

Nup358 interacts with APC and plays a role in cell polarization

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Summary

Asymmetric localization of adenomatous polyposis coli (APC) to the ends of a subset of microtubules located in the leading edges is essential for the establishment of front-rear polarity during cell migration. APC is known to associate with microtubules in three ways: through interaction with the plus-end tracking protein EB1, direct binding through a C-terminal basic region, and through interaction with the plus-end motor kinesin-2. Here we report that the middle region of APC has a previously unidentified microtubule plus-end-targeting function, suggesting an additional microtubule-binding mode for APC. Through the same region, APC interacts with Nup358 (also called RanBP2), a microtubule-binding nucleoporin. Ectopic expression of the middle region of APC is sufficient to recruit endogenous Nup358 to the plus ends of

microtubules. Furthermore, our results indicate that Nup358 cooperates with kinesin-2 to regulate the localization of APC to the cell cortex through a nuclear-transport-independent mechanism. Using RNA interference and a scratch-induced wound-healing assay we demonstrate that Nup358 functions in polarized cell migration. These results reveal a more active role for structural nucleoporins in regulating fundamental cellular processes than previously anticipated.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/122/17/3113/DC1>

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Introduction

The microtubule cytoskeleton plays pivotal roles in cell movement, intracellular trafficking, mitosis and cell polarity. Microtubules, which are polymers of α - and β -tubulin subunits, alternate stochastically between growth and shrinkage phases (Desai and Mitchison, 1997). These dynamic microtubules are transiently stabilized when captured by cellular structures including cell cortex and kinetochores. Such a remodeling of microtubule cytoskeleton is important for coordinating the polarization process that involves establishment and maintenance of distinct membrane domains, segregation and positioning of organelles, and targeted delivery of cell fate determinants (Gundersen, 2002). A group of proteins that accumulate at the plus ends of microtubules, collectively referred to as +TIPs, are implicated in regulating microtubule plus-end dynamics and stabilization (Akhmanova and Steinmetz, 2008). Interestingly, APC associates with plus ends of a subset of microtubules present at the cell cortex and is implicated to have a role in regulating aspects of microtubule function and polarization (Barth et al., 2008; Bienz, 2002; Mimori-Kiyosue et al., 2000; Nathke et al., 1996). APC is also a tumor suppressor that is truncated in most colorectal cancers and has a well-established role in negatively regulating Wnt signaling by mediating the degradation of β -catenin (Bienz and Clevers, 2000; Nathke, 2004; Polakis, 2000). However, its role in microtubule reorganization and cell polarity is not well understood.

In a study involving live imaging of cells microinjected with fluorescently labeled APC monoclonal antibodies, the microtubule ends associated with endogenous APC were shown to have increased growth stability as compared with non-APC-decorated microtubules (Kita et al., 2006). This indicates that APC at cell extensions might favor net microtubule growth. Further experiments also suggested that the interaction of APC with microtubules could

occur independently of its association with EB1 (Kita et al., 2006). Although the exact mechanism remains elusive, the asymmetric localization of APC at the leading edges in different cellular contexts appears to be regulated by the small GTPases Cdc42, Rac1 and Rho acting through downstream effectors that include the Par3/Par6/APKC polarity complex, IQGAP1 and mDia (Etienne-Manneville and Hall, 2003; Henderson and Fagotto, 2002; Watanabe et al., 2004; Wen et al., 2004). Recently, Wnt signaling has also been reported to control the localization of APC and cell polarity (Schlessinger et al., 2007). Interestingly, glycogen synthase kinase 3 β , which is a component of the Wnt signaling pathway and involved in the phosphorylation of β -catenin, also phosphorylates APC and negatively regulates its microtubule-binding functions (Zumbrunn et al., 2001). In addition, other Wnt pathway players are also known to regulate microtubule dynamics and cell polarity (Salinas, 2007; Schlessinger et al., 2007); however, the details of how Wnt signaling and cell polarization are coordinated remain unclear.

During mitosis, APC is targeted to the kinetochores in a microtubule-dependent manner, and depletion of APC has been shown to impair chromosome segregation, implicating a role for APC in regulating kinetochore-microtubule interactions (Fodde et al., 2001; Green et al., 2005; Kaplan et al., 2001). Nup358, a Ran-binding nucleoporin (Saitoh et al., 1996; Wu et al., 1995), has a similar microtubule-dependent localization and a regulatory function at the kinetochores (Joseph et al., 2002; Joseph et al., 2004; Joseph, 2006). Nup358 contains many distinct motifs that are required for protein-protein interactions. Two internal repeats present in the C-terminal region of Nup358 interact with SUMO and Ubc9, the SUMO-specific E2 enzyme, and possesses both *in vitro* and *in vivo* SUMO E3-like activity (Kirsh et al., 2002; Pichler et al., 2002; Reverter and Lima, 2005; Saitoh et al., 1997). Recent studies suggest

that the mitotic function of Nup358 includes SUMOylation and regulation of topoisomerase II and borealin, two proteins involved in chromosome alignment and segregation (Dawlaty et al., 2008; Klein et al., 2009).

In interphase, Nup358 localizes to the cytoplasmic face of the nuclear pore complex and is expected to play a role in nucleocytoplasmic transport. Using an *Xenopus* egg extract system, it was reported that depletion of Nup358 did not affect the import of nuclear localization signal (NLS) containing protein cargos into the nucleus (Walther et al., 2002). Studies in mammalian cells have shown that Nup358 plays a supporting role in CRM1-mediated nuclear export of proteins (Bernad et al., 2004; Hutten and Kehlenbach, 2006). RNAi-mediated depletion of *Drosophila* Nup358, however, revealed a role for Nup358 in the export of mRNA (Forler et al., 2004). A recent study indicated that Nup358 facilitates α - and β -importin-mediated nuclear import of proteins in mammalian cells (Hutten et al., 2008). Collectively, these results suggest that Nup358 might play a regulatory role in nucleocytoplasmic transport.

Earlier, we showed that Nup358 associates with and regulates interphase microtubules through its N-terminal leucine-rich-region to control cell migration (Joseph and Dasso, 2008). Owing to the similarities between APC and Nup358, with respect to their localization and regulation both in interphase and in mitosis, we

hypothesized that these two proteins are functionally related. Here we report that Nup358 interacts with APC and regulates its asymmetric accumulation at the cell edges. Furthermore, our results suggest a role for Nup358 in cooperating with APC to control cell polarity during directional migration.

Results

APC associates with Nup358 in vivo

Immunoprecipitation of HEK293 cell lysate using Nup358 antibodies, followed by western blot analysis of APC, indicated that Nup358 interacts with APC in vivo (Fig. 1A). To explore the connection between APC and Nup358 further, we exogenously expressed red fluorescent protein-tagged full-length APC (RFP-APC-FL) and analyzed the distribution of endogenous Nup358. RFP-APC-FL localized to the cortical clusters, as observed with endogenous APC (Mimori-Kiyosue et al., 2000). Interestingly, endogenous Nup358 was recruited to these clusters, whereas Nup358 showed a diffused staining at the cell edges in control RFP-expressing cells (Fig. 1B). Closer examination confirmed that Nup358 localized to individual microtubules that were positive for RFP-APC-FL near the cell periphery (supplementary material Fig. S1). Moreover, a similar staining of endogenous Nup358 in cells expressing RFP-APC-FL was observed with another antibody against Nup358 (Yokoyama et al., 1995) (supplementary material

