Wnt5a signaling controls cytokinesis by correctly positioning ESCRT-III at the midbody

Katsumi Fumoto*, Koji Kikuchi†, Hidetoshi Gon and Akira Kikuchi§

Department of Molecular Biology and Biochemistry, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita 565-0871, Japan

*These authors contributed equally to this work
†Present address: Department of Molecular Pharmacology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
§Author for correspondence (akikuchi@molbiobc.med.osaka-u.ac.jp)

Summary

Wnts activate at least two signaling pathways, the β-catenin-dependent and -independent pathways. Although the β-catenin-dependent pathway is known to contribute to G1–S transition, involvement of the β-catenin-independent pathway in cell cycle regulation remains unclear. Here, we show that Wnt5a signaling, which activates the β-catenin-independent pathway, is required for cytokinesis. Dishevelled 2 (Dvl2), a mediator of Wnt signaling pathways, was localized to the midbody during cytokinesis. Beside the localization of Dvl2, Fz2, a Wnt receptor, was detected in the midbody with the endosomal sorting complex required for transport III (ESCRT-III) subunit, CHMP4B. Depletion of Wnt5a, its receptors, and Dvl increased multinucleation. The phenotype observed in Wnt5a-depleted cells was rescued by the addition of purified Wnt5a but not Wnt3a, which is a ligand for the β-catenin-dependent pathway. Moreover, depletion of Wnt5a signaling caused loss of stabilized microtubules and mislocalization of CHMP4B at the midbody, which affected abscission. Inhibition of the stabilization of microtubules at the midbody led to the mislocalization of CHMP4B, while depletion of CHMP4B did not affect the stabilization of microtubules, suggesting that the correct localization of CHMP4B depends on microtubules. Fz2 was localized to the midbody in a Rab11-dependent manner, probably along stabilized microtubules. Fz2 formed a complex with CHMP4B upon Wnt5a stimulation and was required for proper localization of CHMP4B at the midbody, while CHMP4B was not necessary for the localization of Fz2. These results suggest that Wnt5a signaling positions ESCRT-III in the midbody properly for abscission by stabilizing midbody microtubules.

Key words: Wnt5a, Cytokinesis, Frizzled, ESCRT, Midbody

Introduction

Wnt is a secreted ligand that regulates various cellular functions, including proliferation, differentiation, migration, and adhesion (Logan and Nusse, 2004). There are 19 Wnt members in the human and mouse genomes, and these Wnts activate at least two signaling pathways, the β-catenin-dependent and -independent pathways (Kikuchi et al., 2009; Sato et al., 2010). The β-catenin-dependent pathway contributes to G1–S transition by expressing cyclin D1 and c-Myc in a β-catenin/T cell factor (Tcf)-dependent manner, and abnormal accumulation of β-catenin causes tumorigenesis by disrupting cell cycle control (Kikuchi et al., 2009; MacDonald et al., 2009). In addition, it has been reported that low-density-lipoprotein receptor-related protein 6 (LRP6), which is a co-receptor for the β-catenin-dependent pathway, is efficiently phosphorylated by a mitotic cyclin Y-dependent protein kinase, Pftk, and that β-catenin-dependent transcription is enhanced in mitosis (Davidson et al., 2009). Expression of conductin (also referred to as Axil and Axin2), which is a target gene of the β-catenin-dependent pathway, was regulated in a cell-cycle-dependent manner, with its highest expression level occurring during mitosis, and overexpression of conductin caused chromosomal instability in colon cancer cell lines (Hadjihannas et al., 2006). Dishevelled (Dvl), a cytoplasmic mediator of Wnt signaling pathways, as well as LRP6 and a Wnt receptor Frizzled 2 (Fz2), was demonstrated to be involved in mitotic spindle orientation (Kikuchi et al., 2010). These results suggest that the β-catenin-dependent pathway not only promotes G1–S progression but also regulates mitotic progression. On the other hand, it remains unclear whether the β-catenin-independent pathway functions in cell cycle regulation.

From anaphase to telophase, an actomyosin-based contractile ring in the midzone drives the ingress of the cleavage furrow between the two reforming nuclei, resulting in the formation of the midbody, which is a narrow anti-parallel microtubule-enriched intercellular bridge connecting the two daughter cells, during cytokinesis (Hu et al., 2012; Steigemann and Gerlich, 2009). The intercellular bridge persists until abscission splits the daughter cells apart. An endosomal sorting complex required for transport (ESCRT) has been implicated in diverse cellular processes, such as endosomal membrane sorting and cytokinesis (Peel et al., 2011). ESCRTs and vascular protein sorting 4 (Vps4) are sequentially localized to the midbody to mediate fission events (Wollert et al., 2009). During cytokinesis, ESCRT-I protein tumor-susceptibility gene 101 (Tsg101) and apoptosis-linked gene 2-interacting protein X (ALIX) interact with a non-canonical coiled-coil region of centrosomal protein of 55 kDa (Cep55) (Carlton and Martin-Serrano, 2007). Cep55 is localized to the center of the midbody where it binds to the centralspindlin subunit mitotic kinesin-like protein-1 (Mklp1) (Fabbro et al., 2005). The ESCRT-III subunit charged multivesicular body protein (CHMP) binds to the
microtubule-severing enzyme Spastin and accumulates on a region adjacent to Tsg101 to lead to complete abscission (Elia et al., 2011; Morita et al., 2007). Vps4 functions to dissociate ESCRT-III after membrane scission is completed (Hurley and Hanson, 2010). All of these ESCRTs and Vps4 are essential for cytokinesis, because their depletion leads to cytokinetic failure, which is assessed by an increase in multinucleated cells.

