The Role of the Golgi Protein GM130 in Cell Polarity and Tumorigenesis

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Abstract
The Golgi apparatus is linked to the establishment of cell polarity, but the mechanism that allows the organelle to control cell polarity still remains unknown. Research in the area focused primarily on signaling from the plasma membrane to understand how polarity is established, and the small GTPase Cdc42 was identified as a main regulator of this process. Interestingly Cdc42 is mainly localized at the Golgi, thus we investigated the possibility that the Golgi regulates Cdc42 activity and by this mechanism it regulates polarity. We identified a GM130–RasGRF complex as a regulator of Cdc42 at the Golgi. Silencing GM130 results in RasGRF-dependent inhibition of the Golgi pool of Cdc42, but does not affect Cdc42 at the cell surface. Therefore, this is a specific mechanism to control a spatially restricted pool of Cdc42. Furthermore, active Cdc42 at the Golgi is important to sustain asymmetric front–rear Cdc42-GTP distribution in directionally migrating cells. We propose that the Golgi delivers active Cdc42 to the leading edge of migrating cells, thereby establishing asymmetry in Cdc42 activation at the plasma membrane and promoting directional migration. Two further observations supported a possible role for GM130 in cancer. First, concurrent to Cdc42 inhibition, silencing GM130 also results in RasGRF-dependent Ras-ERK pathway activation. Second, depletion of GM130 is sufficient to induce E-cadherin downregulation, indicative of a loss in cell polarity. We found that GM130 expression is frequently lost in colorectal and breast cancer patients. Whether the loss of GM130 solely affects polarity, or whether it affects other processes relevant for tumorigenesis remains unclear. To further investigate the role of GM130 in cancer, we analyzed the effect of GM130 depletion in a panel of breast cancer cells lines looking at processes linked to tumor progression such as survival, proliferation, adhesion, migration and invasion. We show that depletion of GM130 does not drastically affect survival, proliferation and adhesion. However, GM130 depleted cells show increased cellular velocity and increased invasiveness though matrigel, therefore supporting the view that alterations of polarity contribute to tumor progression. These findings establish a previously unrecognized role for a GM130–RasGRF–Cdc42 connection in regulating polarity and tumorigenesis.
Zusammenfassung


Introduction
Cell polarity

Cell polarity is the process that aims at generating asymmetry into a cell in order to produce and maintain functionally specialized domains. By compartmentalizing signaling, these domains control many cellular processes such as proliferation, migration and differentiation. In multicellular organisms, cell polarity is necessary for the correct development of the organism, as it shapes the three-dimensional organization of tissues and, ultimately, the entire organism. Different cell types exhibit different types of polarity, according to the function they have in the organism. Neuronal cells establish an axon and several dendrites necessary to transmit signals to the neighbor cells. Immune cells migrate following gradients, thus establishing a frontal leading edge and a retractile rear, then, when they recognize a danger for the body, they form the so called immunological synapse to exert their function of immune surveillance, thus changing their polarity. Stem cells need to divide asymmetrically in order to maintain homeostasis, therefore they polarize during cell division to give rise to two different daughter cells. Epithelia act as barriers between different compartments and epithelial cells have to function as selective filters for nutrients and signaling molecules. To do so, they establish an apical membrane and a baso-lateral membrane, divided by structures called Tight Junctions (TJs) that form physical barriers between neighbor cells. All these different forms of polarity are controlled by the same set of proteins that are distributed in three highly conserved complexes: Par, Crumbs and Scribble. The Par complex is composed by Par3, Par6 aPKC and Cdc42. The Crumbs complex is composed by Crumbs, PALS1 and PATJ. The Scribble complex is composed by Scribble, DLG and LGL (a complete list is shown below in Table 1). Those complexes are interacting with each other in agonistic and antagonistic ways. In epithelial cells, they control via mutual exclusion their subcellular localization: Crumbs is apical, Par is located in the sub-apical region at the tight junctions (TJ) and Scribble is baso-lateral. Mislocalization of one protein belonging to any of these complexes will most probably affect also the other two polarity complexes. Polarity is fundamental for the establishment and maintenance of epithelia. Tight Junctions are critical to the function of epithelia and their formation is coordinated by the Par and Crumbs complexes. Par3, Par6 and Crb3 are among the first proteins clustering at the plasma membrane together with Cadherins. Those clusters, called primordial adhesions, will establish connections with the actin cytoskeleton and subsequently evolve to form Tight Junctions (that define the border between apical and baso-lateral membrane) and Adherent Junctions, that will link the lateral...
membranes of neighbor cells. Par and Crumbs proteins act as scaffolds to recruit all the components of TJs and if the recruitment of Par and Crumbs proteins is disturbed, TJs as well as epithelia do not form. Polarity is also required for epithelial homeostasis, as polarity proteins control signaling processes that shape the collective response of epithelia to external stimuli, such as the need to proliferate in response to an injury and to stop proliferation at the right moment. Polarity proteins control all these processes and, therefore, play a pivotal role in epithelial homeostasis. Since epithelia are the tissue of origin of the majority of cancers, the processes that regulate their behavior are of clinical interest. Loss of polarity was recently proposed to be causal to cancer, but the mechanism behind this hypothesis is still not completely understood. One possible link between cancer and epithelia lies in the way epithelial homeostasis is maintained. Epithelia in vivo retain a certain stemness that allows them to renew. In order to maintain a stem cell population, epithelial stem cells need to undergo asymmetric cell division, thus they need to differentially segregate cell fate determinants (such as the Drosophila Numb and Prospero). Polarity proteins are needed to correctly segregate these proteins. Therefore, loss of polarity could impair the ability of stem cells to divide asymmetrically and this was shown to cause cancer in Drosophila. Proofs that polarity controls cell division also in mammals and that alterations of this regulation could cause cancer are still missing, but recently the polarity related protein PAR3L was implicated in asymmetrical division and epithelial homeostasis in a mouse model, thus giving polarity proteins a role in asymmetric cell division also in mammals.
Table 1 - Nomenclature of polarity proteins in Drosophila, C.elegans and mammals (adapted from Assémat et al.\textsuperscript{10})

<table>
<thead>
<tr>
<th>Complex</th>
<th>Drosophila melanogaster</th>
<th>Caenorhabditis elegans</th>
<th>Mammals</th>
</tr>
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<tbody>
<tr>
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<td>DmPar6</td>
<td>Par6</td>
<td>Pard6A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pard6B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pard6G</td>
</tr>
<tr>
<td>Bazooka</td>
<td>Par3</td>
<td></td>
<td>Pard3A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pard3B</td>
</tr>
<tr>
<td>DmaPKC</td>
<td>PKC3</td>
<td></td>
<td>PRKCζ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PRKCι</td>
</tr>
<tr>
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<td>Crb1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eat-20</td>
<td>Crb2</td>
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<tr>
<td>Stardust</td>
<td>TAG117</td>
<td>C50F2.8</td>
<td>Crb3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y55B1BR.4</td>
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<td></td>
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<td></td>
<td>LLGL2</td>
</tr>
</tbody>
</table>

**Par complex**

The first polarity proteins to be discovered were the components of the Par complex. They were identified by Kemphues et al. in 1988\textsuperscript{11} in a screening done in C.elegans embryos. After fertilization, the cells divide asymmetrically, giving rise to daughter cells which have different division times, different size and that ultimately will have different fates. Deletion of a group of 6 genes was found to disrupt this asymmetry, resulting in embryonic death. Those genes were called Par (for Partition defective). Par proteins localize asymmetrically within the embryo, with Par1 and 2 at the posterior cortex, Par3 and 6 at the anterior cortex and Par4 and 5 diffused in the cytoplasm. Later, another protein called PKC3 (atypical protein kinase C, aPKC) was included in the Par complex. PKC3 was found to directly interact with Par3, this interaction playing a pivotal role in the establishment of polarity in C. elegans embryos\textsuperscript{12}. 

