding occurs and to assess its importance to abscission.

Our results show that the septins are required for release of the midbody/midbody ring as well as for timely cytoplasmic isolation. In septin-inhibited embryos, cytoplasmic isolation eventually occurs, but the midbody/midbody ring ends up in an aberrant configuration protruding into the posterior cell (Fig. 8). Thus, when the septins are absent, a septin-independent process is able to bring about cytoplasmic isolation, albeit in an aberrant way that does not result in the midbody/midbody ring being properly enveloped within the cell–cell boundary. In septin-depleted embryos, the ESCRT machinery is recruited, but the midbody/midbody ring fails to release. An appealing possibility is that release fails because
Scaffolding of abscission by the midbody ring  •  Green et al.

Remarkably, all of the events that we monitored during both stages of abscission, cytoplasmic isolation, shedding of membrane and midbody ring components, and midbody/midbody ring release, all succeeded when we prevented midbody assembly by inhibiting the microtubule-bundling protein PRC1/SPD-1. This result suggests that the midbody is not an essential component of the diffusion barrier between daughter cells; the midbody ring can close the hole between the daughter cells sufficiently to block cytoplasmic diffusion. Midbody microtubules are also proposed to scaffold the recruitment of membrane trafficking components that narrow the intercellular bridge before scission (Schiel and Prekeris, 2013) and of the ESCRT machinery to promote scission (Fededa and Gerlich, 2012; Agromayor and Martin-Serrano, 2013). Our results suggest that the midbody ring, in the absence of midbody microtubules, is sufficient to direct remodeling of the intercellular bridge.
bridge to allow it to attain a conformation conducive to cleavage
and to recruit the ESCRT machinery that releases the midbody/
midbody ring. Previous work in HeLa cells showed that PRC1^{Δ6-1} deple-
tion results in an abscission defect (Mollinari et al., 2005). These
authors reported that furrowing proceeded to completion,
and cells remained connected by an intercellular bridge for ~1 h,
the usual time between formation of the intercellular bridge but
in the execution of the final scission event (Mollinari et al.,
2005). Additional work using higher resolution assays for the
different steps in abscission will be needed to determine which
step in abscission is blocked in the absence of PRC1 and whether
remodeling of the intercellular bridge in human cells is affected
by the absence of midbody microtubules.

In human cells, centralspindlin recruits CEP55, which re-
cruits the ESCRT machinery to promote scission (Fabbro et al.,
2005; Zhao et al., 2006; Morita et al., 2007; Carlton et al., 2008;
Lee et al., 2008; Bastos and Barr, 2010). Because C. elegans (and Drosophila)
lack CEP55 homologues, the ESCRT machinery is likely recruited in
these systems either by direct binding to centralspindlin or an-
other midbody ring component. Direct binding to centralspindlin
is an attractive possibility, as centralspindlin is recruited directly
to the midbody ring with similar timing to the ESCRT complex
in PRC1^{Δ6-1}-depleted embryos (Fig. 3 C). As centralspindlin
also transitions from the midbody to the midbody ring in human
(Mollinari et al., 2005; Estey et al., 2010; Caballe and Martin-
Serrano, 2011). Consistent with this finding, cell–cell boundaries
have previously been shown to form and remain closed, even
in the presence of chromatin obstructions (Bembeneck et al., 2013).
The fact that we did not see regression of cell–cell boundaries
under any of the conditions we tested suggests that redundant
mechanisms are in place to ensure the successful formation of sta-
ble cell–cell boundaries in intact organisms. The nature of these
mechanisms will be an interesting area to explore in future work.

Abscission phase in vivo
Our work provides a detailed molecular analysis of abscission in
an organismal context. One of our most interesting findings is how
robust abscission is in the C. elegans embryo. Cell–cell bounda-
ries form and remain stably closed in cells lacking midbody micro-
tubules, depleted of the ESCRT machinery, and depleted of the
septins, all conditions previously shown to result in abscission failure and reopening of the intercellular bridge in vertebrate cells
(Mollinari et al., 2005; Estey et al., 2010; Caballe and Martin-
Serrano, 2011). Consistent with this finding, cell–cell boundaries
have previously been shown to form and remain closed, even
in the presence of chromatin obstructions (Bembeneck et al., 2013).