Progress in the understanding of the roles of ESCRTs in cytokinesis promoted us to analyze the relationship between Wnt signaling and ESCRTs during cytokinesis, because we previously found that Dvl localizes to the midbody (Kikuchi et al., 2010). The β-catenin-independent pathway was originally shown to regulate cytoskeletons, thereby coordinating cell motility and polarity (Veeman et al., 2003). Among 19 Wnt family members, Wnt5a is a representative ligand that activates this pathway. When Wnt5a binds to Fz and receptor tyrosine kinase-like orphan receptor 1/2 (Ror1/2), which function as co-receptors in the β-catenin-independent pathway, Dvl mediates Wnt5a signaling to regulate various cellular functions including adhesion, migration, invasion, and gene expression (Kikuchi et al., 2012). Extending our previous observation of the localization of Dvl2 to the midbody (Kikuchi et al., 2010), we found here that Fz2 is also localized to the midbody at telophase and that its localization is different from Dvl2 but similar to CHMP4B, an ESCRT-III subunit. Depletion of Wnt5a, Fz2, Ror2, or Dvl increased multinucleated cells and caused destabilization of midbody microtubules. These molecules were also required for the proper localization of CHMP4B at the midbody. In addition, Fz2 formed a complex with CHMP4B in a Wnt5a-independent manner. These results suggest that Wnt5a-mediated β-catenin-independent pathway is involved in the positioning of ESCRT-III in the midbody properly, thereby leading to abscission.

Results

Dvl2 is localized to the midbody at telophase

HA–Dvl2 and GFP–Dvl2 overexpressed in HeLaS3 and USOS cells, respectively, were concentrated to the center of the midbody during telophase (Kikuchi et al., 2010) (Fig. 1A; supplementary material Fig. S1A). Endogenous Dvl2 was also localized to the same region as ectopically expressed Dvl2 in HeLaS3 and a human breast carcinoma cell line, MDA-MB-435 cells (Fig. 1B; supplementary material Fig. S1A). The view of midbody morphology by electron microscopy reveals that anti-parallel microtubules are interdigitated and end at the center of the midbody, thereby creating the tight overlap region, where microtubule staining is blocked (Elad et al., 2011). This region is referred to as the stem body. Therefore, we used the term ‘stem body’ to indicate the central dark area of the midbody.

Molecules required for cytokinesis, such as motor proteins and microtubule associating proteins, are also accumulated at the midbody from telophase to abscission (Elad et al., 2011; Glotzer, 2009; Steigemann and Gerlich, 2009). Mklp1, a centralspindlin subunit, is present in the stem body, where it promotes midbody microtubule bundling (Sellitto and Kuriyama, 1988). In late telophase the localization of Mklp1 and GFP–Tsg101, an ESCRT-I subunit, became restricted to the stem body and they showed similar localization to GFP– or HA–Dvl2 (Fig. 1C). CHMP4B, an ESCRT-III subunit, accumulated on both sides of the stem body (Elia et al., 2011), and its localization was different from that of Dvl2 (Fig. 1C). GFP–Dvl2 at the intercellular bridge moved asymmetrically into one daughter cell after abscission (Fig. 1D) and was colocalized with mCherry-LC3 (supplementary material Fig. S1B). LC3 is known to be localized to the midbody during abscission and is required for lysosomal degradation of midbody ring after abscission (Pohl and Jentsch, 2009).

The accumulation of HA–Dvl2 to the stem body was reduced in Wnt5a-, Fz2-, or Ror2-depleted HeLaS3 cells but not in
LRP6-depleted cells (Fig. 2A,C; supplementary material Fig. S2A), while the expression of HA–Dvl2 was still observed in the cytosol of dividing Wnt5a-depleted cells as well as control cells (Fig. 2A). Endogenous Dvl2 localized to the midbody also decreased, whereas its total levels were not changed in Wnt5a-depleted cells as compared to control cells (Fig. 2B,D; supplementary material Fig. S2B). Therefore, it seems that a defect in the stem body localization of Dvl2 in Wnt5a- and its receptor-depleted cells does not reflect merely a failure of expression of Dvl2. The midbody localization of Dvl2 was restored by expression of mouse Wnt5a, which is resistant to siRNA for human Wnt5a, in Wnt5a-depleted cells (Fig. 2B,D; supplementary material Fig. S2C). These results suggest that Wnt5a signaling through Fz2 and Ror2 is required for the localization of Dvl2 to the stem body and is involved in cytokinesis.

**Fz2 is localized to a different region of the midbody from Dvl2**

Fz functions as a seven-transmembrane receptor for Wnt, and Fz2 has been shown to be involved in both the β-catenin-dependent and –independent pathways (Sato et al., 2010) (supplementary material Fig. S3A). While HA–Fz2 was localized to the cell surface membrane from prometaphase to anaphase, it accumulated at the midbody in early telophase (Fig. 3A; supplementary material Fig. S3B). At late telophase, FLAG– or HA–Fz2 was observed at both sides of the stem body clearly and showed the similar localization to CHMP4B (Fig. 3B; supplementary material Fig. S3B). However, FLAG–Fz2 showed localization different from Mklp1, GFP–Tsg101, and GFP–Dvl2 (Fig. 3B). FLAG–Fz2 was detected at the midbody when cells were stained with anti-FLAG-antibody before fixation and permeabilization (supplementary material Fig. S4A,B), indicating that the localization of Fz2 was not as a result of cytoplasmic aggregation by its overexpression but that Fz2 was present on the cell surface. Under the same staining conditions, FLAG–Dvl2 was not detected on the cell surface (supplementary material Fig. S4C). Receptor-tyrosine kinase-like orphan receptor 2 (Ror2), which is known as a receptor for Wnt5a (Green et al., 2008), was not clearly accumulated on the midbody but distributed throughout the cell cortex, including the intercellular bridge region (supplementary material Fig. S5).