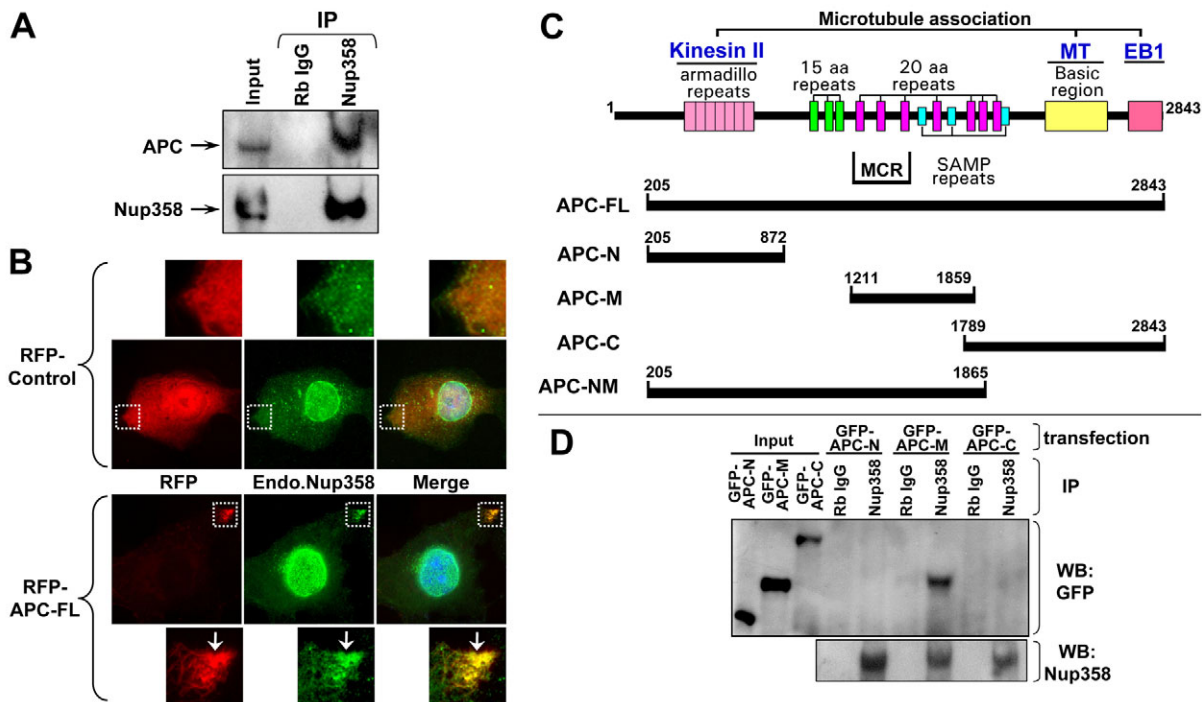


Fig. 1. Nup358 associates with APC in vivo. (A) APC co-immunoprecipitates with Nup358. HEK293 cell lysate was subjected to immunoprecipitation (IP) using anti-Nup358 antibodies and the immunoprecipitate was probed for the presence of APC by western blotting. Rabbit IgG (Rb IgG) was used in control IP. (B) Endogenous Nup358 is recruited to a subset of microtubule plus ends that are proximal to the cell cortex by the exogenously expressed APC. COS-7 cells were transfected with pDsRed-Ex-C1 vector (RFP) or pDsRed-APC-FL (RFP-APC-FL) (red) and analyzed for the localization of endogenous Nup358 (Endo. Nup358; green) using specific antibodies. DNA was visualized by Hoechst staining (blue). Magnified views of the marked regions confirm co-localization of endogenous Nup358 with RFP-APC-FL at the cell cortex (arrows in bottom panel). (C) Schematic representation of APC with different domains and of the constructs used in this study. The corresponding amino acids are indicated in numbers. APC is known to associate with microtubules in three ways as indicated: through interaction with kinesin-2 mediated by the ARM domain of APC, through interaction with EB1 and through a direct binding mediated by the C-terminal basic region of APC (MT). Mutation cluster region (MCR) represents the region that is mutated in most colorectal cancers (Polakis, 2000). (D) The middle region of APC is required for association with Nup358. Different fragments of APC were transiently expressed in HEK293 cells as GFP-fusions and the lysate was subjected to IP using anti-Nup358 antibodies. The immunoprecipitates were probed with GFP antibodies to identify the region of APC that is required for interaction with Nup358. Rabbit IgG was used for control IPs.

Fig. S2). Taken together, these data suggest that Nup358 specifically associates with microtubules in an APC-dependent manner.

Middle region of APC possesses both plus-end-targeting and Nup358-binding functions

To delineate the region within APC that is required for the interaction with Nup358, HEK293 cells transiently expressing different fragments of APC (Fig. 1C) were subjected to immunoprecipitation with Nup358 antibodies. The middle region (1211-1859 amino acids), hereafter called APC-M, was specifically detected in the Nup358 immunoprecipitate (Fig. 1D), indicating that this region is sufficient for the association with Nup358.

Notably, transient expression of RFP-GST-APC-M in mammalian cells resulted in its localization to distinct comet-like structures in the cytoplasm. To test whether they represented the plus ends of growing microtubules, we co-stained with antibodies against EB1, a prototypical +TIP. The result confirmed that APC-M co-localized with EB1 at the tips of growing microtubules, suggesting that this region has an independent plus-end-targeting function (Fig. 2A, arrows). A closer examination, however, shows that the localization of EB1 is distinct from that of RFP-GST-APC-M (Fig. 2A, arrows). EB1 had a stronger localization to the distal ends than to the proximal region of microtubules, giving a comet-like appearance, whereas RFP-GST-APC-M showed a more-or-less uniform localization at the plus ends, with partial overlap with EB1. The functional significance, if any, of this spatial difference in the localization of EB1 and APC-M at the microtubule plus ends is unclear. Furthermore, live-cell imaging studies indicated that the dynamics of RFP-GST-APC-M at the microtubule ends is similar to that of EB1 (supplementary material Movie 1).

As the middle region of APC is also involved in interaction with Nup358, we examined the localization of endogenous Nup358 in cells expressing RFP-GST-APC-M. Immunostaining analysis showed that endogenous Nup358 co-localized with RFP-GST-APC-M at the ends of microtubules (Fig. 2B, arrows), indicating that this region of APC is sufficient to recruit endogenous Nup358 to microtubules. By contrast, the C-terminal region of APC (2560-2843 amino acids) consisted of the EB1-binding domain localized to the plus ends of microtubules as expected but failed to recruit endogenous Nup358 to the microtubule tips (supplementary material Fig. S3A,B), confirming the specificity of the *in vivo* interaction between the middle region of APC and Nup358.

Previously, expression of the middle region of APC was shown to downregulate the level of endogenous β -catenin in SW480 colorectal cancer cells (Munemitsu et al., 1995). However, we found no significant difference between the levels of endogenous β -catenin in COS-7 cells expressing RFP-GST or RFP-GST-APC-M (data not shown). This ruled out the possibility that microtubule plus-end-localization and recruitment of endogenous Nup358 by APC-M are due to an indirect effect caused by changes in β -catenin levels. Based on these results, we conclude that the middle region of APC has both independent microtubule-plus-end-targeting and Nup358-binding functions.