Introduction – Cell Polarity - 8
Homologs of the Par proteins were lately described also in Drosophila and in mammals. It is now evident that the complex Par6-Par3-aPKC is well conserved among the vast majority of the animal kingdom.

**Par6**

Mammals have three Par6 homologues: Par6A (also called Par6C), Par6B and Par6D (also called Par6G). These proteins share similar structure, with 2 conserved domains: a CRIB (Cdc42-Rac Interaction Binding) domain that binds preferentially to active Cdc42, and one PDZ (PSD-95–Discs Large–zona occludens-1) domain that binds to Lgl2, Par3 and other proteins. Par6 is one of the first polarity proteins to be recruited to nascent TJs in response to locally activated Cdc42 or Rac. Once Par6 is at TJs, it recruits the kinase aPKC. This interaction is fundamental because aPKC activity will determine the localization of Par3 and Lgl2 (the latter belongs to the Scribble complex). Those two polarity proteins compete for the same binding site on Par6 and only when Lgl2 gets phosphorylated by aPKC, it loses its specificity for Par6, thus allowing the binding between Par6 and Par3. Pals1, a component of the Crumbs complex, is another binding partner of Par6, and the two proteins mutually regulate their localization to TJs. Comparison of the Par6 isoforms revealed that all Par6 interact with the same affinity with aPKC, Par3 and Lgl. However, Par6A does not interact with Pals1, whereas Par6B does. Gao et al. observed that overexpression of Par6A does not interfere with polarity, whereas Par6B overexpression destroyed TJs and proposed that the exceeding Par6B disrupts polarity by sequestering Pals1 to locations other than TJs. In this respect, it is interesting to note that Par6B is often overexpressed in tumors, while Par6A is not. It could be speculated that overexpression of Par6B confers the tumor an advantage that Par6A overexpression doesn’t offer and this selective advantage could be the loss of polarity mediated by Par6B.

**Par3**

Mammals have two Par3 genes, Par3A and Par3B. Par3 proteins have three PDZ domains and, as it is the case for Par6, they function as scaffolds. Par3 proteins bind to transmembrane proteins localized at the TJs, called Junctional Adhesion Molecules (JAMs). JAMs are recruited at newly formed cell-cell contacts before Par3; thus, they might be the anchor for Par3 localization. Given that Par3 binds to both Par6 and aPKC, JAM dependent localization of Par3 could contribute to correctly localize the whole Par complex by recruiting Par3. In addition to aPKC, Par6 and JAMs, Par3 binds to several other proteins, often implicated in the
control of processes other than cell polarity. One of its most important partners is the Rac1 specific GEF Tiam1 \cite{2,13,21}. The functional significance of this interaction has been object of controversies. In an epithelial polarity model (MDCK cells), Par3 knockdown was reported to impair TJ formation because of an increased Rac1 activation. Par3 would therefore serve as a localized inhibitor of Tiam1 \cite{21}. However, Mertens et al. \cite{2} showed that Tiam1 mediated activation of Rac1 is necessary for TJ formation in keratinocytes. In this latter model, Tiam1 was suggested to act upstream of Par3, and Rac1 activation would promote the recruitment of the Par complex to TJ. Cells KO for Tiam1 show strong impairment in TJ assembly, underscoring the relevance of Rac signaling for TJ formation. Finally, Xue et al used fluorescence resonance energy transfer (FRET) microscopy to study the spatio-temporal dynamics of Rac1 on TJ \cite{22}. In cells depleted of Par3, Rac1 resulted to be overall more active, but its localization was mainly cytosolic, whereas in the presence of Par3 the activation was restricted to cell-cell contacts \cite{22}. This observation shows that spatial restriction of signaling can be more important than the magnitude of the signal itself. In a neuronal polarity model, the binding between Par3 and Tiam1 was shown to be dependent on Cdc42 activation. When Cdc42 is activated, it recruits Par6, which then will bind to Par3 and aPKC. Par3 will subsequently bind to Tiam1, leading to localized activation of Rac1. Failure in localized activation of Rac1 will impair axonal growth \cite{13}. It is generally accepted that Tiam1 functions downstream of Par3, however, it was shown that Tiam1 can be recruited to TJs independently on the Par complex and thus, it is possible that Tiam1 recruitment to TJs locally activates Rac that will then recruit the Par complex there \cite{23}. In this scenario, the Par complex would sustain its own recruitment to TJ in a positive feedback mediated by Tiam1.

Among the numerous binding partners of Par3, worth of notice is the recently identified ASPP2 \cite{24,25}. ASPP2 is a pro apoptotic protein that binds to p53 and promotes the transcription of several pro apoptotic genes \cite{26}. Par3 interacts with ASPP2 at the level of TJs and this interaction is necessary for maintenance of TJ. Depletion of ASPP2 results in mislocalization of Par3 and loss of apico-basal polarity \cite{24,25}. Polarity proteins have the function to shape cell processes according to the needs imposed by the extracellular environment and it is interesting to note how processes that were previously not connected at all often converge on polarity proteins. The interaction between Par3 and ASPP2 opens the exciting possibility that the Par complex could regulate p53 function. Par3 is often downregulated in cancer and
placing p53 downstream of Par3 could explain how loss of Par3 provides an advantage for tumor cells.

**aPKC**

aPKC is the only protein with a kinase activity belonging to the Par complex, thus it is the only protein that actively transmits signals from the Par complex to downstream effectors. Mammals possess two aPKC genes, aPKCα/ι and aPKCζ. Those genes encode two proteins of about 75 kDa and function as kinases. Unlike the other PKCs, aPKCα/ι and aPKCζ are not activated by Ca2+, by DAG or PE.

aPKC is implicated in the control of several cellular processes. In the context of polarity, aPKC regulates the localization of Par3 and Lgl2. These two proteins are phosphorylated by the kinase and the phosphorylation status of Par3 and Lgl2 will determine which of the two proteins will interact with Par6 \(^4,27\). Once Lgl2 is phosphorylated, it loses its affinity toward Par6, thus allowing the binding between Par6 and Par3. In addition, phosphorylation of Lgl2 confines the protein to the baso-lateral membrane, and this is another critical event for the correct establishment of epithelial polarity \(^27\). In front-rear polarity, the same mechanism is used to recruit Par3 to the leading edge and to restrict Lgl2 away from the par complex. In addition, during cell migration aPKC controls endocytosis of integrins via the protein Numb \(^28\). Nishimura et al. reported that aPKC dependent phosphorylation of Numb regulates the localization of Numb. When Numb becomes phosphorylated, it loses its affinity for membranes. This in turn reduces the endocytic rate of integrins, which finally results in impaired migration \(^28\). Other functions of aPKC are not due to its kinase activity, but to its function as scaffold. aPKC contributes to the polarization of the exocyst by interacting with the protein Kibra, that in turn recruits the exocyst to the leading edge of migrating cells \(^29\). The binding between aPKC and Kibra was proven to be important for correct localization of both aPKC and the exocyst and perturbations in such interaction disturb cell migration. Finally, aPKC interacts with the E3 ubiquitin ligase Smurf1. Smurf1 selectively leads to degradation of RhoA and this event is key for correct cell migration. In fact, aPKC localizes Smurf1 at the leading edge of migrating cells, causing local degradation of RhoA and preventing the formation of stress fibers that would alter the actin cytoskeleton and impair migration \(^30\).
Table 2 – List of some of the substrates of aPKC with a role in polarity