Materials and methods

C. elegans strains
C. elegans strains (genotypes listed in Table S1) were maintained at 20°C. As the gfp::air-2 transgene in OD448 is subject to silencing, experiments
with this line were performed by singling adult OD448 hermaphrodites after confirming that they were positive for the GFP::AIR-2 marker and using L4 progeny from these animals for experiments. GFP::AIR-2 expression was verified in the pronuclei of dissected embryos before midbody filming.

RNAi
Double-stranded RNAs (dsRNAs) were prepared by using oligonucleotides (oligos) containing T3 or T7 promoters (listed in Table S2) to amplify regions from genomic N2 DNA. PCR reactions were cleaned (QIAGEN) and used as templates for T3 and T7 transcription reactions (25 µl; Ambion), which were cleaned using a MEGAClear kit (Ambion) and combined. RNA eluted with 50 µl H2O was mixed with 3X injection buffer (25 µl; 1 x 20 mM KPO4, pH 7.5, 3 mM K-Citrate, pH 7.5, and 2% PEG 6000) and annealed by heating at 65°C for 10 min followed by 27°C for 30 min. dsRNA
was injected into L4 hermaphrodite worms 48 h before imaging. For photo-
activation experiments, the gonaids of dsRNA-injected worms were rein-
jected with CMN2B2-caged carboxy-G-9dohamine dextran (Invitrogen
and Molecular Probes) 43 h later (5 h before imaging).

Photoactivation experiments
Adult OD58 (Audhya et al., 2005) hermaphrodites, either injected 43 h
previously with dsRNA or uninjected controls, were injected in both go-
as with a 10,000 molecular weight dextran conjugate of CMN2B2-
caged carboxy-G-9dohamine dissolved in injection buffer (20 mM KPO4,
pH 7.5, and 2% PEG 6000) and annealed by heating at 65°C for 10 min followed by 27°C for 30 min. Worms were injected into L4 hermaphrodite worms 48 h before imaging. For photo-
activation experiments, the gonaids of dsRNA-injected worms were rein-
jected with CMN2B2-caged carboxy-G-9dohamine dextran (Invitrogen
and Molecular Probes) 43 h later (5 h before imaging).
Plots of furrow diameter versus time
Plots of furrow diameter versus time (Fig. 1 A, Fig. 4 A, and Fig. S4 A) were generated by collecting 12-plane z series (2.5-µm intervals) at 20-s time intervals of embryos from the strain OD58 during the course of the first embryonic division on a deconvolution microscope (DeltaVision) equipped with a CCD camera [CoolSNAP]. DeltaVision software was used to rotate the data from the central region of the embryo containing the contractile ring by 90° and generate a maximum intensity projection and to measure the diameter of the hole at each time point.

Midbody/midbody ring release experiments
One-cell stage embryos were dissected from gravid adult hermaphrodites from the strains OD449, OD138, OD1268, OD448, OD178, OD868, and OD579 in M9 medium [Brenner, 1974] and were mounted for imaging by transferring them to a 2% agarose pad with a mouth pipette and covering them with a 18 × 18-mm coverslip. Images were acquired with either (a) an confocal system (Revolution XD; Andor Technology) with a confocal scanner unit (CSU-10; Yokogawa Corporation of America) mounted on an inverted microscope (TE2000-E; Nikon) equipped with a 60×, 1.4 NA Plan Apochromat oil objective lens or (b) a microscope (Axio Observer.Z1; Carl Zeiss) equipped with a CCD camera (ORCA-ER; Hamamatsu Photonics) with 2 × 2 pixel binning. Embryos were imaged every 20 s in a single image plane, manually adjusting to keep the midbody in focus, until ~800 s after furrow initiation. To track the dynamics of the midbody before and at midbody release, an 8 × 1–µm z series was then collected every 20 s until the embryos reached the four-cell stage. Single planes containing the midbody remnant were identified and stitched together to generate movies using MetaMorph software. Live imaging of microtubules [Fig. 3 A] was performed by acquiring a central eight-plane z series (1-µm steps) at 20-s time intervals throughout the first and second division; a single image plane, containing microtubules, at each time point was selected and stitched together to track changes at the midbody. Timing of membrane shedding onset and midbody/midbody ring release was gauged relative to (a) furrow initiation, which we define as the first dimpling of the plasma membrane, or (b) anaphase of the second division in the anterior (AB) cell, which was scored on the basis of the distinct round to squarelike shape change in the plasma membrane boundary.