Localization of APC-M and recruitment of endogenous Nup358 to plus ends of microtubules occur independently of EB1

Localization of APC-M to the plus ends of microtubules prompted us to investigate whether EB1 is required for the localization. COS-7 cells were co-transfected with control or EB1-specific siRNAs and RFP-GST or RFP-GST-APC-M, and analyzed for the

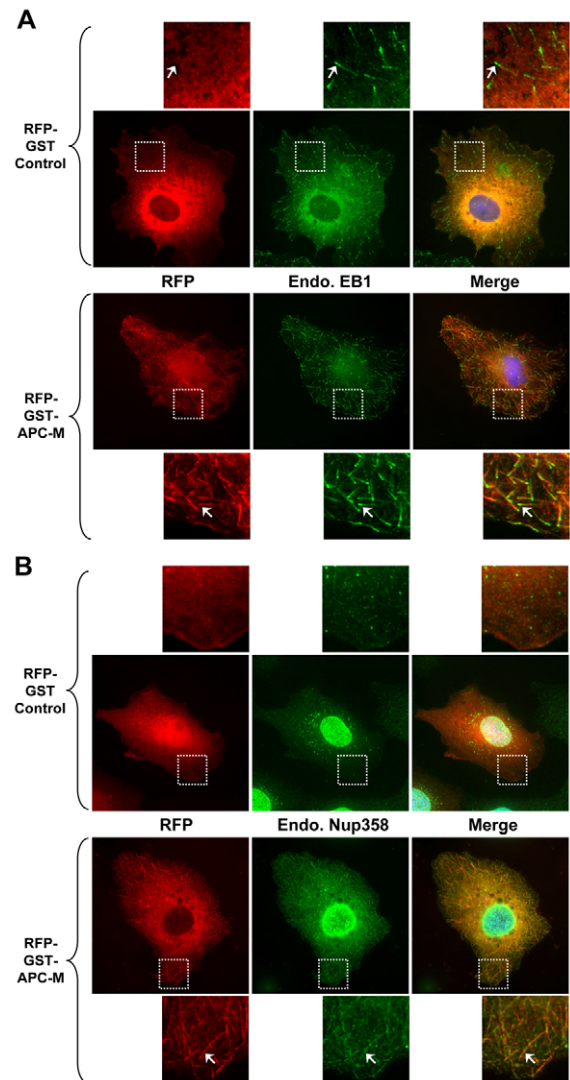


Fig. 2. The middle region of APC has a microtubule plus-end-targeting function and is sufficient to recruit endogenous Nup358 to plus ends. (A) APC-M localizes to the growing plus ends of microtubules. COS-7 cells transfected with RFP-GST or RFP-GST-APC-M (red) were fixed and stained for endogenous EB1 (green) using specific antibodies. DNA was visualized by Hoechst staining (blue). Closer views of the marked regions reveal partial co-localization of RFP-GST-APC-M with EB1 at the microtubule tips (arrows, bottom panel), whereas RFP control showed no co-localization with EB1 (arrows, upper panel). (B) Endogenous Nup358 co-localizes with APC-M at the plus ends of microtubules. RFP-GST or RFP-GST-APC-M (red) transfected COS-7 cells were analyzed for endogenous Nup358 (green) localization. DNA was stained with Hoechst (blue). Bottom panel shows magnified views of the marked regions and arrows indicates co-localization of Nup358 and APC-M at microtubule plus ends.

localization of APC-M. EB1 siRNA specifically knocked down the level of EB1 in COS-7 cells (Fig. 3A). Interestingly, APC-M localized to the plus ends of microtubules both in control and EB1 siRNA-treated cells, indicating that EB1 is dispensable for its localization (Fig. 3B). Moreover, the ability of APC-M to recruit endogenous Nup358 to the microtubule ends was unaffected in the absence of EB1 (Fig. 3C). Based on the results, we conclude that the localization of APC-M and recruitment of endogenous Nup358 to microtubule ends are not dependent on EB1.

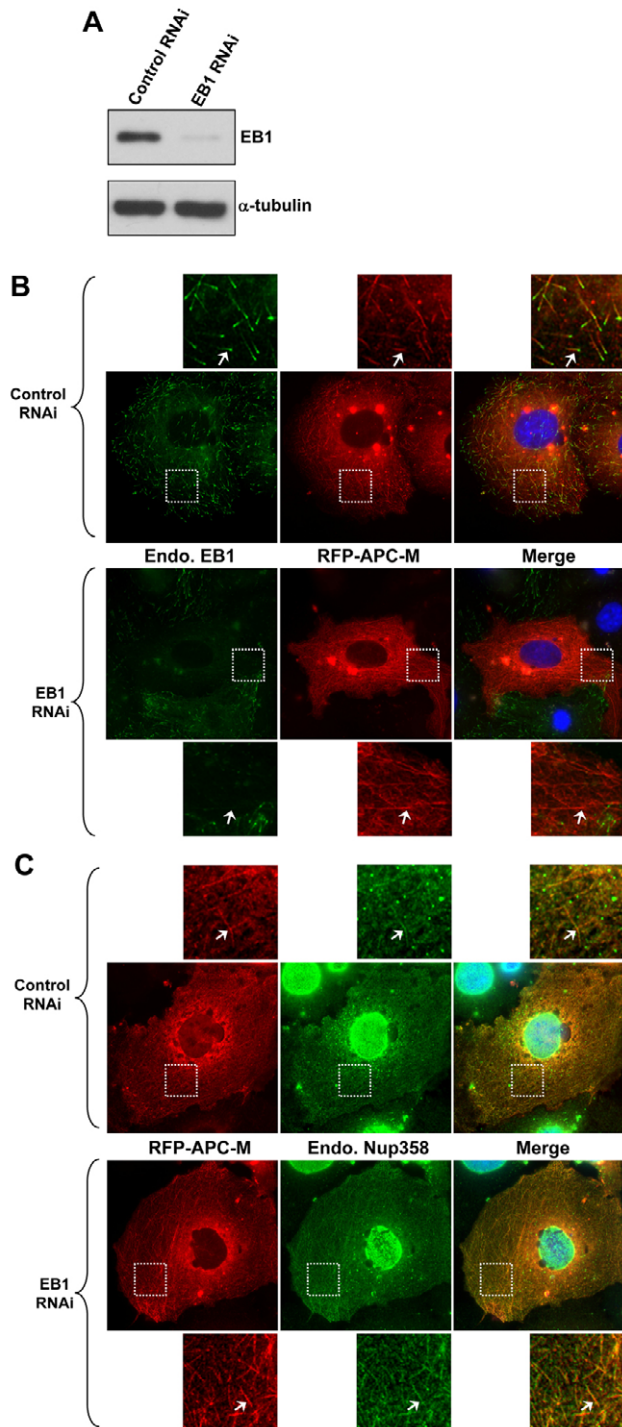


Fig. 3. Localization of APC-M and recruitment of endogenous Nup358 to microtubule plus ends are independent of EB1. (A) COS-7 cells were transfected with control (Control RNAi) or EB1-specific (EB1 RNAi) siRNAs for 64 hours and the level of EB1 was analyzed by western blotting. α -tubulin was used as loading control. (B) The cells were co-transfected with control or EB1 siRNA and RFP-GST or RFP-GST-APC-M, and 64 hours later stained for endogenous EB1 using specific antibodies. Arrows in magnified views of the marked regions show localization of RFP-GST-APC-M and endogenous EB1. Note that APC-M localizes to microtubule ends in EB1-depleted cells. (C) Cells as treated in B were fixed and stained for endogenous Nup358. Arrows in magnified views of the marked regions indicate co-localization of RFP-GST-APC-M and endogenous Nup358 both in control and EB1 RNAi cells.

Interaction between Nup358 and APC does not require axin and β -catenin

APC-M has four β -catenin-binding 20 amino acid repeats and two axin-binding SAMP repeats (Fig. 1C). We wished to explore the possibility that axin and/or β -catenin might be involved in the interaction between APC and Nup358. To investigate whether axin mediated the interaction between APC and Nup358, we performed Nup358 immunoprecipitation from HT29, a colorectal cancer cell line that expresses a truncated APC protein that retains amino acids 1-1555 (containing part of APC-M but lacking all axin-binding domains). Truncated APC from HT29 could be co-immunoprecipitated with Nup358 (Fig. 4A), suggesting that the interaction between APC and Nup358 does not require axin.