Structured illumination microscopy (SIM) images into three dimensions (3D) revealed that HA–Dvl2 made web-like structures, extending into the interior of the stem body and that HA–Fz2 formed discontinuous ring structures along both sides of the stem body (Fig. 3C). Characterization of the organization of

---

**Fig. 2. Wnt5a signaling is required for localization of Dvl2 to the midbody.**

(A) HeLaS3 cells transiently expressing HA–Dvl2 were treated with siRNA for Wnt5a and the cells were stained for HA (green) and β-tubulin (red). siWnt5a-1 is an siRNA-directed against human and mouse Wnt5a. (B) Parental HeLaS3 were treated with siRNA for Wnt5a and the cells were stained for Dvl2 (green) and β-tubulin (green). For the rescue experiment, HeLaS3 cells stably expressing mouse Wnt5a were treated with siRNA for human Wnt5a. siWnt5a-2 is an siRNA against human Wnt5a only. (C,D) The percentage of cells in which HA–Dvl2 (A, n=50) or endogenous Dvl2 (B, n=50) accumulated at the stem body was quantified. The results are shown as means ± s.e.m. from three independent experiments. *P<0.01. Scrambled siRNA-transfected cells were used as a control. Inserts are enlarged views of the areas within the dashed lines. Scale bars: 10 μm (A,B); 5 μm (inserts).
ESCRTs by SIM showed that Cep55, which associates with microtubules, is observed as a solid-disk like structure in the midbody and that CHMP4B forms distinctive ring-like structures which associate with the cell surface membrane (Elia et al., 2011). The similarity in localization and structure between Fz2 and CHMP4B suggests their functional relationship.

Failure of Wnt5a signaling increases multinucleated cells

There are three Dvl homologs in mammals, Dvl1, Dvl2, and Dvl3 (Wharton, 2003) (hereafter referred to collectively as Dvls). To clarify the roles of Dvls in cytokinesis, the number of multinucleated cells, which are a hallmark of cytokinesis defects, was counted in Dvls-depleted cells. Because Dvls were required for prometaphase and metaphase progression (Kikuchi et al., 2010), only the multinucleated cells that have the same size of nuclei were counted to exclude ones induced by lagging chromosomes. Depletion of either Dvl1, Dvl2, or Dvl3 alone by two kinds of siRNA for each Dvl in HeLaS3 cells increased multinucleated cells (Fig. 4A; supplementary material Fig. S6A), and expression of GFP–mouse Dvl2 rescued the phenotype of Dvl2-depleted cells (Fig. 4A; supplementary material Fig. S6B). The extent of the phenotype induced by depletion of all Dvl1, Dvl2, and Dvl3 was similar to or stronger than that by the depletion of each Dvl (Fig. 4A). Therefore, HeLaS3 cells in which all Dvls were depleted were used in the following studies.

It was examined whether Wnts and their receptors are involved in cytokinesis. Depletion of Fz2 or Ror2 increased multinucleated cells but that of LRP6 did not (Fig. 4B). Depletion of Wnt5a in HeLaS3 cells also increased multinucleated cells, and purified Wnt5a rescued the phenotype in a dose-dependent manner, but purified Wnt3a, which is a representative ligand for the β-catenin-dependent pathway, did not (Fig. 4C), suggesting that
Wnt5a signaling is involved in cytokinesis specifically. Depletion of Wnt5a or Dvl s in mouse embryonic fibroblasts (MEFs) also increased multinucleated cells (Fig. 4D; supplementary material Fig. S6C), indicating that Wnt5a signaling is required for cytokinesis in non-cancer cells.

**Wnt5a and Dvl stabilize microtubules at the midbody**

Because Wnt5a and Dvl are involved in the stability and polarized organization of microtubules (Ciani et al., 2004; Matsumoto et al., 2010; Schlessinger et al., 2007), it was examined whether they have a role in microtubule organization at the midbody. In control cells, thick stabilized microtubule bundles that are heavily detyrosinated, which was detected by anti-Glu-tubulin antibody, were observed in the intercellular bridge (Fig. 5A,B). On the other hand, thin microtubules were observed and the staining of Glu-tubulin was reduced in Wnt5a- or Dvls-depleted cells (Fig. 5A,B). EB1 tends to be concentrated to the microtubule plus-ends and its staining at the cell periphery was reduced (Matsumoto et al., 2010) (supplementary material Fig. S7). During cytokinesis EB1 was concentrated at the midbody in control cells as described (Elad et al., 2011). In contrast, EB1 staining at the midbody was decreased in Wnt5a- or Dvls-depleted cells (Fig. 5A,C). These results suggest that the number of overlapping microtubules at the stem body is reduced in Wnt5a- or Dvls-depleted cells, resulting in decreased assembly of the midbody microtubules. There were more than 90% of cells with an intercellular bridge at shorter than 20 μm in control cells, while more than 50% of cells had the intercellular bridge at longer than 20 μm by depletion of Wnt5a or Dvls (Fig. 5D). Therefore, the length of the intercellular bridge was elongated in Wnt5a- or Dvls-depleted cells, suggesting that they failed to separate into two daughter cells because of the abscission defect.

Time-lapse imaging of GFP–β-tubulin-expressing HeLaS3 cells showed that the midbody appears to be formed normally both in control and Wnt5a-depleted cells. However, in Wnt5a-depleted cells midbody microtubule bundles became thinner in time, the intercellular bridge remained connected prior to completion of cytokinesis, and the length of microtubules was elongated consistently with the results from fixed cell assays (supplementary material Movies 1, 2). In addition, the duration time from telophase to abscission was delayed (Fig. 5E). Taken together, Wnt5a and Dvl could be required for the stabilization of midbody microtubules.