<table>
<thead>
<tr>
<th>Substrate of aPKC</th>
<th>Function of phosphorylation</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Par3</td>
<td>Unknown</td>
<td>4, 20</td>
</tr>
<tr>
<td>Lgl2</td>
<td>P-Lgl2 loses affinity for Par6</td>
<td>4, 27, 31</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Inactivation</td>
<td>32</td>
</tr>
<tr>
<td>Numb</td>
<td>Loss of membrane localization (reduction in integrin endocytosis)</td>
<td>28</td>
</tr>
<tr>
<td>Par1 (MARK)</td>
<td>Loss of membrane localization (inactivation)</td>
<td>33</td>
</tr>
<tr>
<td>JAM-A</td>
<td>Targeting to TJ</td>
<td>34</td>
</tr>
<tr>
<td>p21</td>
<td>Inactivation</td>
<td>35</td>
</tr>
<tr>
<td>RhoGDI</td>
<td>Release of Rac1</td>
<td>36</td>
</tr>
</tbody>
</table>

Par complex in cell migration and invasion

The Par complex was the first polarity complex to be linked to cell migration. Etienne-Manneville et al. 37, showed that Cdc42-GTP is recruited to the leading edge in a dynein independent manner. Active Cdc42 in turn recruits Par6 and aPKC to the leading edge. The Cdc42-Par6-aPKC complex is necessary to reorient the centrosome and the Golgi Apparatus towards the leading edge (LE). Reorientation of Golgi and centrosome leads to polarization of secretion towards the LE and this is a key event to sustain directed movement of cells. Vesicles traffic from the Trans Golgi Network (TGN) to the LE on microtubules, supplying the plasma membrane with lipids and proteins necessary for migration. The Par complex is necessary for this process because it promotes the stabilization of microtubules pointing to the LE with several mechanisms working in parallel. First, APC, a protein interacting with the plus end of microtubules, is stabilized at the LE. Second, DLG localizes to the LE in an aPKC dependent manner. Third, Par3 together with Tiam1 can interact directly with microtubules and stabilize them. APC stabilization is dependent on Cdc42. Cdc42 is activated at the leading edge, probably by integrins and Src signaling. Active Cdc42 then recruits Par6, Par3 and aPKC, building the Par complex. The interaction between Cdc42 and Par6 activates aPKC, which will in turn phosphorylate and inactivate GSK3β 32. Inactive GSK3β will not be able to promote the degradation of APC, resulting finally in the local stabilization of APC at the leading edge. APC contains an EB1 domain that mediates its interaction with the plus ends of microtubules. DLG is another microtubules binding protein that is also recruited to the LE in a manner dependent on aPKC activity. DLG interacts with APC and this interaction is necessary to stabilize microtubules. Perturbing the Par complex affects the localization of both APC and DLG, but a
constitutively active GSK3β, that impairs recruitment of APC, does not affect DLG recruitment to the leading edge, meaning that APC and DLG are recruited via two different mechanisms 38. Par3 localizes to the leading edge via its binding to Par6 and there, together with Tiam1, it stabilizes microtubules 39. It was shown that Par3 interacts also with the motor protein dynein, the interaction being necessary for the correct orientation of Golgi and centrosome 40. It could be proposed that by acting on microtubules, dynein will generate a pulling force that will lead to reorientation of Golgi and centrosome. Depletion of Par3 results in impaired migration 40 and impaired ability of the cells to follow a chemoattractant 39. On the contrary, invasion through a 3D environment was enhanced in the absence of Par3 41. This was however not dependent on the motility of cells, but on increased secretion of matrix metalloproteases (MMPs) that contributes to cell invasion by degrading the extracellular matrix (ECM) and thus allowing the cells to go through the ECM 41.

In addition to controlling vesicle trafficking, the Par complex controls also endocytic processes via aPKC. As previously discussed, aPKC phosphorylates Numb, a protein normally localized to clathrin coated pits (CCPs), that is necessary for endocytic recycling of β1 and β3 integrins 28. Phosphorylation of Numb reduces integrins endocytosis by displacing Numb from the plasma membrane. During migration, aPKC is activated at the leading edge and, thus, Numb will be selectively inactivated at the leading edge, but it will still function in the rest of the plasma membrane. This will lead to accumulation of integrins at the leading edge and this mechanism will contribute to maintenance of directional migration 28.