To investigate whether β -catenin is involved, we used two cell lines that differ in their endogenous β -catenin levels: CHO-K1 with no detectable level of β -catenin and HCT-116, a CRC cell line that has higher levels of β -catenin owing to a mutation in its phosphorylation site (Fig. 4B). We predicted that if β -catenin has any role in mediating interaction between APC and Nup358, the ability of exogenously expressed APC or its fragments to recruit endogenous Nup358 to the cell cortex should be compromised in either of the cell lines. However, we found that endogenous Nup358 was efficiently recruited to the cell cortex by RFP-APC-FL or RFP-APC-NM (RFP-tagged N-terminal and middle regions of APC) in both cell lines (Fig. 4C,D, arrows). Taken together, the results suggest that β -catenin is not essential for the *in vivo* interaction between APC and Nup358. However, we cannot rule out the possibility that both axin and/or β -catenin could modulate the interaction between Nup358 and APC *in vivo*.

Nup358 cooperates with kinesin-2 to regulate APC localization

Previous studies have shown that APC requires interaction with kinesin-2 for its localization to clusters (Jimbo et al., 2002). The C-terminal basic region and the EB1-binding region are dispensable for APC clustering at the cell cortex, whereas binding of kinesin-2 is essential but not sufficient for this localization (Jimbo et al., 2002; Zumbunn et al., 2001). In addition to the N-terminal ARM repeats, through which APC interacts with kinesin-2, the middle region also is essential for localization of APC to the cell cortex (Jimbo et al., 2002). Because APC interacts with Nup358 through its middle region, it is possible that this association is crucial for APC localization. Kinesin-2 is a heterotrimer containing two motor subunits (KIF3A and KIF3B) and a non-motor accessory subunit (KAP3) (Hirokawa, 1998). To test the possible interaction between Nup358 and kinesin-2, the Nup358 immunoprecipitate was probed with KAP3 and KIF3A/B antibodies. Nup358 showed specific interaction with KAP3 and KIF3A (Fig. 5A). However, KIF3B was not recognized in either the input or the immunoprecipitate by the kinesin-2 antibody that was used. Together, these results suggest that Nup358 might exist in a larger *in vivo* complex containing APC and kinesin-2.

The above results point to the intriguing possibility that a trimolecular interaction between Nup358, APC and kinesin-2 is essential for proper recruitment and/or stabilization of APC at the cell cortex. In agreement with this idea and as previously reported (Jimbo et al., 2002), individual APC fragments that possessed either the kinesin-2-binding region alone (APC-N; 205-872 amino acids) or the Nup358-interacting domain alone (APC-M; 1211-1859 amino acids) showed significantly reduced localization to cell edges, whereas the fragments that harbored both kinesin-2- and Nup358-binding regions (APC-NM; 205-1865 amino acids) were localized to clusters in a similar way to the full-length APC (Fig. 5B). APC-

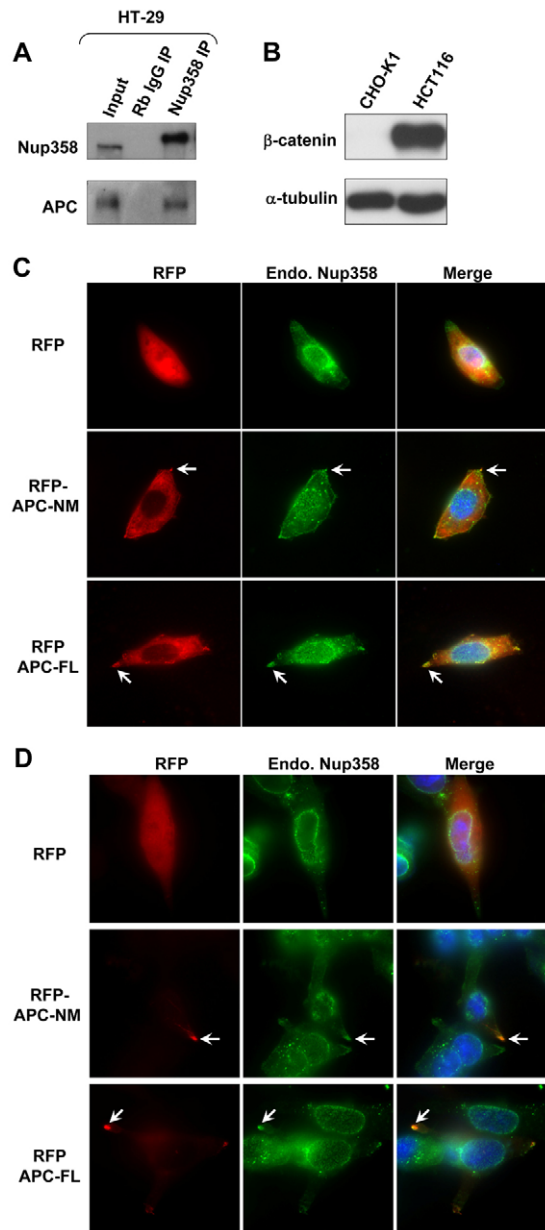


Fig. 4. Recruitment of endogenous Nup358 to cell cortex by RFP-APC-FL or RFP-APC-NM does not depend on axin or β -catenin levels. (A) HT29 cell lysates were subjected to immunoprecipitation using Rabbit IgG (Rb IgG IP) or Nup358 (Nup358 IP) antibodies. The immunoprecipitates were analyzed for the presence of APC by western blotting. HT29 expresses a truncated APC protein that retains 1-1555 amino acids. (B) CHO-K1 and HCT116 cells were lysed and analyzed for the level of β -catenin by western blotting. α -tubulin was used as loading control. (C) CHO-K1 and (D) HCT116 cells were transfected with RFP, RFP-APC-FL or RFP-APC-NM (red) and were stained for endogenous Nup358 (green) using specific antibodies. DNA is represented in blue. Arrows indicate co-localization of APC and Nup358. Note that endogenous Nup358 co-localizes with expressed RFP-APC-FL or RFP-APC-NM both in CHO-K1 and HCT-116 cells, which have a significant difference in their β -catenin levels.

NM also recruited endogenous Nup358 to the clusters in almost all expressing cells. Notably, localization of endogenous KAP3 to the clusters was substantially enhanced when RFP-APC-NM or RFP-APC-FL was exogenously expressed, as compared with RFP control (supplementary material Fig. S4). Because both APC-NM

and APC-FL also recruited the endogenous Nup358 to cortical clusters (Fig. 5B), our data are consistent with the formation and stabilization of a tri-molecular complex between APC, Nup358 and kinesin-2 at the cell cortex.

To test whether Nup358 is required for APC localization, Nup358-depleted cells were immunostained for the distribution of endogenous APC (Fig. 5C,D). In control siRNA-treated cells, in addition to the accumulation at the leading edges, APC localized to a spot near the nucleus, possibly indicating the Golgi apparatus. In Nup358-depleted cells, APC was redistributed in the cytoplasm; APC showed reduced clustering at the cell cortex, but was concentrated around the perinuclear region. Western blot analysis showed that there was no substantial difference between the levels of APC in control and Nup358 siRNA-treated cells (supplementary material Fig. S5). These results indicate a role for Nup358 in the cytoplasmic distribution of APC, particularly in its localization to the cell cortex. As expected, depletion of KAP3 also reduced the number of cells with APC clusters at the cell extensions (Fig. 5C,E). Additionally, APC-M overexpression, which is expected to interfere with the interaction between endogenous APC and Nup358, substantially affected the formation of APC clusters at the cell cortex (Fig. 5F,G, arrows). From these results, we conclude that polarized accumulation of APC at the leading edges requires functional interaction with both kinesin-2 and Nup358.