To examine whether microtubule stabilization is required for the localization of Dvl, EB1 was depleted to destabilize microtubules. The staining of microtubules and Glu-tubulin was indeed decreased in EB1-depleted cells (Fig. 5B). Consistent with the results, ~36% of cells had the intercellular bridge at longer than 20 μm by depletion of EB1 (Fig. 5D). In addition, the localization of HA–Dvl2 to the stem body was reduced in EB1-depleted cells (Fig. 5F). Therefore, the localization of Dvl and the stabilization of microtubules at the midbody might be mutually dependent.
Wnt5a signaling regulates the proper localization of ESCRT-III at the midbody

Midbody microtubules are assembled at the stem body, and some centrosomal proteins are translocated to the midbody on microtubules by kinesin family proteins to control abscission (Sagona et al., 2010). Because the loss of Wnt5a signaling perturbs the stabilization of midbody microtubules, it was examined whether Wnt5a signaling is required for the localization of cytokinesis regulating machineries, such as centralspindlin and ESCRTs. Depletion of Wnt5a or Dvl3 in HeLaS3 cells did not affect the localization of Mklp1 and GFP–Tsg101 to the stem body (Fig. 6A,B). Although CHMP4B–GFP was still located to the midbody in Wnt5a- or Dvl3-depleted HeLaS3 cells, the correct localization of CHMP4B–GFP on both sides of the stem body was disrupted (Fig. 7A,B). The same results were observed in Fz2- or Ror2-depleted but not in LRP6-depleted cells (Fig. 7B). Consistently, endogenous CHMP4B was localized to both sides of the stem body in control cells as well and its correct localization was impaired in Wnt5a- or Dvl3-depleted cells (Fig. 7C,D).

SIM images showed that endogenous CHMP4B is observed as double ring structures at the stem body in control cells (Fig. 7E). In contrast, CHMP4B was mislocalized and observed as dot-like structures in the stem body in Wnt5a- or Dvl3-depleted cells (Fig. 7E).

In contrast, CHMP4B was mislocalized and observed as dot-like structures in the stem body in Wnt5a- or Dvl3-depleted cells (Fig. 7E).

As shown in Fig. 5A, Wnt5a and Dvl were required for the stabilization of microtubules at the midbody. (A) Wnt5a- or Dvl3-depleted HeLaS3 cells were stained for Glu-tubulin (red), EB1 (green) and β-tubulin (gray). Since the fluorescence intensity of EB1 on the midbody was higher than that in cytoplasmic regions in control cells (see also C), cytoplasmic EB1 signals were almost invisible when images were taken under dynamic range. However, EB1 was actually on microtubule tips as shown in supplementary material Fig. S7. (B,C) Fluorescence intensity of Glu-tubulin (B) and EB1 (C) in the midbody of Wnt5a-, Dvl3- or EB1-depleted HeLaS3 cells (n=40) were measured and normalized to perinuclear fluorescence intensity. Fluorescence intensity is shown as arbitrary units. *P<0.001. (D) The length of the intercellular bridge in Wnt5a-, Dvl3- or EB1-depleted HeLaS3 cells (n=50) was measured and is shown as a frequency histogram. (E) Control or Wnt5a-depleted cells that transiently expressed GFP–α-tubulin were observed by time-lapse imaging from telophase to completion of abscission, at 5 min intervals. Duration was measured by monitoring GFP–α-tubulin (n=15). Black bars indicate the average duration. (F) Left panels: EB1-depleted HeLaS3 cells that transiently expressed HA–Dvl2 were stained for HA (red) and β-tubulin (green). Middle panel: lysates of EB1-depleted cells were probed with anti-EB1 and anti-β-tubulin antibodies. Right panel: the percentage of cells (n=50) in which transiently expressed Dvl2 was localized to the stem body was quantified. *P<0.01. Scrambled siRNA-transfected cells were used as a control. Scale bars: 10 μm (A,F).
phosphorylation of LRP6, resulting in activation of the β-catenin-dependent pathway that is required for G2/M transition (Davidson et al., 2009). We also reported that Fz2, LRP6, and Dvl2 are required for mitotic spindle orientation, which is an event in early mitotic phase (Kikuchi et al., 2010). The present study showed a novel function of Wnt5a signaling, which activates the β-catenin-independent pathway, in cytokinetic abscission by positioning ESCRT-III at the midbody through the stabilization of midbody microtubules. Our findings provide the first evidence that the Wnt5a-mediated β-catenin-independent pathway functions in later mitotic phases.

**Microtubule-dependent localization of Fz2 and Dvl2 to the midbody is regulated by Wnt5a**

Overexpressed and endogenous Dvl2 were localized to the stem body at telophase. In addition, Fz2 accumulated on the stem body at early telophase and relocated to both sides of the stem body at late telophase. We further observed the localization of Dvl and Fz2 by using SIM microscopy and demonstrated that they show unique structures in the midbody. Dvl was not merely accumulated to the interior of the midbody but made web-like structures. This structure might contribute to make open interphase to bind to other proteins that are involved in abscission. As previously reported, Dvl oligomerizes via its DIX domain (Kishida et al., 1999; Schwarz-Romond et al., 2007), and therefore it is intriguing to speculate that oligomerization might be required for the structure formation and efficient stabilization of midbody microtubules. SIM image also revealed that Fz2 forms discontinuous ring structures at late telophase. This structure strikingly resembles with CHMP4B at the midbody (Elia et al., 2011), and we demonstrated that the similarity in the localization of Fz2 and CHMP4B is related with their roles in cytokinesis (see below).

