Linking cytoplasmic dynein and transport of Rab8 vesicles to the midbody during cytokinesis by the doublecortin domain-containing 5 protein

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Summary
Completion of mitosis requires microtubule-dependent transport of membranes to the midbody. Here, we identified a role in cytokinesis for doublecortin domain-containing protein 5 (DCDC5), a member of the doublecortin protein superfamily. DCDC5 is a microtubule-associated protein expressed in both specific and dynamic fashions during mitosis. We show that DCDC5 interacts with cytoplasmic dynein and Rab8 (also known as Ras-related protein Rab-8A), as well as with the Rab8 nucleotide exchange factor Rabin8 (also known as Rab-3A-interacting protein). Following DCDC5 knockdown, the durations of the metaphase to anaphase transition and cytokinesis, and the proportion of multinucleated cells increases, whereas cell viability decreases. Furthermore, knockdown of DCDC5 or addition of a dynein inhibitor impairs the entry of Golgi-complex-derived Rab8-positive vesicles to the midbody. These findings suggest that DCDC5 plays an important role in mediating dynein-dependent transport of Rab8-positive vesicles and in coordinating late cytokinesis.

Key words: Rab8 vesicles, Cytokinesis, Cytoplasmic dynein, Doublecortin domain-containing protein 5 (DCDC5), Midbody

Introduction
Cytokinesis is the final step of mitosis, whereby one parent cell is split into two daughter cells in a process that requires generation of new membrane barriers. This process consists of several morphological stages: specifying and positioning the cleavage site, cleavage-furrow formation and constriction, spindle midzone formation, and cell separation or abscission. Although it is crucial for successfully completing mitosis, cytokinesis has been relatively less investigated than earlier mitosis-related events. The slow progress in this area might be because many proteins play dual roles in early and late mitotic events, and cellular analyses have traditionally focused on the early mitotic roles. Likewise, cytoplasmic dynein, the main retrograde motor, as well as its many subunits and accessory and interacting proteins have been implicated in regulating several stages of mitosis (reviewed by Sharp et al., 2000c). When mitosis is initiated, dynein is involved in regulating nuclear envelope breakdown (NEBD), where it acts together with interacting proteins such as LIS1 and NDEL1, and microtubules (MTs) (Hebbar et al., 2008; Salina et al., 2002). Dynein has been also implicated in spindle formation and mitosis (Echeverri et al., 1996; Gaetz and Kapoor, 2004; Gaglio et al., 1997; Goshima and Vale, 2003; Vaisberg et al., 1993). Following NEBD, the correct positioning of dynein and its interacting proteins LIS1, NDE1 and NDEL1 on kinetochores, astral MTs and cortical sites assist in moving polarized chromosomes toward the minus ends of spindle MTs, as well as moving the spindle poles to the position of the spindle within the cell (Busson et al., 1998; Coquelle et al., 2002; Cottingham and Hoyt, 1997; Faulkner et al., 2000; Gonczy et al., 1999; Grishchuk et al., 2007; O’Connell and Wang, 2000; Pfarr et al., 1990; Sharp et al., 2000a; Sharp et al., 2000b; Yan et al., 2003; Yingling et al., 2008). The role of dynein in later stages of mitosis is implied by the phenotypes induced following suppression of several subunits of dynein. Knockdown of the LIC2, Roadblock 1 and LC8 subunits impaired the completion of cytokinesis, at least in part by affecting endosome distribution (Palmer et al., 2009) (reviewed by Eggert et al., 2006). The role of dynein during cytokinesis is probably similar to that of other mitotic motors at earlier stages of mitosis. Mitotic motors play at least three distinct roles: (1) crossbridging and sliding MTs relative to adjacent MTs or other structures; (2) transporting specific mitotic cargoes along the surface lattice of spindle (or midbody) MTs; and (3) regulating MT assembly dynamics and coupling movements with regard to MT growth and shrinkage (reviewed by Sharp et al., 2000c).