Immunofluorescence
Immunofluorescence was performed by dissecting 5–10 worms with a scalpel in a 2.2-µl drop of water on a slide coated with 1 mg/ml polylysine (P1524, Sigma-Aldrich). Coated slides were dried on a hot plate and baked for 30 min in a 100°C oven before use. Dissected worms were covered with a 10-mm square coverslip, and the slide was immersed in liquid nitrogen. For storage, embryos were dried with a Kimwipe (Kimberly-Clark), avoiding the region with the adhered cells, and OD297 in M9 medium before storing at 20°C. After 20 min, the slides were transferred to a 100°C oven before use. Dissected worms were covered with a 18 × 18–mm coverslip. Images were acquired with either (a) an Olympus 60×, 1.4 NA Plan Apochromat oil objective lens (Olympus) mounted on a DeltaVision system that included a microscope (BX70; Olympus) equipped with a camera (CoolSNAP CCD). Images in Fig. 2 A were acquired on a sweep field confocal microscope (TiE; Nikon) equipped with a CCD camera (CoolSNAP H2Q) using a Nikon 60×, 1.4 NA Plan Apochromat oil objective lens. Acquisition parameters were controlled by Elements software [Nikon]. For embryo immunofluorescence, ~100 z sections at 0.2-µm steps were acquired, three-dimensional datasets were computationally deconvolved using softWoRx software [Applied Precision], and relevant sections were used to generate maximum intensity projections. Line scans (20 × 70 pixels; Fig. 3 E) were drawn across the embryo center, and mean intensity values for each pixel position were recorded using MetaMorph software. Pixel intensities were normalized by dividing by the mean fluorescence intensity in the cytoplasm near the cell periphery (mean of the first and last 10 pixel values along the trace) before plotting intensity versus line scan position.

Online supplemental material
Fig. S1, related to Fig. 1, illustrates loading of embryos with dextran, GFP–Aurora B8, and GFP–CYK-7 in control embryos and that the first division midbody/midbody ring ends up in the EMS cell. Fig. S2, related to Fig. 2, shows GFP–Aurora B8 and Myosin 68021/GFP in ESCRT-I/II/III (RNAi) embryos. Fig. S3, related to Fig. 5, shows release of MKLP17244 in control and PRC17 (RNAi) embryos. Fig. S4 shows furrow diameter versus time in septin53/54 (RNAi) embryos, Myosin 68021/GFP in septin53/54 (RNAi) embryos, and persistent diffusion of photoactivated probe in a septin53/54 (RNAi) embryo. Table S1 shows the staging table used in this study. Table S2 shows oligos used for dsRNA production. Video 1, related to Fig. 1 B, shows dextran photoactivation before cytokinesis onset and after apparent closure. Videos 2, 3, and 4, related to Fig. 1, show MKLP17244 (Video 2), Aurora B8, and CYK-7 (Video 4) during abscission. Video 5, related to Fig. 2, shows GFP–ESCRT-61 (Video 5) during abscission. Video 6, related to Fig. 2, shows Aurora B8 and CYK-7 in ESCRT-I/II/III (RNAi) embryos. Video 7, related to Fig. 3, shows chromosomes and spindle microtubules in control and PRC17 (RNAi) embryos. Video 8, related to Fig. 5, shows Aurora B8 and CYK-7 in PRC17 (RNAi) embryos. Video 9, related to Fig. 6, shows MKLP17244 and CYK-7 in septin53/54 (RNAi) embryos. Video 10, related to Fig. 7, shows that ESCRT-521 depletion leads to a distended intercellular bridge. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201306036/DC1.

We thank members of the Oegema and Desai laboratories for helpful suggestions.

R. Green was supported by a fellowship from the American Cancer Society (PF-06-254-01-CCG). K. Oegema and A. Desai receive salary and additional support from the Ludwig Institute for Cancer Research. J. Audhya is funded by National Institutes of Health (GM088151).

Submitted: 7 June 2013
Accepted: 9 October 2013

References