**Par complex in cancer**

Loss of polarity is proposed to be cause of cancer, but no clear mechanism has been elucidated to explain how this should work. The role of polarity proteins in tumor progression is clearer and polarity proteins are often classified as oncogenes or tumor suppressors 22, 41. All Par complex proteins have been described to be altered in several kinds of cancers. They are somehow unique among the polarity proteins because, contrary to Scribble and Crumbs proteins, Par proteins can act as tumor suppressor or as oncogenes, depending on the context. Even though the Par complex was first described in Drosophila because of its role in asymmetric division, whether it is important in asymmetric division also in mammals is still not clear. The role of Par3 in asymmetric cell division was assessed using the mammary gland regeneration model.
To assess the role of polarity in cancer, an in vivo model is needed and a model that is often used is the mammary gland regeneration system. This model is based on the ability of the mammary gland to completely regenerate, following the transplant of mammary cells (MECs) into a cleared fat pad. MECs can be genetically engineered and then reimplanted, thus making it possible to evaluate the contribution of a specific protein or of a mutation in a developmental process (the growth of the organ) and in cancer. The mammary gland is composed by bilayered epithelial ducts. There is a luminal epithelial layer (cells expressing cytokeratin 8 and cytokeratin 18) surrounded by a contractile myoepithelial layer (cells expressing cytokeratin 14 and smooth muscle actin). Even though there are no specific markers for mammary stem cells, the terminal parts of the ducts, termed terminal end buds (TEB), were identified as the niche of such stem cells. Those regions are normally rich in cells double positive for K8 and K14. Par3 depletion resulted in complete failure in the regrowth of the organ. However, this was not dependent on loss of stem cells, as the TEB of Par3 knockdown organs were bigger than normal and contained more double positive cells. Instead, the polarity related protein Par3L was found to be important in asymmetric cell division and stem cell maintenance in breast morphogenesis. Par3L does not interact with aPKC or Par6, but it interacts with and inhibits LKB1 (homologous to Drosophila Par4). LKB1 is a tumor suppressor in mammals and its deletion results in development of the cancer-prone Peutz-Jeghers syndrome (PJS). Interestingly, LKB1 is frequently targeted for inactivation in several cancers. LKB1 activates AMPK and TSC2, thus leading to inactivation of the mTor pathway, that will make the cell cycle progress slower. Another recent study identified a link between LKB1 and YAP/TAZ signaling. In this study, Mohseni et al. showed that mutations in LKB1 resulted in hyperactivation of YAP/TAZ and subsequent translocation of these two transcription factors to the nucleus. LKB1 phosphorylates the Par1 homologues MARK1, 3 and 4. MARK will then phosphorylate MST1/2, then LATS1/2, and this will finally result in YAP phosphorylation (meaning inactivation). In this optic, it seems plausible that Par3L plays a role in cancer initiation via inactivation of LKB1 that will result in increased YAP translocation to the nucleus. However, this speculation needs to be verified experimentally. Interestingly, Scribble forms a complex with LKB1, MARK, MST1/2 and LATS1/2 and mislocalization of Scribble also results in alterations in YAP phosphorylation. Crosstalk between Scribble and Par proteins could therefore be important to regulate YAP/TAZ activity and this is of special
interest because YAP/TAZ nuclear translocation was shown to be sufficient to generate epithelial stem cells in breast cancer. Par3 has been extensively studied in different cancer models. Depending on the context, Par3 can act as tumor suppressor or as an oncogene. In a skin cancer model, Par3 knockout mice developed significantly less papillomas in response to chemically induced Ras transformation. Par3 depletion resulted in increased apoptosis and reduced proliferation that was explained by a defect in aPKC-mediated ERK activation. However, Par3 depleted mice developed more keratoacanthomas than wild type mice due to abnormal ERK signaling. In fact, Par3 depleted cells had consistently more P-Raf in a vesicular compartment instead that at the membranes. Low levels of Par3 were also observed in keratoacanthomas developing in wt mice, suggesting that Par3 depletion is a mechanism common to keratoacanthomas. Raf signaling appears to be critical in the development of keratoacanthomas. It was observed that several chemotherapeutic agents functioning by inhibiting constitutively active B-Raf gave rise to keratoacanthomas in a significant number of patients because of a paradoxical activation of Raf in cells with a wild type Raf.

In breast cancer, Par3 behaves as a tumor suppressor. In the presence of oncogenic signaling, Par3 depletion causes faster growth of the primary tumor and higher incidence of metastasis. McCaffrey et al. showed that Par3 depletion together with constitutively active Ras or NICD results in a delocalization of P-aPKC, which in turn results in altered signaling, such as hyperphosphorylation of Stat3 that leads to increased secretion of the matrix metalloprotease 9 (MMP9). Silencing MMP9 or inhibiting Stat3 signaling completely abolished the increased invasiveness of Par3 depleted cells both in vitro and in vivo. Xue et al. proposed an alternative mechanism that could lead to increased invasiveness of Par3 depleted cells. Par3 depletion leads to hyperactivation of Rac via mislocalization of Tiam1 and this results in decreased stability of E-cadherin at the cell-cell contact sites. Therefore, Par3 depleted cells are less cohesive and more prone to invade the surrounding tissue. Common to both studies is the observation that Par3 is altered (lost or mislocalized) in human breast cancers. Furthermore, both studies concluded that Par3 depleted cells become more invasive without undergoing EMT. Par3 depletion increases cell proliferation, but at the same time it also induces apoptosis. Expression of an oncogenic gene can overcome the apoptotic phenotype induced by loss of Par3, thereby unmasking the pro-proliferative effect of a loss of Par3.
Even if Par3 is often downregulated in tumors, it is not clear what mechanism leads to loss of Par3 expression. There are some evidences that Par3 is a target of EMT factors. Par3 was shown to be downregulated upon TGFβ stimulation via upregulation of miRNA-491-5p. TGFβ, a potent EMT inducer, stimulates the release of miRNA-491-5p, that will in turn silence Par3 expression. Early in EMT, TJ are disassembled and polarity is lost. Therefore, it is expected that some TJ related proteins are affected by EMT inducers. However, Par3 depletion alone does not result in EMT induction, matching the in vivo observations that Par3 depletion promotes invasion in the absence of EMT. On the other hand, Par3 depletion potentiates ad its overexpression decreases the ability of TGFβ to induce EMT. TGFβ influences also Par6 signaling. Par6 colocalizes and interacts with the TGFβ receptor I (TβRII) at TJs. Both TβRI and TβRII can phosphorylate Par6 on a highly conserved Serine (S345). This phosphorylation is necessary for TGFβ mediated disruption of TJs, as it is needed to recruit the E3 ligase Smurf that will target RhoA for degradation. Strikingly, overexpression of an unphosphorylatable mutant of Par6 strongly inhibited lung metastasis in breast cancer model and partially restored epithelial characteristics in mesenchymal cells in vitro. Par6B is often amplified in breast cancer, whereas Par6A and G are not frequently altered. Overexpression of Par6B, but not of Par6A, was proposed to affect cell polarity, therefore it could be speculated that polarity loss is needed to promote cancer. However other studies failed to see defects in polarity upon overexpression of either Par6A or B and furthermore, Par6A is sometimes overexpressed in lung cancer, implying that another mechanism should exist downstream of Par6 to promote cancer. Interestingly, overexpression of Par6A or B results in increased cell proliferation. This effect requires binding to both Cdc42 and aPKC and is mediated by increased and sustained ERK activation. The interaction between Par6A and PKCι was shown to be important for Rac driven transformation of non-small cell lung cancer (NSCLC). An inhibitor that interferes with the binding between Par6A and aPKC (aurothiomalate) blocks PKCι-dependent signaling to Rac1 and inhibits transformed growth of NSCLC cells. Knockdown of either Par6A or PKCι results in less active Rac1, which in turn will result in less active ERK and less production of MMP10. The expression of MMP10 downstream of PKCι happens also in an in vivo mouse model and correlates with poor survival. Of the two aPKCs, PKCι is the most commonly altered in cancer. PKCι is an oncogene for pancreatic cancer, lung cancer, ovarian cancer, and many other types of cancer. It was also shown that PKCι activation is a key event downstream of Ras induced oncogenesis.
in colon $^{61}$ and in lung $^{62}$. The signaling downstream of PKCι which is of crucial importance for cancer progression appears to be the same in all cancer types. PKCι, or better, the interaction between Par6 and PKCι activates Rac1. Rac1 signals then to MEK and ERK via its effector PAK1. ERK signaling will in the end drive transformation $^{63}$. Interestingly, the Par complex activates Rac via recruitment of the Rac GEF Ect2 $^{63}$. In MDCK, Ect2 was reported to interact with the Par complex components and to activate aPKC $^{64}$. Ect2 was however dispensable for TJ formation and apico-basal polarity $^{64}$. The gene coding for Ect2 is on chromosome 3q26, in the same region of the PKCι gene PRKCI and, interestingly, this region is often amplified in cancer $^{57}$ $^{63}$. Not only PKCι is downstream of Ras; there is evidence that PKCι could also play a role downstream of PI3K, another well known driver of cancer. In breast cancer cell lines, mutations in PI3K correlate with PKCι activation $^{65}$. Knockdown of PKCι results in decreased proliferation and increased senescence in PI3K transformed cells, but not in cells where PI3K is not mutated $^{65}$. Therefore, aPKC could play a role downstream of PI3K in mediating the balance between proliferation or senescence. A substrate of aPKC is p21, a regulator of the cell cycle and inducer of senescence $^{65}$. PKCι phosphorylates and inactivates p21. Thus, silencing of aPKC could result in more active p21 that will induce senescence $^{65}$.