The final step of cytokinesis, namely, abscission, requires serial dynamic events involving ingression of the contractile ring. This process depends on actin and its associated motor myosin, and the formation of an intracellular bridge (also termed midbody) filled with antiparallel arrays of microtubules (reviewed by Guizetti and Gerlich, 2010). Proper abscission requires adding a new plasma membrane during closure of the cleavage furrow (Maiato et al., 2004). Vesicle trafficking is also needed for cleavage furrow ingression. Two types of vesicles are known to participate in the abscission: Golgi-complex- and recycling-endosome-derived vesicles. Vesicles of Golgi-complex origin are characterized by the presence of the small GTPase proteins Rab6 or Rab8. By contrast, Rab11 is found in recycling endosome-derived vesicles, and its inactivation leads to a prolonged midbody stage and eventually to furrow regression (Wilson et al., 2005; Yu et al., 2007).
Our research focuses on elucidating the role of doublecortin-domain-containing protein 5 (DCDC5), a member of the doublecortin (DCX) superfamily of proteins (Coquelle et al., 2006; Reiner et al., 2006). Mutations in the X-linked gene DCX or its interacting protein LIS1 result in lissencephaly or subcortical band heterotopia (SBH) (Caspi et al., 2000; des Portes et al., 1998; Gleson et al., 1998; Reiner et al., 1993). DCX is a neuronal MT-associated protein (MAP) (Horesh et al., 1999) that binds to MTs in a unique position in between the protofilaments through a defined DCX domain (Moore et al., 2004; Sapir et al., 2000; Taylor et al., 2000). Furthermore, both LIS1 and DCX are found to complex with dynein and to regulate centrosomal-nuclear coupling in migrating neurons by a dynein-dependent pathway (Shu et al., 2004; Tanaka et al., 2004; Tsai et al., 2007; Tsai et al., 2005). DCX domains are conserved in evolution and define a superfamily of proteins of which eleven members are present in mammals (Coquelle et al., 2006; Reiner et al., 2006; Sapir et al., 2000). Several members of the DCX superfamily play important roles during mitosis. DCX knockout mice exhibit deficits in proliferation of neuronal progenitors (Pramparo et al., 2010). Doublecortin-like kinase regulates the formation of the mitotic spindle and the progression of the cell cycle in developing neuroblasts (Shu et al., 2006). In addition, mutations in the worm doublecortin-like kinase ortholog, Zyg-8, affect anaphase spindle positioning (Gonczy et al., 2001). Our results suggest a mitotic role for DCDC5, a member of the DCX superfamily. DCDC5 is specifically expressed during mitosis. The reduced DCDC5 expression levels compromised cell viability and increased the number of multinucleated cells. In particular, the relative position of the midbody was affected, cytokinesis duration was extended and Rab8-positive vesicles failed to enter the midbody. Importantly, DCDC5 directly interacts with cytoplasmic dynein and Rab8. Therefore, we propose that DCDC5 assists in dynein-dependent transport of Rab8 vesicles to the midbody during cytokinesis.

Results

**DCDC5 is a mitotic protein**

The subcellular localization of endogenous DCDC5 was studied by immunostaining HeLa cells with DCDC5-specific antibodies. Whereas no immunostaining was noted in interphase cells (Fig. 1A–A'), DCDC5 exhibited a dynamic expression pattern during mitosis (Fig. 1B–K'; supplementary material Movies 1–3 of three-dimensional mitotic images). During prophase, punctate DCDC5 immunostaining appeared (Fig. 1B–B'), which condensed around centrosomal areas. During metaphase, DCDC5 was highly enriched on a subset of spindle MTs and decorated cortical sites (Fig. 1C–C'), but it was absent in astral MTs. When anaphase began, DCDC5 was present on a subset of MTs and part of the protein was distributed in a punctate manner (Fig. 1D–D'). During telophase, the punctate pattern was still visible and DCDC5 was highly concentrated along midbody-forming MTs (Fig. 1E–E'). By contrast, during late cytokinesis, DCDC5 was distributed more uniformly; however, some enrichment was observed along the midbody (Fig. 1G–G'). DCDC5 colocalized preferentially with stable MTs, as indicated by co-immunostaining with anti-acetylated tubulin antibodies (Fig. 1H–H'). We used Pearson’s correlation coefficient (PCC) to evaluate the colocalization between DCDC5 and acetylated tubulin. The colocalization was high (Fig. 1I–I', PCC = 0.77±0.02), whereas its colocalization with α-tubulin was moderate (Fig. 1J–J', PCC = 0.65±0.01). Following methanol fixation or live imaging of GFP-tagged DCDC5 (supplementary material Fig. S1A–A' and B) DCDC5 was also detected in the Flemming body, which is the contractile ring within the midbody. In mitotic HeLa cells a similar dynamic expression pattern of GFP-tagged DCDC5 was evident: during metaphase DCDC5 decorated the spindle and later the fluorescent protein was highly enriched in the midbody (supplementary material Movie 4). The specificity of DCDC5 antibodies was examined during metaphase. DCDC5 antibodies that were preincubated with the antigen used to generate them practically abolished DCDC5 immunoreactivity (supplementary material Fig. S2A–D). In addition, we showed by mass spectrometry (MS) analysis that these antibodies specifically immunoprecipitated DCDC5 (data not shown). Moreover, the mitosis-specific expression of DCDC5 was corroborated by western blot analysis using synchronized HeLa cells collected at different stages of the cell cycle (Fig. 1K), and the subsequent detection of enriched amounts of DCDC5 immunoprecipitated from mitotic HeLa cell extracts (Fig. 1L). Furthermore, a short-hairpin RNA (shRNA) sequence specific for DCDC5 reduced the apparent DCDC5 immunostaining in mitotic cells by 65% (supplementary material Fig. S2F–H). DCDC5 cDNA was readily amplified only from control mitotic cells using semi-quantitative RT-PCR, whereas a weak signal was obtained from control interphase cells, suggesting that the mitotic expression of DCDC5 is regulated at the mRNA level. No PCR product was obtained from interphase or mitotic HeLa cell lines expressing DCDC5 shRNA sequences (supplementary material Fig. S2E). These results indicate that DCDC5 is a mitotic specific protein.