Nup358 regulates polarized accumulation of APC in a nuclear-transport-independent manner

APC has been reported to be a nucleo-cytoplasmic shuttling protein and exported from the nucleus in a CRM1-dependent manner (Henderson and Fagotto, 2002). Nup358 binds to components of the nuclear transport machinery and has been implicated in nucleo-cytoplasmic transport of proteins, although the exact role is far from clear (Bernad et al., 2004; Hutten and Kehlenbach, 2006). Thus, it is possible that the failure of APC to accumulate at the cell cortex in the absence of Nup358 could be due to defective export of APC from the nucleus. To test this, we treated cells with leptomycin B (LMB), an inhibitor that blocks the CRM1-dependent export of macromolecules (Fukuda et al., 1997), and examined for the localization of APC by indirect immunofluorescence. In contrast to the previous report, we observed no detectable accumulation of APC in the nucleus after LMB treatment for 20 hours. Also, LMB had no significant effect on the clustering of APC at the cell cortex as compared with the control cells (supplementary material Fig. S6). In these experiments, accumulation of p53 in the nucleus due to inhibition of its CRM1-dependent nuclear export was monitored to ascertain the activity of LMB (Freedman and Levine, 1998). Additionally, treatment of cells with siRNA oligonucleotides against Nup358 resulted in no detectable difference in the distribution of APC between nucleus and cytoplasm (Fig. 5D). Collectively, these data argue for a nuclear-transport-independent role for Nup358 in the accumulation of APC at the leading edge. Also, at least in Vero cells, CRM-1-mediated export of APC might not be a rate-limiting or regulatory step in the localization of APC to the cell cortex.

Nup358 plays an essential role in polarized cell migration

The functional consequence of the reduced APC clusters due to Nup358 depletion was explored by a wound-migration assay. Control cells or Vero cells treated with Nup358-specific siRNA were allowed to migrate into a scratch-induced wound and the centrosome positioning within the cell was analyzed by immunostaining with

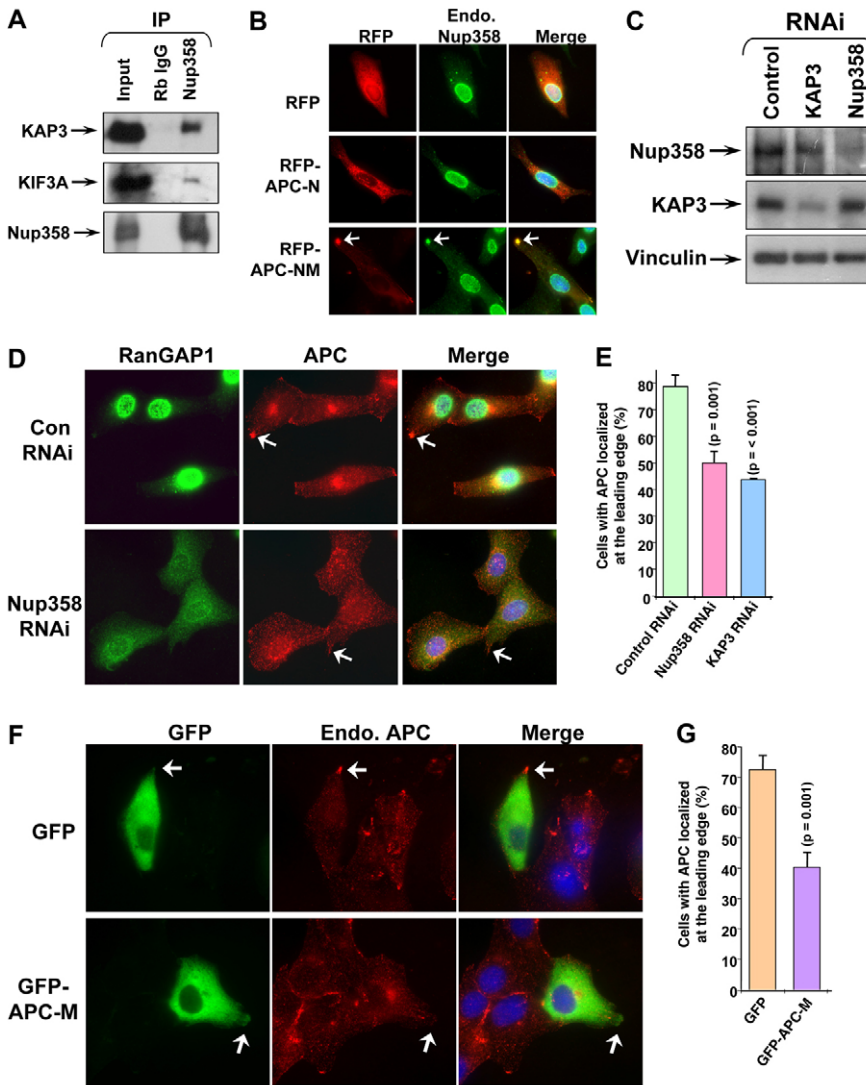


Fig. 5. Interactions between Nup358, APC and kinesin-2 are required for APC to accumulate at the leading edge. (A) Nup358 interacts with kinesin-2 *in vivo*. The Nup358 immunoprecipitate (IP) from HEK293 cell lysate was probed by western blotting for the presence of KAP3 and KIF3A/3B using specific antibodies. Rabbit IgG (Rb IgG) was used in the control IP. (B) Presence of both kinesin-2- and Nup358-binding regions are required for APC cluster localization. Vero cells were transfected with RFP, RFP-APC-N or RFP-APC-NM (red) and stained for endogenous Nup358 (Endo. Nup358; green). DNA was visualized by Hoechst staining (blue). Arrows indicate the APC localization in cell extensions. (C) RNAi-mediated knockdown of Nup358 and KAP3 in Vero cells. Cells were treated with control, Nup358-, or KAP3-specific siRNA oligonucleotides (RNAi) and western blotting was performed to analyze the extent of knockdown. Vinculin was used as loading control. (D) Nup358 and KAP3 RNAi impair APC clustering in Vero cells. Cells were treated with control, Nup358- or KAP3-specific siRNAs and stained for endogenous APC (red) using specific antibodies. Redistribution of RanGAP1 (green) from the nuclear membrane to the cytoplasm was monitored to identify the Nup358 knocked-down cells (Joseph et al., 2004). DNA is represented in blue. Arrows indicate the APC localization in cell extensions, which is significantly reduced in Nup358-depleted cells (lower panel). (E) Quantitative graphic representation of APC cluster formation in Vero cells after Nup358 or KAP3 knockdown as compared with control siRNA-treated cells. The results were representative of three independent experiments. Error bars indicate standard deviations; $n=100$; P -values versus control cells are indicated, calculated using the Student's t -test. (F) APC-M acts in a dominant-negative manner to reduce APC accumulation at the cell cortex. Vero cells were transfected with GFP or GFP-APC-M (green) and stained for endogenous APC (red). DNA is in blue. (G) Quantitative representation of GFP or GFP-APC-M overexpressing cells that were positive for localization of endogenous APC to cell extensions. The results were representative of three independent experiments. Error bars indicate standard deviations; $n=100$; P -values versus control cells are indicated, calculated using the Student's t -test.

anti- γ -tubulin antibodies. Cells at the wound edges were considered polarized if the centrosome was located in front of the cell nucleus towards the direction of the wound. In Nup358-depleted cells, centrosome polarization was significantly reduced as compared with the control siRNA-treated cells (Fig. 6A,B). Moreover, a similar effect was observed with a different siRNA directed against Nup358 (Joseph and Dasso, 2008) (data not shown). These results suggest that Nup358 plays an important role in cell polarization during directional migration.