**Crumbs complex**
The mammalian genome encodes three Crumbs isoforms (Crb1-3), which are single-pass transmembrane proteins, with an extracellular domain rich in EGF-repeats and a cytosolic tail that interacts with other components of the Crumbs complex. The link between epithelial polarity and Crb was first described in Drosophila epithelia, where Crb was exclusively confined to the apical membranes $^{66}$. Mutations of this protein caused drastic defects in the epithelium and were embryonically lethal. Conversely, overexpression of Crb was sufficient to confer apical characteristics to all the plasma membrane of Drosophila epithelial cells $^{67}$. Even more strikingly, the cytoplasmic tail of Crb was shown to be the mediator of this effect $^{67}$. Two more proteins were found to have functions similar to Crb: Stardust $^{68}$ and Dpatj (also called Disc Lost) $^{69}$. Further characterization of these two proteins showed that they form a complex with Crb and are required to establish epithelial polarity.

**CRB**
Humans have 3 Crbs, named Crb1-3 and they differ by tissue distribution. Crb1 is expressed in brain, cornea and retina and mutations in this protein are associated with degenerative pathologies of the retina $^{70}$. Crb2 is more widely expressed $^{71}$. Also this protein plays a role in
retinal development, as mutations or depletions of Crb2 are associated with retinal degeneration \(^ {71, 72}\). Both Crb1 and Crb2 have a large extracellular domain with several EGF-like domains and laminin repeats, a single transmembrane domain and a short 37 amino acid intracellular C-terminus containing one FERM (4.1, ezrin, radixin, moesin) and one PDZ protein-binding motif (ending with the aminoacids ERLI) \(^ {73}\). Crb3, instead, does not possess a large extracellular domain. However, its cytosolic tail is strongly conserved and it binds Pals1 (protein associated with Lin-7) and PATJ (Pals1 associated tight junction), the mammalian orthologues of Stardust and Dpatj \(^ {74}\). In addition to the FERM and the PDZ/ERLI domain, Crb3 has an SH3 domain \(^ {75}\). Crb3 is widely expressed in all epithelial tissues \(^ {75}\) and it localizes apically to the TJs \(^ {75, 76}\). Overexpression of Crb3 results in expansion of the apical membrane and delayed assembly of TJs \(^ {75, 76}\). Furthermore, MDCK cells overexpressing Crb3 were unable to correctly form cysts when growing in a 3D matrix \(^ {76}\), meaning that Crb3 overexpression disturbs apico-basal polarity. This could be due to the fact that Crb3 overexpression leads to uncontrolled expansion of the apical membrane, at the expenses of the baso-lateral membrane that does not form. The effect of Crb3 on TJ and apico-basal polarity was dependent on its ERLI domain. There are two proteins known to bind to this domain: Pals1 \(^ {76}\) and Par6 \(^ {75}\). However, it is still not clear which of these two proteins plays a major role downstream of Crb3. Par6 also interacts directly with Pals1 \(^ {15}\). Hurd et al. showed that Crb3 can control Par6 localization in a Pals1 dependent manner \(^ {15}\). Thus, Crb3 could function via recruiting the Par complex to TJs. Crb3 was also shown to recruit the Par complex to cilia, where Crb3 and the Par protein work together in the process of ciliogenesis \(^ {77}\). A splice variant of Crb3 was described to localize to cilia and at the spindle poles during mitosis \(^ {78}\). This variant of Crb3 has a C-terminal sequence ending with CLPI (CRB3-CLP) instead of the highly conserved ERLI domain. Crb3-CLP does not bind to Pals1 or to Par6 \(^ {78}\). Its knockdown leads to both a loss of cilia and a multinuclear phenotype associated with centrosomal and spindle abnormalities. Importin β1 was found to be a specific interaction partner of Crb3-CLP responsible for these abnormalities \(^ {78}\).

**Pals1**

Pals1 (protein associated with Lin-7) is the mammalian homolog of Drosophila’s Std. It is also known as MPP5 (membrane-associated palmitoylated protein 5) and belongs to the family of membrane-associated guanylate kinase (MAGUK) proteins. MAGUKs are scaffold proteins that contain PDZ, SH3 (Src homology 3), and guanylate kinase (GUK) domains. Pals1 also
contains two L27 domains: an L27C domain that binds mLin-7 and an L27N domain that binds to the PDZ domain of PATJ and is needed for localization of Pals1 to TJs. Pals1 therefore localizes to TJs via PATJ and serves as a bridge between PATJ and Crb3. Pals1 interacts with and stabilizes PATJ. Loss of Pals1 leads to polarity defects, but it is not possible to conclude if these defects are caused by the depletion of Pals1 or by the concomitant reduction in the levels of PATJ. As previously discussed, Pals1 interacts with Par6 and it appears that binding to Par6 and PATJ is mutually exclusive.

**PATJ**

PATJ (Pals1 associated tight junction) contains 10 PDZ domains in tandem and is a parologue of MUPP1 (multi PDZ domain protein). PATJ is the orthologue of the Drosophila protein Dpatj (or Disc Lost) and it maintains the same structure of Disc Lost. PATJ and MUPP1 localize to TJs and their localization was shown to be dependent on binding to other TJ proteins such as JAMs and ZO-3. Even though MUPP1 and PATJ share most of their interaction partners, MUPP1 appears to be dispensable for polarity establishment, whereas PATJ is of crucial importance to recruit both the Crumbs complex and the Par complex to TJs. PATJ and, with lower affinity, MUPP1, bind to Par6. The binding of PATJ to Par6 seems to be important for Par6 localization at TJs and depletion of PATJ results in mislocalization of Par6. Interestingly, overexpression of a constitutively active Cdc42 rescued TJ formation in the absence of PATJ. However, TJs were not concentrated apically, but they were extended laterally. From these observation a model could be drawn where the Crumbs complex and the Par complex are recruited to TJs and there the Crumbs complex restricts the localization of the Par complex. PATJ could participate in the recruitment of the Par complex, but active Cdc42 is upstream of it, meaning that the Crumbs complex is not crucial for Par complex localization.

Consisted with the above mentioned importance of Pals1 in epithelial polarity is the observation that Pals1 controls the stability of MUPP1 and PATJ. MUPP1 competes with PATJ for the binding to Pals1 and binding to Pals1 increases the stability of MUPP1 and PATJ. Therefore, when MUPP1 is downregulated, PATJ is stabilized and vice versa. Accordingly, downregulation of Pals1 will cause a decrease on protein level of both MUPP1 and PATJ. The close connection between these proteins hints that amplification or depletion of only one of these proteins, conditions encountered for example in cancer, will also affect the levels of the others, probably affecting cell polarity.
Crumbs complex in cell migration and invasion

Despite its major role in establishment of apico-basal epithelial polarity, the role of the Crumbs complex in cell migration (i.e. front rear polarity) has not been yet deeply investigate. It was described that PATJ binds to Par3, but this binding could be indirect, via the PATJ to Par6 interaction. Depletion of PATJ results in mislocalization of Par3 and Par6, with subsequent defect in directed migration. Accordingly, depletion of MUPP1, which increases PATJ levels, resulted in increased Par3 at the leading edge. Depletion of Pals1 also resulted in migratory defect, but Crb3 was fully dispensable for the process. Most likely, the Crumbs complex is not a major regulator of front-rear polarity; however, some proteins belonging to the complex may have a function in migration.