**DCDC5 is a microtubule-associated protein (MAP)**

DCDC5 is a member of the doublecortin superfamily of proteins. The doublecortin (DCX) domains bind to and actively polymerize MTs (Horesh et al., 1999; Moore et al., 2004; Sapir et al., 2000), and serve as scaffolds for several protein interactions (Coquelle et al., 2006; Reiner et al., 2006). Because DCDC5 has two DCX domains, we determined whether DCDC5 is also a microtubule-associated protein (MAP). Recombinant DCDC5 co-assembled with polymerized MTs was enriched in the pellet in the presence of Taxol (Fig. 2A). Following nocodazole treatment, which results mainly in disassembled tubulin dimers, both DCDC5 and tubulin were found in the supernatant. The interaction between DCDC5 and tubulin was further examined by expressing biotin-tagged DCDC5 in transfected cells (Fig. 2B). A prominent tubulin signal was found with affinity-purified biotinylated DCDC5 but not with affinity-purified biotinylated GFP (Fig. 2C). These results suggest that DCDC5 can interact both with polymerized MTs and with tubulin subunits. Consistent with this, we previously demonstrated that one DCX domain of DCDC5 was sufficient to promote MT polymerization (Coquelle et al., 2006). Collectively, these data and the above colocalization studies suggest that DCDC5 is a mitotic MAP.

**DCDC5 knockdown increased apoptosis and the number of multinucleated cells**

DCDC5 expression levels were reduced in HeLa cells using stable or transient transfections of DCDC5 shRNA. The reduced levels of DCDC5 increased cellular apoptosis, as manifested by immunostaining with anti-activated caspase-3 antibodies.
(compare Fig. 3A with B). The proportion of activated caspase-3-positive cells after DCDC5 shRNA treatment, increased by more than 77% (supplementary material Table S1). These results were corroborated using non-synchronized cells sorted according to their DNA content (Fig. 3C). The proportion of cells in the subG1 fraction within a population of DCDC5-shRNA-treated cells increased by 2.85-fold (from 6±0.8% to 16.95±3.1%; P<0.01). In addition, there was a modest decrease in the proportion of cells in the G1/G0 phases (P<0.05). These findings were supported by a significant decrease (32%) in cell viability,
determined using the MTT assay (supplementary material Table S1). Furthermore, the percentage of multinucleated cells almost doubled following DCDC5 knockdown (supplementary material Table S1). A similar increase in the relative numbers of multinucleated cells was also observed during live imaging of control and treated cells (from 8.6±0.6%, n=25, to 25.6±10%, n=28, respectively). Collectively, these data suggest that DCDC5 plays an essential role in cytokinesis.

**DCDC5 knockdown affects midbody positioning and mitosis progression**

Next, we investigated the relative position of the midbody and the cell body during cytokinesis by immunostaining (Fig. 3D–F). Whereas in control cells the midbody is typically found in the middle of the dividing cells, the position of the midbody changed following reduction of DCDC5 (Fig. 3D–E’, summarized in the graph in F), revealing a significant deviation from the midline (P<0.0001 using the Mann–Whitney t-test; DCDC5-shRNA-treated cells 3.5±0.29 mm versus the control 1±0.13 mm, n=52 and 41, respectively). This striking finding prompted us to investigate the progression of DCDC5-shRNA-treated cells throughout mitosis using time-lapse microscopy (Fig. 3G–I; supplementary material Movies 5, 6, 7, 8). To this end, HeLa cells were transfected with a fluorescent histone marker, H1E–GFP, to enable us to clearly evaluate mitosis progression. Cells treated with DCDC5 shRNA exhibited a significant delay in the progression from metaphase to anaphase in comparison with controls (41±4 minutes, n=80 versus 22±2 minutes, n=76; DCDC5 shRNA and control, respectively, P=0.0007 using an unpaired t-test with Welch’s correction; Fig. 3G–H). In addition, cytokinesis completion differed between the control and shRNA-treated cells. Whereas the control cells divided properly, giving rise to two daughter cells (Fig. 3M), DCDC5-shRNA-treated cells either fused to form multinucleated cells (Fig. 3Q) or separated after an extended time (Fig. 3U). The observed cell fusion events might explain the increase in multinucleated cells following DCDC5 reduction. DCDC5-shRNA-treated cells spent approximately 50% more time in cytokinesis compared with control cells (211±16 minutes versus 141±4 minutes, n=23, 30, respectively, P<0.0001, unpaired t-test with Welch’s correction; summarized in Fig. 3U). To determine whether the shift in midbody position from the centre of the cell causes mislocalization of known midbody proteins, we examined the localization of Polo kinase-1 (Plk1). However, no change in the localization of Plk1 within the contractile ring of the midbody was detected following DCDC5 reduction (supplementary material Fig. S3). These results suggest that DCDC5 is required for cells to progress through mitosis because it promotes proper localization of the midbody during cytokinesis.
Fig. 3. See next page for legend.
DCDC5 and membrane transport