N- and C-terminal fragments of Nup358 affect APC localization and cell polarity

To understand the molecular basis of the interaction between Nup358 and APC further, we co-transfected COS-7 cells with RFP-GST-APC-M and constructs expressing different fragments of Nup358 as GFP fusions (Fig. 7A) and analyzed whether any fragment co-localized with APC-M. GFP-BPN localized to the interphase microtubules as reported previously (Joseph and Dasso, 2008) and, interestingly, APC-M partially co-localized with BPN on microtubules, whereas BPM (901-2219 amino acids) and BPC (2220-3224 amino acids) showed diffused staining in the cells, with no co-localization with APC-M (Fig. 7B, arrow). In many coexpressing cells, APC-M often decorated

the entire microtubules along with BPN (1-900 amino acids). These results possibly suggest that the middle region of APC interacts with the N-terminal region of Nup358. However, we could not detect interaction between APC-M and BPN in immunoprecipitation assays (data not shown), indicating that APC-M and BPN could localize to microtubules independently of each other or that the interaction between APC and Nup358 could occur in the context of polymerized microtubules *in vivo*.

To explore whether any regions of Nup358 interfered with the formation of APC clusters, Vero cells were transfected with GFP vector control, GFP-BPN, GFP-BPM or GFP-BPC and analyzed for the localization of endogenous APC to the cell edges. Both BPN and BPC significantly interfered with APC localization as compared with GFP control, whereas the middle region of Nup358 (BPM) did not show any discernible effect on the extent of APC cluster formation at the cell edges (Fig. 7C,D). To investigate whether overexpression of these regions affected cell polarization during migration, Vero cells transfected with different fragments of Nup358 were subjected to the scratch-induced cell migration assay. Consistent with interference in the localization of APC upon overexpression, BPN and BPC fragments significantly affected cell polarity during motility (Fig. 7E,F). These data show that BPN and

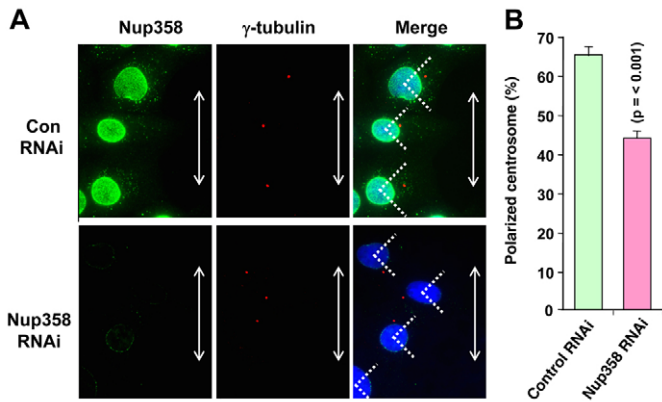


Fig. 6. Nup358 RNAi impairs polarization during directed migration. (A) Vero cells were subjected to control or Nup358-specific siRNA treatment. The monolayer of cells was then scratched and four hours later immunostained with Nup358 (green) and γ -tubulin (red) specific antibodies. DNA is stained with Hoechst (blue). We scored centrosome polarization as positive when centrosomes were localized within a 90° sector (borders are marked in white dotted lines) facing the wound (wound is indicated by double-edged arrow). (B) Quantitation of centrosome polarization in cells treated with the indicated siRNA. The results were representative of three independent experiments. Error bars indicate standard deviations; $n=100$; P -values versus control cells are indicated, calculated using the Student's t -test.

BPC interfered with APC localization to the cell cortex in a dominant-negative manner and further support a direct role for Nup358 in regulating APC and polarized cell migration.

Discussion

Previous studies have shown that localization of APC to the cell cortex is dependent on dynamic microtubules (Mimori-Kiyosue et al., 2000; Nathke et al., 1996). APC is known to associate with microtubules in three ways: by direct binding to microtubules and by indirect binding through kinesin-2 and EB1. Here, we show that the middle region of APC, which has an established role in β -catenin signaling, has an independent microtubule plus-end-targeting function as well, thus defining an additional mode of microtubule interaction. Localization of APC-M to the plus ends of microtubules that were positive for EB1 indicates that APC-M associates with the growing ends of microtubules. Could this interaction have any functional role in the localization of APC to cell extensions? At steady state, endogenous full-length APC is detected on a subset of microtubules near the cell edges. It is possible that, through its middle region, APC might interact with the plus ends of all growing microtubules. However, perhaps owing to the difference in the dynamics of association between endogenous APC and the ends of different microtubules, APC is detected only on a subset of microtubules at steady state. Consistent with the idea that APC interacts with the plus end of all growing microtubules, it has been shown that at least in a small proportion of COS-7 cells expressing GFP-APC, APC colocalizes with EB1 in the interior part of the cells (Langford et al., 2006). What determines the dynamic difference between various microtubules as far as APC association is concerned is far from clear. In any case, the plus-end-targeting function of APC-M appears to be important in order for APC to eventually accumulate at the cell cortex. The results from our studies certainly indicate a role for Nup358 and kinesin-2 in achieving the asymmetric accumulation of APC on a specific set of microtubule plus ends. It is possible that other plus-end-binding proteins also play a role in this process. Results from

this and other studies indicate that, at least, EB1 might not be directly involved in this regulation (Kita et al., 2006).

Our results show that the nucleoporin Nup358 interacts with APC and regulates its localization to the cell cortex, and thereby controls cell polarity. Given that Nup358 is implicated in nucleo-cytoplasmic transport, mislocalization of APC in Nup358 RNAi-treated cells could be an indirect effect of impaired nucleo-cytoplasmic transport of APC itself or any protein(s) that might be involved in its localization. However, our results suggest that the distribution of APC between the nucleus and cytoplasm appeared to be more or less similar in control and Nup358 siRNA-treated cells, except that within the cytoplasm APC failed to localize to the cell cortex in the absence of Nup358. Additionally, APC is a large protein and recently it has been shown that there could be many inter- and intra-molecular interactions that might determine the localization and function of this molecule (Li et al., 2008; Li and Nathke, 2005). Therefore, it is possible that these interactions could have been involved in the localization of at least some of the expressed APC fragments in our studies. Nevertheless, our results strongly suggest a direct role for Nup358 in the localization of APC to the leading edge, as discussed below.

Nup358, similar to APC, associates with interphase microtubules and stabilizes them (Joseph and Dasso, 2008). The co-immunoprecipitation and immunofluorescence data presented here support the idea that Nup358 physically interacts with APC. Both Nup358 RNAi and expression of a dominant-negative fragment of APC (APC-M), which is expected to interfere with the interaction between endogenous APC and Nup358, impair the ability of endogenous APC to localize to cell edges. Additionally, the N- and C-terminal fragments of Nup358 also affect the localization of APC to cell edges and cell polarization in a dominant-negative manner. Moreover, Nup358 physically interacts with kinesin-2, a microtubule motor implicated in APC localization. Also, the results provided here suggest that a tri-molecular complex containing APC, Nup358 and kinesin-2 is involved in the localization of APC to cell cortex and in regulation of cell polarity.

The mechanism by which the N- and C-terminal regions of Nup358 interfere with APC localization and cell polarization is still unknown. One possibility is that BPN affected APC localization by interfering with the microtubule association of endogenous Nup358 and/or APC. We failed to detect any interaction between APC-M and fragments of Nup358 using immunoprecipitation assays, possibly indicating that the interaction might involve multiple regions of Nup358. Alternatively, the association of Nup358 with APC requires other proteins that might mediate and/or stabilize the interactions. Further studies are in progress to explore the nature of the interaction between Nup358 and APC, which is expected to shed more light on the mechanistic details of how Nup358 contributes to the targeting of APC to the cell cortex. Nevertheless, these results demonstrate that Nup358 plays a role in polarity, at least in part, by regulating the localization of APC.