Crumbs complex in cancer

The notion that the Crumbs complex regulates cell growth is based on the observation that it regulates the Hippo pathway, a cascade that senses cell density and thereby mediates contact inhibition of cell proliferation by regulating the localization of the transcriptional regulators YAP/TAZ. YAP/TAZ are controlled by a kinase cascade comprising MST1/2 and LATS1/2. At low cellular densities, YAP/TAZ are unphosphorylated and localize to the nucleus. At high density, the Hippo pathway kinase LATS1/2 leads to phosphorylation and subsequent cytoplasmic localization of YAP/TAZ. This is the mechanism at the basis of contact-mediated inhibition of cells. TJ integrity is linked to YAP/TAZ translocation to the nucleus. When TJs are destroyed by Ca^{2+} depletion from the culture medium, the YAP/TAZ associated protein SMAD translocates to the nucleus. SMAD becomes cytoplasmic as soon as TJs reassemble. Interestingly, Pals1, MUPP1 and PATJ were found as interaction partners of YAP/TAZ in a mass-spectrometry based screening. The binding of these proteins to YAP/TAZ was further validated, but no direct interaction was found between Crb3 and YAP/TAZ. Surprisingly, however, depletion of Pals1, MUPP1 or PATJ did not impair YAP/TAZ recruitment to the Crumbs complex components whereas depletion of Crb3 did. A possible explanation for this observation is that when the Crumbs complex is intact at TJs, it can sequester YAP/TAZ there, preventing their translocation to the nucleus and multiple interactions between YAP/TAZ and several Crumbs complex components are needed in order to sequester the transcription factors. Consistently with this hypothesis, depletion of Crb3 from a monolayer of cells resulted in nuclear relocalization of YAP/TAZ and subsequent proliferation of cells that were before non-proliferating. However, there is in vivo evidence that Crb3 does not take
part in the process of tumor initiation because Crb3 depleted cells do not become tumorigenic when implanted in mice. Karp et al. selected cells with tumorigenic abilities in vivo and compared the gene expression of these cells with the expression of their parental non-tumorigenic cell lines. Unexpectedly, different tumorigenic cell lines showed similar changes in their expression profiles and common to all was the loss of Crb3. These cells exhibited an EMT signature, thus the authors speculated that those tumor cells underwent EMT in vivo. Crb3 overexpression in the tumorigenic cell lines partially restored apico-basal polarity in vitro. When the tumorigenic cells were then retransfected with an empty backbone or with Crb3 and reimplanted in mice, surprisingly, no difference was observed in the size of primary tumors or in their ability to invade the surrounding tissue. Deeper analysis revealed that Crb3 expression was not detectable anymore after 3 weeks from the reimplant, suggesting that the tumor selected for cells where Crb3 was lost. However, when the Crb3 expressing cells were injected in the tail vein of the mice, no colonization of internal organs was observed, in strong contrast with cells expressing an empty plasmid that colonized almost all organs in the abdominal cavity. Crb3 could therefore play a role in the process of extravasation or, alternatively, could render the cells more resistant in the circulation. Consistent with the data on Crb3 and EMT progression is the observation that Crb3 is repressed by at least two transcription factors known to induce EMT. The transcription factor Zeb1 efficiently represses several polarity proteins, such as LGL2, Crb3 and PATJ. The transcription factor Snail directly binds to the promoter of Crb3 and silences it. In addition to reducing Crb3 on a transcriptional level, Snail induces post-translational modifications such as glycosylation and sialylation on Crb3, resulting in reduced half-life of the polarity protein.

**Scribble complex**

Three proteins compose the Scribble complex: Scrib, LGL, DGL. The main function of this complex is to act as scaffolds to restrict apical components to the apical membranes. Interestingly, LGL (letal giant larvae) and DLG (lethal disks large) were identified as tumor suppressor genes in Drosophila. Scribble, LGL and DGL are not physically connected, but they are functionally linked.
**Scribble**

Scribble (Scrib) was first identified in Drosophila in a screen for maternal mutations affecting epithelial morphogenesis. Embryos depleted of Scrib had a corrugated cuticle that was riddled with holes. The epidermis became multilayered and cells from the epidermis lost their contacts with the underlying tissues. In Scrib deficient embryos, Crb was localized throughout the plasma membrane. Together with Crb, several other apical proteins were mislocalized in Scrib deficient embryos, but the localization of several basal proteins did not change. Therefore, Scrib is necessary to restrict apical proteins to the apical membrane. The human homologue of Drosophila’s Scribble is also called Scribble (hscrib) or VARTUL. It was found in a screening for targets of ubiquitin mediated degradation by the E6 protein of the human papillomavirus. Scrib is a large LAP (LRR and PDZ domains) protein localized at cell-cell junctions. It has 16 LRR (Leucine Rich Repeats), 2 LAP domains and 4 PDZ domains. Scribble localizes on the baso-lateral membrane together with E-Cadherin. It colocalizes with DLG, however it is not clear whether the two proteins directly interact. Scribble interacts with several TJ proteins, such as ZO-1, E-Cadherin and β-catenin. Scribble and E-Cadherin mutually control their localization. Depletion of E-cadherin from MDCK cells results in mislocalization of Scribble, whereas re-expression of E-cadherin in the breast cancer cell line MDA-MB-231 induces basal localization of Scribble. On the other hand, Scribble depletion disrupts E-cadherin mediated cell-cell adhesions. However, more than controlling E-Cadherin localization, Scribble controls coupling of E-Cadherin to cytoskeletal components such as α and β catenin. It is possible to rescue the cell-cell contact defect induced by Scribble depletion by overexpressing an E-Cadherin-α catenin fusion protein. Even if E-Cadherin is still baso-lateral in the absence of Scribble, several cytoplasmic vesicles positive for E-Cadherin and Golgi markers appear. E-Cadherin interacts with p120-Catenin and loss of this interaction promotes internalization of E-Cadherin, with subsequent degradation of the protein in lysosomes. Both the interaction between E-Cadherin and p120-catenin and the targeting of E-Cadherin vesicles to lysosomes are dependent on Scribble. Therefore, Scribble depletion results in accumulation of intracellular E-cadherin at the level of the Golgi apparatus. It is tempting to speculate that Scribble could be implicated in trafficking and targeting of post Golgi vesicles. In agreement with this hypothesis, Scribble was shown to regulate the endocytic recycling of the G protein-coupled thyroid stimulating hormone receptor (TSHR) via the Arf GAP GIT1. Scribble recruits the Rac GEF β-PIX that directly interacts with GIT1. The
ternary complex Scribble- β-PIX-GIT1 was described to be important also for exocytosis in neurons and in epithelia. The role of Scribble in apico-basal polarity has been studied both in vitro and in vivo. In a 3D culture model using MCF10A, depletion of Scribble induced a mild defect in apico-basal polarity. When growing in a 3D environment, MCF10A spontaneously organize in rounded, polarized structures called cysts or acini. These spheres are initially filled with cells, then the cells inside the lumen of the cysts undergo apoptosis and are extruded from the structure, resulting in cysts with empty lumen. The remaining cells will be polarized with their apical membrane facing the lumen of the acini. When Scribble knockdown cells were used to produce such cysts, cells filling the lumen did not undergo apoptosis, resulting in acini with altered morphology. Therefore, a role of Scribble in regulating apoptosis was proposed.