Abscission depends on a proper supply of vesicles to the midbody, which allows for membrane sealing before cell separation (Fielding et al., 2005; Montagnac et al., 2008; Pohl and Jentsch, 2008; Skop et al., 2001; Skop et al., 2004; Wilson et al., 2005). The involvement of Rab small GTP-binding proteins in the regulation of membrane trafficking is well documented (Balch, 1990). Therefore, we investigated the localization of several Rab-positive vesicles during cytokinesis. First, we visualized Rab11, which marks recycling endosome vesicles. No difference in the localization of Rab11–GFP vesicles was detected in HeLa cells expressing DCDC5 shRNA (supplementary material Fig. S4A–D′) and no interaction with DCDC5 was observed (supplementary material Fig. S4E). Next, we examined Golgi-complex-derived secretory vesicles using Rab8. Analysis of fixed cells expressing DCDC5 shRNA immunostained with anti-Rab8 antibodies revealed a significant reduction in Rab8-positive vesicles in the midbody [75% in control (n=35) versus 33.3% in shRNA-expressing (n=30); Fig. 4A–C′]. Similar results were observed for Rab8–GFP-transfected cells. These results were strengthened by analysis of time-lapse movies of cells expressing DCDC5 shRNA or control shRNA that were transfected with Rab8–GFP. Relative to control cells, the persistence of Rab8–GFP vesicles, which enter the midbody during cytokinesis, was significantly reduced in DCDC5-shRNA-treated cells (supplementary material Movies 9 and 10, respectively). Quantification of this effect revealed that in DCDC5-shRNA-treated cells, Rab8–GFP vesicles entered the midbody for only a fifth of the duration of cytokinesis, whereas in control cells it was present in the midbody for almost half of the period of cytokinesis (P<0.005, unpaired t-test, 22.04±5.79% versus 48.10±5.766%, n=13).

Fig. 3. DCDC5 knockdown in HeLa cells. (A–C) HeLa cells treated with DCDC5 shRNA are more susceptible to apoptosis. (A, B) Representative images of HeLa cells stably expressing a control vector (A) or DCDC5 shRNA (B) immunostained with anti-α-tubulin antibodies (green), anti-activated caspase-3 antibodies (red) and DAPI. Scale bar: 40 µm. (C) HeLa cells stably expressing DCDC5 shRNA or a control vector were subjected to flow cytometry analysis following propidium iodide staining; the left bar in each pair represents control cells and the right one represents DCDC5-shRNA-treated cells. (D–S) Mitosis is affected in HeLa cells treated with DCDC5 shRNA. (D–E′) Representative images of cytokinetic HeLa control (D–D′) and DCDC5 shRNA (E–E′) cells, immunostained with anti-DCDC5 (red) and anti-α-tubulin (green) antibodies. Scale bar: 15 µm. The arrows indicates the midbody; the line in D′,E′ was used for measuring the distance of the midbody from the cell median. (F) Quantification for the midbody position in DCDC5-shRNA-treated (3.5±0.29 mm; n=52) and control-shRNA-treated (1±0.13 mm; n=41) cells. (G–H′) Representative images from time-lapse movies of control (G–G′) and DCDC5 shRNA cells transfected with HA–GFP (H–H′). The selected differential interphase contrast (DIC) images and fluorescence images were taken at the indicated time points: (G,H; metaphase, G′,H′; anaphase). Scale bar: 10 µm. (I) Quantification of the anaphase-to-metaphase transition duration in DCDC5-shRNA-treated (41±4 minutes; n=80) and control-shRNA-treated (22±2 minutes; n=76) cells. (J–U) Selected frames from time-lapse sequences of control shRNA (J–M) or DCDC5 shRNA (N–U)-treated cells at different stages of the cell cycle. Note the distinct stages of the abscission process following different treatments of cells (M,Q,U). Scale bar: 5 µm. (U′) Quantification of anaphase-to-abscission duration, in DCDC5-shRNA-treated (211±16 minutes; n=23) and control-shRNA-treated (114±14 minutes; n=30) cells. ***P<0.0001 (considered extremely significant); *P<0.05 (considered significant).

Next, we investigated whether Rab8 binds to DCDC5. To this end, we performed co-immunoprecipitation (co-IP) experiments with the recombinant proteins. DCDC5 was found to bind both the active (GTP-bound) and inactive (GDP-bound) forms of Rab8 (Fig. 4D). Rab8 (also known as Rab-3A-interacting protein) is a guanine nucleotide exchange factor (GEF) that participates in the polarized delivery of Rab8 vesicles to the cell surface (Hattula et al., 2002). Therefore, a possible interaction between Rab8 and DCDC5 was also investigated (Fig. 4E). Myc-tagged DCDC5 and Rab8–GFP co-immunoprecipitated reciprocally, confirming that DCDC5 interacts with this protein.