The localization of Dvl to the stem body was dependent on microtubules, because depletion of EB1 interfered with it. The localization of Fz2 to the stem body might be also dependent on microtubules, because it required Rab11, which is involved in microtubule-mediated trafficking of endosomal proteins to the midbody (see below). It has been demonstrated that Dvl is recruited to Fz2 on cell surface membranes in response to Wnt3a to activate the β-catenin-dependent pathway in interphase cells (Bilic et al., 2007). Because disruption of microtubules did not affect Wnt3a signaling (Sakane et al., 2010), it is unlikely that the binding of Dvl and Fz2 depends on microtubules in the activation of the β-catenin-dependent pathway. On the other hand, our data showed that Wnt5a, Fz2, and Ror2, but not LRP6, are required for the accumulation of Dvl2 to the stem body, suggesting that Wnt5a signaling positions Dvl2 to the midbody. Therefore, the trafficking of Dvl and Fz2, which depends on microtubules, might be specific in the Wnt5a-mediated β-catenin-independent pathway during cytokinesis.

Evidence has accumulated that Dvl stabilizes microtubules. Neurons from Dvl1 knockout mice developed a dendritic arbor of lesser complexity than neurons from wild-type mice (Rosso et al., 2005) and did not undergo growth cone remodeling to form synapses in response to Wnt probably through a failure of microtubule stabilization (Purro et al., 2008). Moreover, it was reported that Dvl inhibits GSK3β locally, resulting in changes in the phosphorylation levels of GSK3β targets, such as the microtubule-associated protein 1B, thereby regulating the stabilization of microtubules (Ciani et al., 2004) and that

**Discussion**

Emerging evidence suggests interesting roles for Wnt signaling in mitotic progression. Cycling-Y binds to and mediates the

![Fig. 6. Wnt5a signaling is not required for the localization of Mklp1 and Tsg101 to the midbody.](image)

(Fig. 8A,B; supplementary material Fig. S8A). Thick microtubule bundles stained with Glu-tubulin were observed in CHMP4B-depleted cells as well as control cells (supplementary material Fig. S8B), suggesting that the stabilization of microtubules does not require CHMP4B. It is known that vesicular internalization is re-activated in late mitosis for membrane proteins to be trafficked to the midbody (Schweitzer et al., 2005). The Rab11-mediated recycling pathway, which depends on microtubules, is shown to be involved in the midbody accumulation of endosomes (Wilson et al., 2005). The localization of FLAG–Fz2 to the midbody was indeed decreased in Rab11- but not Rab5-depleted cells (Fig. 8A,B; supplementary material Fig. S8A). The absence of FLAG–Fz2 signal did not simply reflect lack of its expression in Wnt5a-, Dvl1-depleted HeLaS3 cells transiently expressing GFP–Tsg101 were stained for GFP in mitotic progression. Cycling-Y binds to and mediates the

![Image](image)
the binding of Dvl to adenomatous polyposis coli gene product (APC) increases the number of microtubules at the cell cortex (Matsumoto et al., 2010). Consistent with these results, our results suggest that Wnt5a signaling through Fz2, Ror2, and Dvl may be involved in the stabilization and bundling of midbody microtubules, resulting in the positioning of Fz2 and Dvl to the midbody. Alternatively, Wnt5a signaling may regulate the localization of some microtubule-interacting proteins, including Cep55, protein regulator of cytokinesis 1 (Prc1), kinesin family member 4 (Kif4), and Mklp2, rather than the stabilization of midbody microtubules itself, because these proteins contribute to the stabilization of midbody microtubules in the process of the midbody assembly by accumulating at the midbody and relocating into different parts of the midbody. Taken together, Wnt5a signaling to position Fz2 and Dvl at the midbody and the stabilization of midbody microtubules might couple functionally to complete membrane scission.

**Similar spatial localization of Fz2 and CHMP4B at the midbody**

The ESCRT-III protein CHMP4B was shown to move dynamically during cytokinesis (Elia et al., 2011). At the initial stage of cytokinesis Cep55, Tsg101, and CHMP4B are assembled at the midbody. Then, CHMP4B relocates outward to the constriction zone by polymerizing into a spiral, leading to the separation of two cells. Our results showed that centralspindlin and ESCRTs were observed in the midbody in Wnt5a- or Dvl-depleted cells where the stabilization of microtubules is decreased. One possible scenario is that their localization is not solely dependent on microtubules or that thin midbody microtubules might be sufficient to assemble them at the stem body, because midbody microtubules still formed intercellular bridges in Wnt5a- or Dvl-depleted cells. However, depletion of Wnt5a or Dvl and destabilization of microtubules impaired the proper localization of CHMP4B to both sides of the stem body at late telophase, thereby causing the failure of cytokinetic abscission. Fz2 showed the similar localization to CHMP4B and both proteins form a complex in a Wnt5a-dependent manner, suggesting that Fz2 directly contributes to the proper localization of CHMP4B. Consistently, depletion of Fz2 impaired the proper localization of CHMP4B, but CHMP4B was not required for the localization of Fz2.

Fz2 was located to the cell surface membranes from prometaphase to anaphase, but it disappeared from there and accumulated on the midbody at early telophase. In late telophase, Fz2 as well as CHMP4B relocated to both sides of the stem body as well as CHMP4B. Vesicular internalization has been shown to be re-activated from anaphase for membrane proteins to be
trafficked to the midbody (Schweitzer et al., 2005). Rab11-containing endosomes have been shown to accumulate in the midbody and are required for cytokinesis (Wilson et al., 2005). The membrane trafficking by Rab11 in cytokinesis is dependent on microtubules through kinesin (Lin et al., 2002; Montagnac et al., 2008). Our results showed that depletion of Rab11 interferes with the midbody localization of Fz2. Therefore, it is intriguing to speculate that Fz2 is internalized in early telophase and accumulates on the midbody in a microtubule-dependent manner probably through Rab11.

It is important for abscission that CHMP4B concentrates on both sides of the stem body with appropriate timing. Wnt5a signaling through Fz2, Ror2, and Dvl might be required for the functional localization of ESCRT-III to both sides of the stem body, and especially Fz2 might have an important role in this event. As a novel function of Wnt5a-mediated β-catenin-independent signaling, it stabilizes midbody microtubules, thereby controlling cytokinesis.