The role of Scribble in polarity was also deeply investigated in vivo, using the mouse mammary gland as model organ. The mammary gland is formed by highly polarized epithelial cells arranged in ducts. It was shown that the correct development of this organ is dependent on the polarity machinery. Accordingly to the proposed role of Scribble in controlling apoptosis in vitro, loss of Scribble resulted in deregulation of the balance between apoptosis and proliferation in vivo, which resulted in disorganized growth of the mammary gland. The same phenotype was observed also in a mouse model expressing the Scribble mutant P305L, where Scribble is mainly localized in the cytosol, meaning that not only the presence but also the correct localization is required for the correct function of the protein. Overall, human Scribble also plays a role in apico-basal polarity. However, its main role is not structural, as it is the case for Crb3 or Par3, which restrict the localization of several proteins to specific domains of the plasma membrane and are required for TJ stability. Instead, Scribble regulates signaling cascades such as ERK and Rac necessary for the correct development of tissues.

There is evidence that mammalian Scribble might play a role also in planar cell polarity (PCP). In fact, Yates et al. showed that in lung epithelia, Scribble interacts with the PCP protein Vangl1. This interaction is required for correct lung morphogenesis. Lungs of a mouse mutant for Scribble (Circletail - crc) have an abnormal shape, with airways missing open lumen. Scribblecrc (not functional) does not dramatically affect apico-basal polarity, as assessed by staining of apical and basal components, but acts through Vangl1 and RhoA on the cytoskeleton and perturbs AJ and TJ. Therefore, Scribble might be required to maintain normal cell-cell contact regions.
DLG
Mammals have 5 different DLG proteins and DLG1 is the most closely related to Drosophila DLG. Like Scribble, DLG is a target of the papillomavirus oncoproteins E6 and E4. DLGs are MAGUK proteins: they have three or more PDZ domains, a SH3 domain and a GUK domain. In addition to the previously mentioned domains, DLG1 has an N-terminal L27 domain. This domain binds to several TJ proteins, such as Lin-2, Lin-7 and MPP7. The interaction with MPP7 is important for targeting of DLG1 to cell junctions. Matsumine et al. showed that DLG1 binds to APC and, indirectly, to β-catenin. Furthermore, DLG and APC were colocalized at the basal membrane in rat colon epithelia. The binding between DLG and APC occurs also in migrating fibroblasts, where the two proteins colocalize at the leading edge and contribute to the stabilization of microtubules. Depletion of DLG or APC impairs microtubules stabilization towards the leading edge and subsequently, cells lose their migratory capacities.

DLG is strongly linked to PI3K signaling. PI3K was described as interaction partner of DLG1, the interaction regulated by the phosphorylation status of DLG. Phosphorylation of DLG1 on one Tyrosine residue results in increased affinity for PI3K and subsequent recruitment of the kinase to lateral membranes, whereas double phosphorylation on Tyrosine and Serine prevents this interaction. The recruitment of PI3K on basolateral membrane is required for AJ assembly and differentiation of human intestinal epithelial cells. DLG interacts also with the lipid phosphatase PTEN. PTEN dephosphorylates PIP3, antagonizing the action of PI3K and the pro survival signaling of the kinase Akt. DLG forms a ternary complex with the two tumor suppressors APC and PTEN and it is interesting to note that the PDZ domains of DLG responsible for binding APC and PTEN are often mutated in cancer, resulting in disruption of the complex. In addition, the binding between DLG and PTEN can be modulated by phosphorylation, as it was shown that phosphorylation of the PDZ domain of PTEN disrupts the interaction with DLG in vitro. These observations confer DLG a central role in controlling PI3K signaling. It would be interesting to know whether DLG can control mTOR and cell growth via its ability to modulate PI3K and to know if this proposed function of DLG is of importance in pathologies like cancer.

LGL
Mammals have two LGL proteins homologues to Drosophila LGL (LGL1 and 2). In addition, two other LGL related proteins have been described: LGL3 (Syntaxin-binding protein-5) and LGL4.
LGL localize to the baso-lateral membrane of epithelial cells and interact with the LRR domain of Scribble. The baso-lateral localization of LGL is mediated by the Par complex. In fact, LGL exists normally in a complex with Par6 and when aPKC phosphorylates LGL, the protein is dissociates from Par6 and localizes baso-laterally. LGL might play a role in establishment of TJ, but not in their maintenance, as overexpression of LGL during the formation of cell-cell contacts destroys polarity, but overexpression in an already confluent monolayer of cells does not have any effect.

**Scribble complex in cell migration and invasion**

Scribble was shown to be important for directed cell migration. In Drosophila, loss of Scrib, Dlg or Lgl are associated with defects in dorsal closure. In epithelial cells, loss of Scribble impairs directional migration and in a mouse model, reduction of Scribble causes defects in wound healing. However, loss of Scribble seems only to slow down migration, not to block the process. Interestingly, cell speed is increased by depletion of Scribble. Scribble localizes at the leading edge of migrating cells. Its depletion or mislocalization leads to loss of Rac1, Cdc42 and the Rho GEF β-Pix from the leading edge, but no defect is observed in scratch induced activation of Rac1, whereas for Cdc42 activation there is no clear consensus. This suggests that cell migration requires a spatio temporal coordination of all the proteins taking part in this process. A possible explanation for the role of Scribble in cell migration is that it acts as a scaffold, bringing together the small GTPases Cdc42 and Rac1 and their GEF β-Pix. However, Osmani et al. analyzed the dynamics of Scribble, β-Pix and Cdc42 recruitment to the leading edge of migrating astrocytes and they concluded that recruitment and activation of Cdc42 are events happening in two separate steps. Furthermore, Qin et al. studied the functional connection between Scribble and E-Cadherin and showed that β-Pix is dispensable for the migratory phenotype of Scribble depleted cells. Impaired directed migration can be instead rescued by expression of an E-Cadherin-α-Catenin mutant. Scribble does not interact directly neither with E-Cadherin, nor with α-Catenin, therefore the mechanism by which Scribble controls cell migration via E-Cadherin still has to be fully elucidated.

Dlg, another component of the Scribble complex, was shown to have similar functions to Scribble in Drosophila. In mammalian cells, localization of Dlg was analyzed in a wound healing assay. Dlg also localizes at the leading edge, at the plus ends tips of microtubules. APC, a
protein implicated in polarity and strongly connected with the Par complex, shares the same localization as Dlg. The binding between APC and DLG is stimulated by a polarization cue and occurs at the plasma membrane. Activation and recruitment of the Par complex results in aPKC mediated phosphorylation of GSK3β. This stabilizes APC and promotes APC interaction with the plus ends of microtubules. DLG interacts with APC via its PDZ domains and this interaction is necessary to stabilize microtubules. Perturbing the Par complex affects the localization of both APC and DLG, but a constitutively active GSK3β, that impairs recruitment of APC, does not affect DLG recruitment to the leading edge. Therefore, localization of DLG appears to be downstream of the Par complex.