A few nucleoporins have been previously implicated in cell polarity, but the molecular mechanism of their action remains elusive (Collin et al., 2008; Schetter et al., 2006). The data presented here, combined with our previous results (Joseph and Dasso, 2008), reveal a novel cytoplasmic function for the nucleoporin Nup358 in regulating cell polarity, at least in part, by modulating the microtubule dynamics and accumulation of APC at the cell cortex. Our results suggest a model wherein Nup358, in co-ordination with kinesin-2, targets APC to the plus ends of microtubules (Fig. 8). We propose that the Nup358-APC-kinesin-2 complex might regulate microtubule dynamics and aid in establishing cell polarity by mediating the capture and

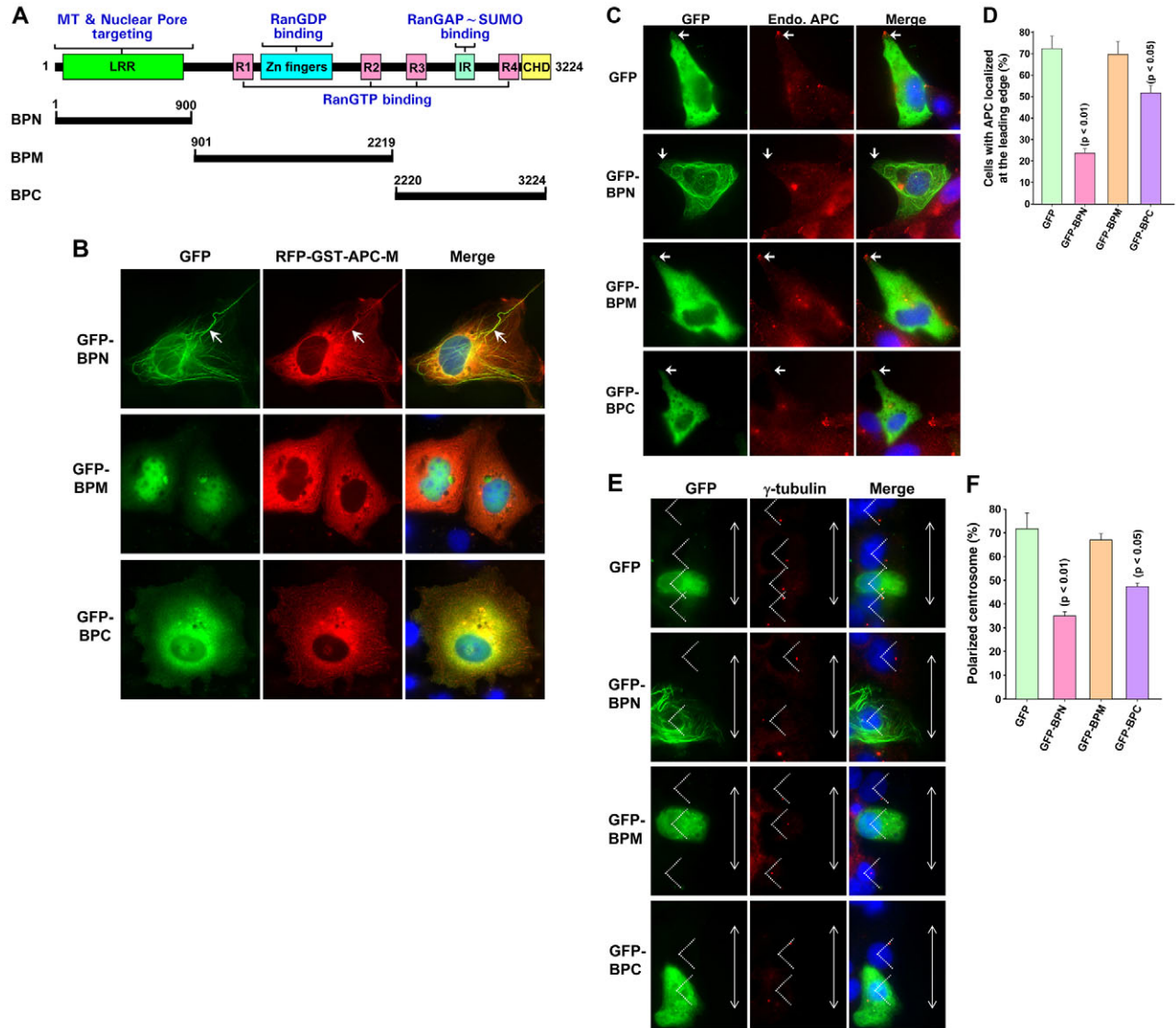


Fig. 7. N-terminal and C-terminal regions of Nup358 negatively affect APC localization and cell polarization. (A) Schematic diagram of Nup358 depicting domains present and different fragments used for this study. LRR, leucine-rich region; R, RanGTP binding domains; IR, internal repeats; CHD, cyclophilin-homology domain. Numbers represent the amino acids (B) COS-7 cells co-transfected with RFP-GST-APC-M (red) and the indicated GFP constructs (green) were subjected to fluorescent microscopy. DNA is represented in blue. Arrows indicate co-localization of APC-M with BPN. (C) Vero cells were transfected with the indicated GFP constructs (green) and stained for endogenous APC (red) using specific antibodies. DNA is stained with Hoechst (blue). Arrows indicate the cell extensions. (D) Quantitative representation of percentage of GFP-positive Vero cells with APC localized to the leading edges after transfection with the indicated constructs. The results were representative of three independent experiments. Error bars indicate standard deviations; $n=100$; P -values versus control cells are indicated, calculated using the Student's t -test. (E) Vero cells transfected with the indicated GFP constructs (green) were subjected to wound-induced migration assay and analyzed for centrosome polarization by staining for γ -tubulin (red) using specific antibodies as in Fig. 6. DNA was stained with Hoechst (blue). (F) Quantitative representation of percentage of GFP-positive cells with polarized centrosomes. The results were representative of three independent experiments. Error bars indicate standard deviations; $n=100$; P -values versus control cells are indicated, calculated using the Student's t -test.

stabilization of microtubules at the cell cortex. In agreement with this notion, both APC and Nup358 have been independently reported to be involved in polarized stabilization of microtubules in migrating cells (Joseph and Dasso, 2008; Kroboth et al., 2007). At steady state, however, we failed to observe the accumulation of endogenous Nup358 at the cell clusters in most cell lines tested, indicating that it might form a transient and dynamic complex with APC. Nup358 could act as a scaffolding protein to localize both APC and kinesin-2 to the microtubule ends proximal to the cell cortex, after which Nup358 might dissociate. However, in some cell lines, such as CHO-K1 and mouse L cells, endogenous Nup358 showed stabilized

accumulation at cell extensions (supplementary material Fig. S7). The extent of accumulation of APC at the cell edges also varied between different mammalian cell lines (data not shown). These observations possibly reflect the difference in the dynamics of both APC and Nup358 at the cell extensions of different cell lines. In any case, our findings indicate that both Nup358 and APC might act in the same pathway that controls cell polarity. The Ran-binding as well as SUMO E3 ligase functions of Nup358 might contribute to its role in regulating APC localization and cell polarity.