Possible mechanism by which Wnt5a signaling regulates cytokinesis

Taken together, one possible model of Wnt5a-regulated cytokinesis is as follows (supplementary material Fig. S9). At early telophase Fz2 is accumulated to the stem body in a Rab11-dependent manner and Wnt5a signaling controls the accumulation of Dvl to the stem body. Accumulated Fz2 and Dvl2 at the midbody enhance the stabilization of midbody microtubules, which contributes to their efficient localization to the midbody. At late telophase Fz2 relocates to both sides of the stem body and Wnt5a signal strengthens the binding of Fz2 and CHMP4B, thereby positioning CHMP4B to both sides of the stem body to lead to complete abscission.

A recent study showed that a secreted extracellular matrix protein, hemicentin, regulates cytokinesis (Xu and Vogel, 2011), giving an example of the notion that a secreted ligand and its receptor-mediated signaling is involved in cytokinesis. Therefore, the present data may give another example of the involvement of secreted ligand-activated signaling in cytokinesis.

Materials and Methods

Materials and chemicals

pCS2/FLAG-Dvl2 and mCherry-LC3 were kindly provided by R. Habas (UMDNJ Robert Wood Johnson Medical School, Piscataway, NJ, USA) and T. Yoshimori (Osaka University, Osaka, Japan), respectively. pEGFP-N1/CHMP4B and pBjMyc/Tsg101 were from M. Maki (Nagoya University, Nagoya, Japan). pEGFP-C1/α-tubulin was from K. Kaibuchi (Nagoya University, Nagoya, Japan). pCS2/FLAG-Fz2 and pEGFPC1-Dvl2, pCGN/Dvl2 and pPGK-neo-Wnt5a were constructed as described (Kishida et al., 2007; Sato et al., 2010). Standard recombinant DNA techniques were used to construct pEGFP-C1/Tsg101 and pCGN-Fz2. GFP-Dvl2 and FLAG-Fz2 cDNAs were cloned into pLV SIN to construct lentiviral vectors (Takara Bio Inc., Shiga, Japan). The lentiviruses were produced in X293T cells by using Lenti-X™ Lentiviral Expression Systems (Takara Bio Inc., Shiga, Japan) according to manufacturer’s instructions.

Both Wnt3a and Wnt5a were purified to homogeneity as described previously (Kishida et al., 2004; Komekado et al., 2007; Kurayoshi et al., 2007). All of the primary antibodies used in this study are listed in supplementary material Table.
S1. Secondary antibodies coupled to horseshadish peroxidase (HRP) were purchased from Jackson ImmunoResearch Laboratories, Bar Harbor, ME, USA and secondary antibodies for immunofluorescence were from Invitrogen, Grand Island, NY, USA.

Cell culture and transfection

HeLaS3, U2OS, X293T, MEF, and MDA-MB-435 were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin. HeLaS3 cells were maintained in DMEM/Ham’s F12 supplemented with 10% FBS and penicillin-strepotmycin. U2OS stably expressing GFP–Dvl2 or FLAG–Fz2, 50,000 parental cells/well in 12-well plates were treated with lentiviruses and 100% methanol for 20 min at 4°C before staining with an anti-FLAG mouse monoclonal antibody (Sigma, St. Louis, MO) and incubated with 10 μg/ml polybrene, centrifuged 1000 rpm for 1 h and incubated for a further 24 h. Then, the cells were selected and maintained in the same medium with parental cells containing 400 μg/ml G418. To generate HeLaS3 cells stably expressing GFP–Dvl2 or FLAG–Fz2, 50,000 parental cells/well in 12-well plates were treated with lentiviruses and 10 μg/ml polybrene, centrifuged 1000 rpm for 1 h and incubated for a further 24 h. Then, the cells were selected and maintained in the same medium with parental cells containing 400 μg/ml G418. MEFs were prepared from E13.5 embryos by a 2T3 protocol (Todaro and Green, 1963) and were maintained in DMEM supplemented with 10% FBS. To express proteins transiently, plasmids were transfected in cells using Lipofectamine LTX (Invitrogen, Grand Island, NY, USA) for HeLaS3 cells and Lipofectamine 2000 for HEK293T cells.

Treatment of cells with siRNA

In analyses with siRNAs for randomized control, Wnt5a, Fz2, LRPS, Rab2, Dvl1, Dvl2, Dvl3, EB1, Rab5, Rab1a, and Rab1b in HeLaS3 cells, the following target sequences were used. Randomized control, 5′-CAGTCCGTTTGGCAGCTGG-3′; Wnt5a-1, 5′-GTTCAAGTGCAGAAGTAT-3′; Wnt5a-2, 5′-CTGTGGATAACCTGTGAAT-3′; Rab2, 5′-GGAGGAGATCTTTGATGAC-3′; Rab1a, 5′-GAGGAAATTTCAGATGAC-3′; Rab5, 5′-CCAAAGTCCAAGCTCGAAT-3′; Dvl1-1, 5′-GTTCAGATGTCAGAAGTAT-3′; Dvl1-2, 5′-CCACATATATCTACACAT-3′; Dvl2-2, 5′-GTTGTTGCTCTGACACACT-3′; Dvl2-3, 5′-CCAGCTATAAAGTCTTCCT-3′; EB1, 5′-GTTGAAATATCTGTCATTAGAC-3′; Rab1b, 5′-AAGGGCAGCTCAGAATAA-3′; Dvl3, 5′-GGAGGATCTTGAGTACG-3′; Dvl5, 5′-GGAGGATCTTGAGTACG-3′; Rab5, 5′-AAGGGCAGCTCAGAATAA-3′; Rab1a, 5′-GGCA-TATGCGCTAAACA-3′; Rab1b, 5′-GCACTCTGGCCTATGAGAAC-3′. The target sequences of siRNA for Dvl1, Dvl2, and Dvl3 in MEF cells were 5′-GGAGGATCTTGAGTACG-3′, 5′-GGAGGATCTTGAGTACG-3′, 5′-GGAAATATCTGTCATTAGAC-3′, respectively.