DCDC5 interacts with the molecular motor cytoplasmic dynein and the interacting proteins

DCX and DCLK have been shown to co-immunoprecipitate with cytoplasmic dynein (Shu et al., 2006; Tanaka et al., 2004). Moreover, several motor proteins, including cytoplasmic dynein were detected in the midbody, suggesting that they might be involved in cell separation (Karki et al., 1998; Nislow et al., 1990; Vernos et al., 1995). Therefore, we postulated that DCDC5 might interact with the main retrograde motor, cytoplasmic dynein. Indeed, recombinant DCDC5 interacted directly with the recombinant dynein intermediate chain (DIC). His-tagged DCDC5 or His-tagged Ndel1, a known dynein interacting protein (Niethammer et al., 2000; Sasaki et al., 2000), was successfully pulled-down with GST–DIC but not with GST (Fig. 5A). Furthermore, the endogenous proteins interacted in synchronized G2/M HeLa cell extracts. DIC co-immunoprecipitated with DCDC5 antibodies and DCDC5 reciprocally co-immunoprecipitated with anti-DIC antibodies (Fig. 5B). DCDC5 immunoprecipitated two additional dynein-interacting proteins, NudC and LIS1, with the strongest signal occurring in the mitotic cell extract (Fig. 5C). Furthermore, DCDC5 interacted with p150Glued1, a core subunit of dynein, which is another major dynein interacting protein (Fig. 5D). Bidirectional co-immunoprecipitation occurred; myc-tagged DCDC5 co-immunoprecipitated GFP–dynactin (p150Glued2) and GFP–dynactin (p150Glued1) co-immunoprecipitated myc-tagged DCDC5. We concluded that DCDC5 can bind to cytoplasmic dynein and its interacting proteins.

DCDC5 links cytoplasmic dynein and Rab8

Our knockdown and physical interaction studies of DCDC5 led to the hypothesis that cytoplasmic dynein might be involved in the trafficking of Rab8-positive vesicles to the midbody during the final stages of mitosis, and that this is mediated by their interaction with DCDC5. We therefore investigated a possible colocalization of these proteins in the midbody region during cytokinesis using cells transfected with GFP–dynactin (p150Glued1) and immunostained with anti-DCDC5 and anti-Rab8 antibodies. To this end, we analyzed super-resolution images of endogenous Rab8, DCDC5 and MTs in the midbodies using the OMX system (Applied Precision, GE Healthcare; Fig. 6A–D′) and found high colocalization between DCDC5 and MTs (PCC=0.77±0.08) and moderate colocalization between DCDC5 and Rab8 (PCC=0.56±0.07). In addition, overexpressed GFP–dynactin (p150Glued1) and endogenous DCDC5 colocalized in the midbody (supplementary material Fig. S5). Furthermore, the role of cytoplasmic dynein in regulating the transport of Rab8-positive vesicles to the midbody was probed by adding a specific inhibitor of dynein, EHNA, after which time-lapse
microscopy was used to monitor the position of GFP-tagged Rab8 vesicles. Adding EHNA resulted in a complete lack of Rab8–GFP-positive vesicles in the midbody (Fig. 6F–H). With this treatment abscission failed and cells remained interconnected with the midbody bridge (in 16 out of 19 movies) or collided to form multinucleated cells (in 3 out of 19 movies).

**Discussion**

**DCDC5 is a mitotic MAP and is dynamically expressed during cell division**

The expression of DCDC5, as well as its subcellular localization, is cell cycle dependent. DCDC5 is the first member of the DCX superfamily of proteins to be identified as being expressed in a mitotic-specific manner. However, two other members of the DCX family, DCX and DCLK, have been previously shown to play a role in cell division (Couillard-Despres et al., 2004; Gonczy et al., 2001; Santra et al., 2010; Shu et al., 2006; Vreugdenhil et al., 2007). DCDC5 is associated with MTs, in particular, with stabilized MTs of the mitotic spindle during metaphase and with midbody MTs during cytokinesis. As expected from a member of the DCX superfamily consisting of two DCX domains, DCDC5 was found to bind directly to polymerized MTs in vitro experiments, again supporting the previous notion that DCDC5 is a MAP.

Knockdown of DCDC5 interferes with the final stage of mitosis

Cells with reduced levels of DCDC5 exhibited prolonged cytokinesis, as well as impairment in the abscission process, thus resulting in cell fusion events. The consequences of these events were decreased cell viability and an increased proportion of apoptotic and multinucleated cells among the DCDC5 knockdown cell population. Multinucleated cells can arise either from early mitotic aberrations, for instance, centrosomal amplification (Brinkley, 2001; Yun et al., 2004) or from defects in cytokinesis (Sagona and Stenmark, 2010; Zhao et al., 2006). Our data are consistent with the interpretation that the multinucleated cells resulted from a failure to complete cytokinesis.

**DCDC5 mediates the delivery of Rab8-positive vesicles to the midbody through a cytoplasmic dynein molecular motor**

It has been established that both the midbody structure and membrane fusion are essential for proper cytokinesis (Albertson et al., 2005; Otegui et al., 2005). However, how different vesicles manage to reach the midbody and contribute to proper abscission is presently poorly understood. In plant cells, the mechanism by which the secretory pathway contributes to cytokinesis is well established (reviewed by Jurgens, 2005; Van Damme et al.,
However, the role of post-Golgi complex secretory vesicles in regulating cytokinesis and abscission of animal cells is not as clear. Rab GTPases define particular routes within the Golgi-complex-derived secretory pathway by controlling vesicle formation, motility, docking, and fusion events (Zerial and McBride, 2001).