Why would a nucleoporin such as Nup358 be involved in regulating cell polarity? Cells could achieve spatial and temporal regulation of

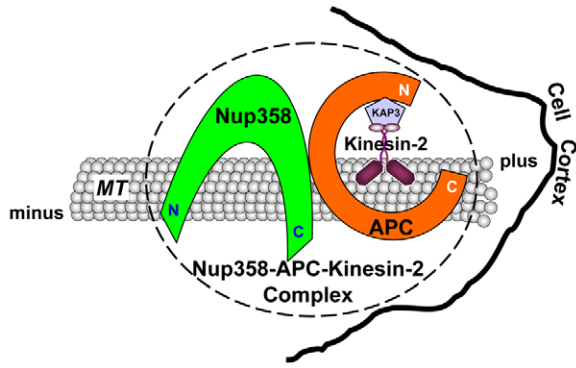


Fig. 8. Model for the function of Nup358 in cell polarity via regulation of APC localization. With the aid of kinesin-2, Nup358 and APC move to the plus ends of growing microtubules (MT). The Nup358-APC-kinesin-2 complex regulates the microtubule plus end dynamics and mediates the capture and stabilization of microtubules by the cortex, after which Nup358 is released.

protein functions during the polarization process in multiple ways. For instance, this could involve regulated trafficking of the protein to a specific region within the cell (Mellman and Nelson, 2008). Availability of the protein at a given time at a defined region within the cell could also be achieved by regulating the localization and translation of its mRNA in a spatio-temporal manner (Shav-Tal and Singer, 2005). Cells might make use of both these mechanisms to generate the asymmetry in protein distribution for establishing and maintaining polarity. While this manuscript was under preparation, APC was shown to play a role in polarized accumulation of a set of specific RNAs at the cell cortex (Mili et al., 2008). Nup358 has already been implicated to have a role in mRNA export in *Drosophila* and mammalian cells (Forler et al., 2004; Hutten and Kehlenbach, 2006), therefore it is tempting to speculate that the interaction between APC and Nup358 might functionally couple mRNA transport to cell polarity. However, further studies are required to explore this possibility.

Previous studies showed that interference with the functions of APC and Nup358 leads to chromosomal segregation defects (Arnautov et al., 2005; Green et al., 2005; Green and Kaplan, 2003; Joseph et al., 2004; Salina et al., 2003). Moreover, APC truncations in colorectal cancers are linked to chromosomal instability (Fodde et al., 2001; Kaplan et al., 2001). Interestingly, cells appear to use similar mechanisms for capture and stabilization of microtubules by kinetochores and by the cell cortex. Our findings that Nup358 and APC might coordinate events to bring about polarization during cell migration raises the possibility that both Nup358 and APC have a similar interdependent role in mediating stable kinetochore-microtubule interactions and chromosome segregation during mitosis. Moreover, a recent study suggests that the mitotic role of Nup358 includes SUMOylation of topoisomerase II and regulation of its activity at the kinetochores (Dawlaty et al., 2008). In a different report, APC has been shown to interact with topoisomerase II and regulate its activity during G2-M transition (Wang et al., 2008). An interesting possibility is that the mitotic roles of APC, topoisomerase II and Nup358 could involve functioning of these players at the kinetochores in a coordinated manner.

Materials and Methods

Cell culture

COS-7, HEK293, Vero, HT-29 and HCT-116 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics in a humidified incubator at 37°C under 5% CO₂. CHO-K1 was grown in Ham's F12 medium with 10% FBS and antibiotics.

Plasmids, transfection and siRNA treatment

Full-length APC constructs were generous gifts from Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and Barry Gumbiner (University of Virginia, Charlottesville, VA). For obtaining RFP-APC-FL (205-2843 amino acids), the *KpnI-BamHI* fragment from the original full-length construct was cloned at the respective sites in pDsRed-Ex-C1 (Clontech). To obtain GFP-tagged or RFP-tagged APC-N (205-872 amino acids), APC-M (1211-1859 amino acids), APC-C (1789-2843 amino acids) and APC-NM (205-1865 amino acids) constructs, appropriate restriction sites were used to release different fragments and these were cloned into pEGFP-C or pDsRed-Express-C1 vectors. For generating RFP-GST and RFP-GST-APC-M constructs, the glutathione *S*-transferase (GST) coding region was PCR-amplified and inserted after the RFP open reading frame using appropriate restriction sites. GFP-tagged versions of Nup358 fragments (GFP-BPN, GFP-BPM and GFP-BPC) have been described earlier (Joseph and Dasso, 2008).

For transfection, cells were grown on glass cover slips for 12-24 hours. Transfections were performed using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions.

Control and Nup358 siRNA treatments were performed in Vero cells as described earlier (Joseph et al., 2004). The target sequence used for synthesizing the siRNA for human KAP3 was 5'-GCATGGATGACCGCTTTA-3' (Qiagen). COS-7 cells were co-transfected with control or EB1 siRNA (Santa Cruz Biotechnology) and RFP-GST or RFP-GST-APC-M constructs using Lipofectamine 2000. After 64 hours the cells were processed for western blot (Fig. 3A) or immunofluorescence (Fig. 3B) analyses.

Antibodies, immunofluorescence and immunoprecipitation

Rabbit polyclonal antibodies against Nup358 have been described earlier (Joseph et al., 2004). The region corresponding to 699-792 amino acids of human KAP3 (a kind gift from Yoshimi Takai, Osaka University, Japan) was sub-cloned into pET30a vector and the recombinant protein was expressed in *Escherichia coli* BL21(DE3)pLysS cells (Novagen) and purified by Ni²⁺ affinity chromatography. Rabbit polyclonal antibodies were raised against the protein and specific antibodies were obtained by affinity purification using the same antigen. Other antibodies used were: rabbit anti-APC (H-290; Santa Cruz Biotechnology), mouse anti-EB1 (BD Biosciences), mouse anti-KAP3 (BD Biosciences), mouse anti-kinesin-2 (Covance), mouse anti-RanGAP1 (Santa Cruz Biotechnology), mouse anti-vinculin (Sigma), mouse anti- γ -tubulin (Sigma), mouse anti-FXFG-containing nucleoporins (mAb414; Covance), mouse anti-GFP (Roche), mouse anti- β -catenin (BD Biosciences), mouse anti- α -tubulin (Sigma) and mouse anti-APC (ALi 12-28; Abcam, Santa Cruz Biotechnology).

For immunofluorescence analysis, cells were fixed 24-48 hours after transfection using methanol alone or methanol:acetone:EGTA, and processed as described previously (Joseph and Dasso, 2008). Hoechst-33342 dye (Sigma) was used to stain the DNA. Images were obtained with a Zeiss Axiovert 200M using a Plan Apochromat 63 \times NA1.4 oil immersion objective. Projection images were generated from optical sections 100 nm apart, with a section thickness of 700 nm, using the Axiovision Extended Focus module. Images were processed further in Adobe Photoshop.

For immunoprecipitation, cells were washed with ice-cold TBS once and lysed in cytoskeleton extraction buffer [10 mM Tris at pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 2 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate containing mammalian protease inhibitor cocktail (Roche Applied Science)]. Protein A dynabeads (Invitrogen) or nSephrose 4 Protein A beads (GE-Healthcare) pre-bound with rabbit anti-Nup358 antibodies or purified rabbit IgG (Vector Laboratories) were incubated with the cell lysate at 4°C for 2 hours. The immunoprecipitate was washed four times with the lysis buffer, eluted in SDS dye and separated on 5 or 6% SDS-polyacrylamide gels. The proteins were transferred to PVDF membrane (Millipore), and western blotting was performed using indicated antibodies.

Wound healing and polarization assay

Vero cells were transfected with the indicated siRNAs or plasmids. The migration assay was performed as described earlier (Joseph and Dasso, 2008). Briefly, after 48 hours of transfection, cells were subjected to serum starvation for 12 hours. Confluent cells were wounded by scratching with a P20 pipette tip and were incubated with DMEM containing 10% FBS for 4 hours. Cells were fixed and analyzed for the orientation of centrosomes using mouse anti- γ -tubulin antibody. Polarization was scored positive if the centrosomes were present in a 90° sector in front of the nuclei of those cells migrating towards the wound (as shown in Fig. 6A).

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