HeLaS3 and MEF cells were transfected with a mixture of siRNAs against genes of interest at 20 nM each using RNAiMAX (Invitrogen, Grand Island, NY, USA) and the cells were used for experiments at 72 h post-transfection. When necessary, for 48 h siRNA transfection, cells were transfected with plasmid cDNA and then the cells were analyzed a further 24 h after transfection.

Cell staining and image analysis

For cell staining and image analysis, cells were seeded on coverslips and fixed for 10 min with cold methanol at −20°C before staining with an anti-β-tubulin antibody and Ph. Individual cells were carefully determined by taking Z-stack images from 0.5 μm-thick sections of area. All processing and measurements were carried out using an LSM510 system with Axiosview. In each experiment multinucleated cells among more than 150 cells, where microtubules and nuclei were visible clearly, were counted.

Immunoblotting and immunoprecipitation

Methods for immunoblotting and immunoprecipitation were described previously (Hino et al., 2005). Briefly, to immunoprecipitate proteins, cells were washed once with PBS and lysed in lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.5% NP40) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) for 10 min on ice. After centrifugation, the supernatant was collected, and incubated with appropriate antibodies and 30 μl of protein-G–Sepharose (50% slurry). The mixtures were placed on a rotary mixer for 1.5 h at 4°C. The beads were then washed four times with lysis buffer and finally suspended in Laemmli’s sample buffer.

Quantitative real-time PCR

Total RNA was isolated from HeLaS3 cells treated with siRNA for Fz2 for 72 h. RNA sample (2 μg) was subjected to reverse transcription using murine leukemia virus reverse transcriptase (PE Applied Biosystems) in a total volume of 20 μl. Quantitative reverse transcription-PCR (RT-PCR) was performed using a LightCycler (Roche Molecular Biochemicals). Aliquots (2.5 μl) of the reverse transcription products were amplified in a reaction mixture (10 μl) containing LightCycler FastStart DNA Master SYBR Green 1, 1 μM primer, and 2 mM MgCl2. Forward and reverse primers were as follows: human GAPDH, 5′-CCTGTGCACTGCAGCTGG-3′ and 5′-CGACAAATCCGTTGACTCC-3′; human Fz2, 5′-GAGCGTGTAGTGGCTG-3′ and 5′-GCTCTGGGTAGCGGAA-3′.

Statistics

Experiments were performed at least three times and the results were expressed as means ± s.e.m. Statistical analysis was performed using StatView-J 5.0 software (SAS Institute Inc.). Differences between the data were tested for statistical significance using t-tests. P-values less than 0.05 were considered statistically significant.

Acknowledgements

We are grateful to Drs R. Habas, M. Maki, and T. Yoshimori for donating cDNAs. We also thank Dr A. Sato for lentiviral production and the Center for Medical Research and Education in Osaka University for imaging using an ELYRA S1 microscope.

Funding

This work was supported by Grants-in-Aid for Scientific Research (A) (2009, 2010 and 2011) [grant number 212490170 to A.K.], for Scientific Research on Priority Areas (2011) [grant number 23112004 to A.K.], and for Young Scientists (B) (2010, 2011) [grant number 22700881 to K.K.] from the Ministry of Education, Science, and Culture of Japan, and by The Nagase Foundation (2011) [to A.K.].

Supplementary material available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.108142/-/DC1

References


Cells Mol. Cell. Biol. 402 J.
palmitoylation and glycosylation of Wnt-5a are necessary for its signalling.
through binding to synaptotagmin.
endocytic recycling compartment to cell surface depends on stable, detyrosinated
(1999). DIX domains of Dvl and axin are necessary for protein interactions and
functions and implication in diseases.
EMBO J. 29, 3470-3483.
EMBO J. 29, 119-129.
**Fig. S1. Localization of Dvl2 to the midbody.** (A) Upper panels: U2OS cells transiently expressing GFP-Dvl2 were stained for GFP (green) and β-tubulin (microtubules, red) at late telophase. An arrow indicates GFP-Dvl2 localized to the stem body. Lower panels: MDA-MB-435 cells were stained for Dvl2 (green) and β-tubulin (red). An arrow indicates endogenous Dvl2 localized to the stem body. (B) U2OS cells stably expressing GFP-Dvl2 were transiently transfected with mCherry-LC3. Live images of the cells were acquired every 3 min for 12 h and two representative still images after abscission are shown. GFP-Dvl2 (arrows) was incorporated with mCherry-LC3 (arrowheads) into a daughter cell. Scale bar, 10 μm.
Fig. S2. Knockdown of Wnt5a and its signaling proteins. (A) Upper panels, lysates of Wnt5a, Ror2 or LRP6-depleted HeLaS3 cells were probed with indicated antibodies. β-Tubulin was used as a loading control. Lower panel, expression of Fz2 mRNA 72 hr after siRNA transfection was determined by quantitative real-time qPCR and normalized to GAPDH expression. (B) Lysates of Wnt5a-depleted HeLaS3 cells were probed with Dvl2 and β-tubulin antibodies. β-Tubulin was used as a loading control. (C) Parental HeLaS3 cells or HeLaS3 cells stably expressing mouse Wnt5a were treated with siRNA for human Wnt5a and were probed with Wnt5a and β-tubulin antibodies. β-Tubulin was used as a loading control. Scrambled siRNA transfected cells were used as a control.
Fig. S3. Localization of Fz2 to the midbody. (A) Outline of the Wnt signaling pathways. (B) Localization of HA-Fz2 at early telophase and late telophase shown in Fig. 3A was quantified. Fluorescence intensity of HA-Fz2 along the midbody (dotted line) was measured. Although a single highest peak was detected in the center of midbody at early telophase as shown in an upper panel (arrow), separated double peaks were detected in late telophase as shown in a lower panel (arrowheads). Fluorescence intensities are shown as arbitrary units.
Treat FLAG-Fz2- or FLAG-Dvl2-expressing HeLaS3 cells with anti-FLAG antibody (to label FLAG-tagged proteins on cell surface)