We hypothesized that DCDC5 is involved in the trafficking of Rab-dependent membrane vesicles to the midbody. The role of the small GTPase Rab8, which marks the Golgi-complex-derived secretory vesicles in cytokinesis, is not as well established; it has been mainly implicated in basolateral membrane transport, cell polarity and ciliogenesis (Ang et al., 2003; Hattula et al., 2002; Nachury et al., 2007). However, it has been shown that Rab8 endosomes are transported along MTs to the midbody ring, suggesting their potential contribution to membrane dynamics during cytokinesis (Pohl and Jentsch, 2008b). Importantly, Rab8 was identified in a large-scale RNA interference (RNAi) screen aimed at identifying all proteins that are required for mitosis in human cells (MitoCheck, http://www.mitocheck.org/cgi-bin/mtc?query=MCG_0004801). The reported phenotypes include multinucleated cells, nuclei that stay together, and cell death; phenotypes similar to those observed following DCDC5 knockdown. Importantly, we showed that DCDC5 binds directly to both activated GTP-bound and inactivated GDP-bound forms of Rab8 and is also found in an immunocomplex with the Rab8 exchange factor Rabin8.

Rab GTPases have been implicated in motor-protein-dependent movement of vesicles on cytoskeletal tracks from...
sites of their formation to sites of fusion (Echard et al., 1998a). Among possible candidate motor proteins that might directly interact with DCDC5, the obvious choice was the main retrograde motor protein, cytoplasmic dynein, because it has been previously demonstrated that two DCDC5 family members, DCX and DCLK, interact with this motor complex (Shu et al., 2006; Tanaka et al., 2004a). Our experiments showed that DCDC5 directly binds the dynein intermediate chain. Moreover, DCDC5 co-immunoprecipitates with additional members of the dynein-dynactin protein complex, including p150 Glued, LIS1 and NudC. It was reported that the dynein motor is more efficiently recruited to acetylated MTs (Dompierre et al., 2007; Friedman et al., 2010), suggesting that enhancing MT acetylation stimulates retrograde transport. Because DCDC5 is a MAP and it colocalizes with acetylated MTs during mitosis, it might serve as a target protein for capturing Rab8-positive vesicles and for ensuring their spatial proximity to stabilized MT tracks and the molecular motor dynein delivery to the midbody.

Consistent with this proposal, we found that following DCDC5 shRNA treatment, as well as specific inhibition of the dynein motor, the delivery of Rab8-positive vesicles to the midbody was significantly reduced. Therefore, our results corroborate the role of Golgi-complex-derived secretory vesicles in regulating cytokinesis in animal cells. However, to date, the plus-end-directed kinesin motors and kinesin-like proteins have been identified as being required for abscission in C. elegans (Raich et al., 1998), Drosophila embryos (Adams et al., 1998) and mammalian cells (Carleton et al., 2006). The dynein–dynactin complex also participates in retrograde membrane trafficking during cytokinesis (Montagnac et al., 2009; Palmer et al., 2009). The orientation of microtubules in the midbody directs membrane trafficking to the cleavage plane. Cryo-electron tomography analysis of midbodies supports the presence of minus-end-directed trafficking by revealing a bundle of MTs that traverse the midbody with their plus ends positioned away from the center of the midbody (Elad et al., 2011). These specific continuous MTs persist to the verge of abscission, and they could provide tracks for dynein-induced trafficking of Rab8 to the midbody for proper completion of cytokinesis.

**DCDC5 and apoptosis**

DCDC5 knockdown induces, on the one hand, prolonged cytokinesis and, on the other hand, an increased number of apoptotic cells. Several proteins are known to be crucial for cell cycle events as well for protecting cells against apoptosis (Verhagen et al., 2001), including BIR domain proteins (BIRPs) such as survivin and cIAP1 (Li et al., 1999; Samuel et al., 2005). Another member of the BIRP family, BRUCE, plays an important role in delivering membrane vesicles to the site of abscission and maintains the integrity of the midbody (Pohl and Jentsch, 2008). The depletion of BRUCE in cultured cells sensitizes against apoptotic stimuli and finally leads to cell death (Hao et al., 2004; Ren et al., 2005). Interestingly, BRUCE localizes to Rab8 tubular endosomes (Pohl and Jentsch, 2008). Decreased delivery of Rab8-positive vesicles to the midbody in DCDC5 knockdown cells might affect proper BRUCE activity in this region and thereby impair its function as an apoptotic inhibitor.

We describe here a novel retrograde motor-dependent mechanism of specific vesicle trafficking involved in the final stages of mitosis. To date, the majority of the studies conducted in the field of vesicular trafficking have focused on the importance of membrane traffic, delivery and fusion with the plasma membrane (Straight and Field, 2000; Finger and White, 2002), whereas less attention has been placed on the potential cargo inside the vesicles that are delivered to the surface of daughter cells. The contents of the vesicles and their membrane...
protein components might be important for stabilizing newly formed membranes in the terminal phase of cytokinesis. Thus, one of the most intriguing future objectives arising from our study would be to investigate the content of Rab8-positive vesicles that is required to enter the midbody for proper cell separation. Resolving this mystery will provide further valuable information about the mechanism underlying cytokinesis completion.

Materials and Methods

Plasmids and antibodies

A full-length cDNA clone of human DCDC5 was obtained from the NITE Biological Research Center, Kisarazu, Japan (clone TESTI400134). The coding sequence of the cDNA was amplified by PCR using the ProofStar DNA polymerase (Quagen, Valencia, CA), digested by XhoI and SalI and subcloned in-frame into the mammalian expression vector pEGFP-N1 (Clontech Laboratories, Montain View, CA); Biotin-GFP was a gift from Niels Galjart, Erasmus University Medical Center, the Netherlands (de Boer et al., 2003).

The following primers were used for the N-terminus: 5'-TCCGCTGAGAATGAGAAACACCATTGTA9-3' and for the C-terminus: 5'-ACGGCAGGCTATTGCTGCTGCTC9-3' or 5'-CGGTCGAGCTCAGCCAAATTCTCTTCTCTTTTTA9-3'. C-terminus myc-tagged DCDC5 was generated by PCR with the following primers: 5'-ATATGTTTACGGCGCCGCTCACAGGTCTCCTGAGAATGACATCTGCTTCGCAAGCAAAATTTCTCTTCTCTTTTTTA9-3' and 5'-TCCGCTGAGCAATGAGAAACACCATTGTA9-3'. The fragment was cloned into a pCAGS vector between XhoI and NotI. His-tagged DCDC5-ricin domain fusion protein was obtained by amplifying nucleotide sequences 2186-2650 and inserted into pRESET-A (Invitrogen, Carlsbad, CA) using 5'-TCCGCTGAGGAGGCTCTCCTTCTGAAATGTTGGA9-3' and 5'-ACCGGGTAC-CAGATGATGATGACACACAGACA9-3'. His-DCDC5 nucleotides (1443-1488) was generated similarly. Rab8-GFP and Rab88-GFP were a gift from Johan Peranen, University of Helsinki, Finland. Rab88-GST was generated by PCR amplification of the Rab8 open reading frame, followed by inserting the fragment into EcoRI–XhoI sites of pGEX-4T1. p533-myc-GFP was kindly provided by Alexander Bershadsky (Weizmann Institute of Science, Rehovot, Israel). pHIE-GFP (Gerlitz et al., 2007) was used as a histone marker in live imaging experiments. Anti-DCDC5 antibodies were generated in rabbits using purified His-tagged DCDC5-ricin domain fusion protein as an antigen.

Monoclonal anti-β-tubulin (DM1A) and anti-acetylated tubulin were obtained from Sigma (Rehovot, Israel). Monoclonal anti-dynein intermediate chain (DIC), monovalent anti-cyclin B1 and polyclonal anti-caspase3 antibodies were obtained from Santa Cruz (CA, USA); polyclonal phospho-histone 3 was purchased from Upstate Biotechnology (Waltham, MA) and monoclonal anti-Rab8 from BD Transduction Laboratories (Franklin Lakes, NJ). Monoclonal anti-NuRD clone c15 was a gift from Junken Aoki, Tokyo University, Japan, and polyclonal anti-GFP was obtained from Jan Faix, Hannover Medical School, Germany. The anti-LIS1 antibody was monoclonal clone 210 (Sapir et al., 1997). Rhodamine-conjugated affipure goat anti-mouse and Cy3-conjugated affipure goat anti-rabbit IgG (H+L) were from Jackson Immunoresearch (West Grove, PA). Alexa Fluor® 488 goat anti-mouse IgG (H+L) was from Molecular Probes (Invitrogen).

Cell culture, synchronization and transfections

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Biological Industries, Kibbutz Be’itar Haemek, Israel) supplemented with 8% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. HeLa cells were synchronized using a thymidine–nocodazole block; cells were collected for further biochemical analysis or washed and released into the cell cycle for 20 minutes in fresh pre-warmed medium in order to obtain cells in telophase to cytokinetic phases. HeLa cells were transfected using the JetPEFTM transfection reagent (Polyplus-transfection, NY).

RNA interference

DCDC5 small interfering RNAs were generated in pSUPER-derivated expression vectors using the following primers: shRNA1, 5'-gattcacaGCTGATTCGCTACGCTATTCGTGGGATGGAAGAAGTCTG9-3' and shRNA2, 5'-gattcacaGCTGATTCGCTACGCTATTCGTGGGATGGAAGAAGTCTG9-3'. Lowercase letters indicate spacer sequences, whereas uppercase letters indicate DCDC5 mRNA sequence.

Flow cytometry

For each sample, 1 × 10⁶ cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, resuspended in 1 ml PBS containing 1 mg/ml RNase and 40 µg/ml propidium iodide (Sigma), incubated in the dark for 30 minutes at room temperature, and finally analyzed using fluorescence-activated cell sorting (FACS) at ~200 cells/second on a LSRRI flow cytometer system (BD Biosciences).