Wash the cells with PBS (to remove non-specifically bound anti-FLAG antibody)

Fix the cells with 100% Methanol

Treat the cells with anti-β-tubulin antibody (B and C) and anti-Dvl2 antibody (C)

Treat the cells with secondary antibodies; red for FLAG-Fz2 or FLAG-Dvl2 (B and C), green for microtubules (B and C), gray for Dvl2 (C)

Fig. S4. Localization of Fz2 to cell surface membrane. (A) An outline of the methods used in this experiment is shown. Before fixation, HeLaS3 cells transiently expressing FLAG-Fz2 (B) or FLAG-Dvl2 (C) were treated with anti-FLAG antibody to label FLAG-tagged proteins on the cell surface. Thirty minutes after incubation, the cells were washed with PBS and fixed with cold 100% methanol. Cells were then stained for β-tubulin (B) or β-tubulin and Dvl2 (C) antibodies.

While FLAG-Fz2, which is localized on the plasma membrane, was detected with anti-FLAG antibody before fixation, FLAG-Dvl2, an intracellular protein, was not detected with anti-FLAG antibody (FLAG Ab) before fixation. Expression of FLAG-Dvl2 was confirmed by treatment of cells with anti-Dvl2 antibody (Dvl2 Ab) after fixation. Ab, antibody. Scale bars, 10 μm.
**Fig. S5. Localization of Ror2.** HeLaS3 cells were stained for Ror2 (green) and β-tubulin (red). Inserts indicate the enlarged image of a dotted rectangular area. Scale bar, 10 μm.
Fig. S6. Depletion of Dvl by siRNA. (A) Lysates of HeLaS3 cells treated with two kinds of siRNAs for Dvl1, 2, and 3 were probed with indicated antibodies. β-Actin was used as a loading control. N.S., non-specific band. (B) GFP-Dvl2 was transiently expressed in Dvl2-depleted HeLaS3 cells and the lysates were probed with anti-Dvl2 and anti-β-actin antibodies. (C) Lysates of Wnt5a or Dvls-depleted MEFs were probed with indicated antibodies. Clathrin and β-tubulin were used as a loading control. Scrambled siRNA transfected cells were used as control.
**Fig. S7. Localization of EB1 in interphase cells.** Wnt5a or Dvl-depleted HeLaS3 cells were stained for EB1 (red) and β-tubulin (green). Scale bar, 10 μm.
Fig. S8. No requirement of CHMP4B for microtubule stability in the midbody. (A) Lysates of CHMP4B, Rab11 or Rab5-depleted HeLaS3 cells were probed with indicated antibodies. β-Tubulin was used as a loading control. (B) CHMP4B-depleted cells were stained for Glu-tubulin (red) and β-tubulin (green). Scale bar, 10 μm.
Fig. S9. Possible model for the involvement of Wnt5a signaling in cytokinesis. See the text.
Table S1. Primary antibodies used in this study

<table>
<thead>
<tr>
<th>Company</th>
<th>Name</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aves Labs</td>
<td>Chicken anti-GFP</td>
<td>IF</td>
</tr>
<tr>
<td>BD Biosciences</td>
<td>Mouse anti-EB1</td>
<td>IB, IF</td>
</tr>
<tr>
<td>Cell Signaling Technology</td>
<td>Rabbit anti-Dvl2</td>
<td>IB, IF</td>
</tr>
<tr>
<td>Cell Signaling Technology</td>
<td>Rabbit anti-Dvl3</td>
<td>IB</td>
</tr>
<tr>
<td>Cell Signaling Technology</td>
<td>Rabbit anti-LRP-6</td>
<td>IB</td>
</tr>
<tr>
<td>Cell Signaling Technology</td>
<td>Rabbit anti-Wnt5a/b</td>
<td>IB</td>
</tr>
<tr>
<td>Medical &amp; Biological Laboratories</td>
<td>Rabbit and mouse anti-HA</td>
<td>IF</td>
</tr>
<tr>
<td>Millipore</td>
<td>Rabbit anti-Glu tubulin</td>
<td>IF</td>
</tr>
<tr>
<td>Molecular Probes</td>
<td>Rabbit anti-GFP</td>
<td>IF</td>
</tr>
<tr>
<td>Novus Biologicals</td>
<td>Rat anti-α-tubulin</td>
<td>IF</td>
</tr>
<tr>
<td>R&amp;D Systems</td>
<td>Goat anti-Ror2</td>
<td>IF</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>Mouse anti-Dvl1</td>
<td>IB</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>Mouse anti-Dvl2</td>
<td>IB, IP</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>Mouse anti-GFP</td>
<td>IB</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>Rabbit anti-Mklp1</td>
<td>IF</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>Rabbit anti-CHMP4B</td>
<td>IB, IF</td>
</tr>
<tr>
<td>Sigma</td>
<td>Mouse anti-β-actin</td>
<td>IB</td>
</tr>
<tr>
<td>Sigma</td>
<td>Mouse anti-β-tubulin</td>
<td>IB, IF</td>
</tr>
<tr>
<td>Wako</td>
<td>Mouse anti-FLAG</td>
<td>IF</td>
</tr>
</tbody>
</table>