MTT assay

HeLa cells were plated at a density of 5 × 10⁴ cells per 100 µl. MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 0.5 mg/ml, Sigma] was added to the wells 48 hours after plating. The plate was incubated for 2 hours at 37˚C, after which, the medium were removed and 170 µl of DMSO was added to each well to dissolve the formazan crystals that had formed. The absorbance at 550 nm was determined using an ELISA (enzyme-linked immunosorbent assay) plate reader.

Immunostaining, time-lapse sequences and image collection

HeLa cells were plated on 13-mm-thick cover slips (Menzel-Glaeser, Braunschweig, Germany). Forty-eight hours after transfection, cells were fixed for 20 minutes at room temperature with 4% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA and 4 mM MgSO4, pH 6.9) buffer and treated as previously described (Coquelle et al., 2006). For methanol fixation, coverslips were incubated in cold methanol for 5 minutes at −20°C. Immunostained cells were visualized using wide-field microscopy (DeltaVision, Applied Precision, CA). As indicated in the legends, a number of 200-300 z-sections were deconvoluted and projected to a maximal intensity projection image using Delta Vision software. Where indicated, we used DeltaVision OMX, a three-dimensional structured illumination microscopy system that provides super-resolution imaging. DeltaVision OMX surpasses the 250-nm resolution limit of conventional microscopy by a factor of two and enables imaging beyond the surface of the coverslip with multiple probes. For time-lapse sequences, HeLa cells were grown on 35-mm-diameter glass-bottom microwell dishes (MatTek, Ashland, MA) overnight and were visualized using the DeltaVision System (Applied Precision, CA). Each recording session lasted from 3 to 6 hours at 37˚C, and frames were taken every 2.5 minutes. Eight 500-nm z-sections were deconvoluted and projected to obtain a maximal intensity projection image for each point time using DeltaVision software. The Pearson correlation coefficient (PCC) was used to quantify the degree of colocalization between fluorophores. We used the Java constraint solver tool JACoP to analyze the PCC’s (Bolte et al., 2006). The dynein inhibitor, 1 mM EHNA [erythry9-(2-hydroxy-3-nonyl)adenine; Sigma, St. Louis, MO] was added before beginning the time-lapse sequences. Images and time-lapse sequences were processed using the DeltaVision system package, or Imaris software (for 3D images; Bitplane). Statistical analysis was conducted using Prism 4 for Macintosh (GraphPad Software, Inc.). The microtubule binding assay was conducted as previously described (Horesh et al., 1999).

Immunoblot and immunoprecipitation

Adherent cultured cells were washed in cold PBS and collected using cell scrapers. Mitotic synchronized cells were collected by the shake off method and cell pellets were obtained. Cell pellets were either flash frozen in liquid nitrogen and stored at −80°C for later lysis or were lysed directly by resuspension in lysis buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 1 mM sodium vanadate supplemented with protease inhibitor cocktail (Sigma). For immunoblots, 40 µg protein was mixed with SDS sample buffer and separated by SDS-PAGE. The protein lysates were incubated with the appropriate antibodies for 2 hours at 4°C. Thereafter, 15 µl (bed volume) of protein A/G agarose (Santa Cruz) pre-blocked in lysis buffer supplemented with 10 µg/ml BSA (Sigma) was added to each sample for another 2 hours. Immunoprecipitated proteins were pelletted by centrifugation and washed three times in lysis buffer. The proteins were eluted from the beads by adding SDS sample buffer, then boiled for 3 minutes, separated on 10% SDS-PAGE, and finally subjected to western blot analysis with the indicated antibodies.

Semi quantitative RT-PCR

RNA from confluent cells in 10-cm dishes of non-synchronized or synchronized HeLa cells was isolated using TRI REAGENT (Sigma). Total RNA (5 µg) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), and 1/25 of the reverse-transcribed material was used in PCR reactions. The DCDC5 cDNA domain transcript was amplified using the following primers: F1 5’-TCCGCTGAGCAGCGTCTCCTGGTAAATGTTGGGA9-3’ and R1 5’-ACGGGTACCATGATGATGATGACACACAGACA9-3’. The GAPDH transcript was amplified using primers F1 5’-ATCGGCACACACCTTCTCAATGAGCTGCG-3’ and R1 5’-CTCCACCTCCTGCTGGTCACACATGC-3’.
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References


Table S1. Phenotype of *DCDC5* shRNA-treated HeLa cells

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Control</th>
<th>DCDC5 shRNA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 3 positive cells</td>
<td>3.5 ± 0.29</td>
<td>6.2 ± 0.65</td>
<td>0.0018</td>
</tr>
<tr>
<td></td>
<td>n (cell number) = 2023</td>
<td>n = 1194</td>
<td></td>
</tr>
<tr>
<td>Viable cells (Normalized)</td>
<td>100</td>
<td>68 ± 0.08</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>n (repeats)=10</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Multinuclear cells (&gt;2 N)</td>
<td>7.4 ± 1.1</td>
<td>14.5 ± 1.2</td>
<td>0.002</td>
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<tr>
<td></td>
<td>n (cell number) = 1268</td>
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