Lgl, the last component of the Scribble complex, has a less prominent role in cell migration. It was shown in epithelial cells in Drosophila that aPKC phosphorylates Lgl and thus controls its confinement to the basolateral membrane. This is however of more importance for asymmetric cell division than for migration. Plant et al. showed that Par6 and aPKC interact with Lgl in a mouse model. Interestingly, Par6 and Lgl were also found colocalizing at the Golgi apparatus. Once directed migration is induced via scratch of a cell monolayer, Lgl can be found localizing both at the Golgi and at the leading edge. If phosphorylation of Lgl by aPKC is inhibited, Lgl is mislocalized, but this only results in a very mild effect in the ability of the cells to polarize. Recently, NMII-A (non-muscle myosin IIA) was described as a new binding partner of LGL. NMII-A binds to LGL only when it is not phosphorylated. In addition, aPKC and NMII-A bind to the same domain of LGL; thus, aPKC and NMII-A compete for their binding to LGL. Once LGL bind to NMII-A, it inhibits NMII-A Filament assembly. In a migrating cell, LGL is at the leading edge with aPKC. Once LGL gets phosphorylated by aPKC, it moves back in the lamellipodium, where it binds to and inhibits NMII-A. This enables the cell to polarize the F-actin necessary to move forward.

**Scribble complex in cancer**
The Scribble polarity proteins are normally described as tumor suppressors. Therefore, their expression is often lost or downregulated in cancer. Several proteins of the Scribble complex are repressed by EMT transcription factors such as Zeb1 and Snail. Zeb1 represses LGL2. In the absence of Zeb1, depletion of LGL2 mimics most of the changes induced by the transcription factor, such as reduced E-Cadherin levels and increased metastases in vivo. Instead, re-expression of LGL2 in tumor cells with high levels of Zeb1 reverts the EMT phenotype and reduces the number of metastasis formed in vivo after the implant of these
cells in nude mice. LGL2 is also repressed by Snail. As reported for Zeb1, LGL2 re-expression can rescue Snail induced EMT and strongly decrease the metastatic power of cells in vivo. The polarity protein DLG is also repressed in cancer, but by an epigenetic mechanism involving hypermethylation of its promoter. DLG and Scribble are targeted for degradation by the HPV protein E6 and this event is thought to be crucial for the progression to cancer induced by HPV infection. Scribble is probably the most studied protein of the Scribble complex. It is frequently downregulated and/or mislocalized in different cancers. Loss or mislocalization of Scribble results in increased ERK signaling. Conversely, overexpression of Scribble decreases MEK and ERK phosphorylation. Dow et al. showed that loss of Scribble is necessary for H-Ras driven transformation of MCF10A cells. In fact, MCF10A cells transformed by constitutively active H-Ras (H-RasV12) expressed less E-Cadherin, formed small filopodia when plated on collagen and were only poorly invasive. However, depletion of Scribble induced a strong invasive phenotype. Conversely, Scribble overexpression inhibited the hyperactivation of ERK, completely restored normal E-Cadherin levels, blocked protrusion of cells expressing H-Ras-V12 and inhibited the ability of cells to form colonies on soft agar. ERK signaling was shown to be the key signaling cascade driving all these events; in fact, chemical inhibition of MEK, but not of JNK and p38, mimicked the effects of Scribble overexpression of MCF10A cells expressing H-Ras-V12. Overexpression of a mutant of Scribble with impaired localization (P305L) was not able to reduce phosphorylation of ERK and, accordingly, did not restore epithelial characteristics of MCF10A cells expressing H-Ras-V12. However, this mutant was still able to suppress anchorage independent growth of cells, meaning that probably Scribble is not the only player downstream of H-Ras. It is not completely clear how Scribble controls ERK phosphorylation. It is known that Scribble interacts directly with ERK and at the same time recruits Protein Phosphatase 1 γ (PP1γ). Despite the evidence that PP1γ dephosphorylates ERK when both proteins are bound to Scribble, the majority of ERK molecules are in the nucleus, so it is not clear how relevant is the impact of Scribble on ERK. Nevertheless, Nagasaka et al. showed that Scribble-mediated ERK signaling is biologically relevant, as a mutant of Scribble lacking the PP1γ interaction domain can no longer repress H-Ras-V12 induced transformation. Scribble depletion cooperates with oncogenic c-Myc to generate breast tumors in a mouse model. It is known that c-Myc induces both proliferation and apoptosis. When Scribble is lost, the pro-apoptotic effect of c-Myc disappears, leaving only the pro-proliferative response.
C-Myc drives apoptosis via activation of the small GTPase Rac1 that signals to JNK. Zhan et al. showed that the scaffolding function of Scribble for the Rac GEF β-Pix is necessary for the c-Myc-induced apoptosis. C-Myc binds directly to the promoters of β-Pix and GIT1, increasing their transcription. If Scribble is lost, however, the increased levels of β-Pix due to c-Myc will not lead to activation of the Rac-JNK pathway, resulting in missed induction of apoptosis. Scribble seems to cooperate specifically with c-Myc and not with other oncogenes. In fact, analysis of c-Myc driven breast tumors revealed low levels of Scribble in many of the samples analyzed, whereas HER2 driven tumors did not show this trend. Surprisingly, though, Scribble is often upregulated in breast cancer and high levels of Scribble correlate with low survival. Deeper analysis shows that even if upregulated, Scribble is often mislocalized in many breast tumors. Mislocalization could be a direct consequence of overexpression or, as it appears to be the case in most tumors, it is caused by mutations in the protein. Even though no recurrent modification of Scribble was observed in tumors, many different mutations were mapped, each of them resulting in mislocalization of the protein. Feigin et al. generated a transgenic mouse expressing a mutant of Scribble known to localize away from the basal membrane (Scrib P305L). Scribble P305L mice develop multifocal hyperplasia in the mammary gland by the age of 1 year. By 2 years of age, hyperplasia progresses to cancer in the majority of the animals. The tumor suppressor PTEN was found as a binding partner of Scribble and it was proposed that mislocalization of Scribble in the cytosol resulted in trapping of PTEN away from the plasma membrane, where the phosphatase can no longer inhibit Akt, Akt and its downstream target mTOR1 will therefore be hyperactive, resulting in faster cell cycle and increased proliferation. Interestingly, Scribble was described to interact also with another phosphatase named PHLPP1 that dephosphorylates Akt. Depletion of Scribble resulted in localization of PHLPP1 away from the membranes and release of the inhibition on Akt. Overall, Scribble acts as signaling hub for MAPK and Akt cascades and its role in tumorigenesis relies on its ability to modulate these signaling cascades.
Figure 1 – Schematic representing the localization of the main polarity proteins in (A) apico-basal polarity and (B) front-rear polarity.


52. Wang, X. et al. Downregulation of Par-3 expression and disruption of Par complex integrity by TGF-β during the process of epithelial to mesenchymal transition in rat proximal epithelial cells. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1782, 51-59 (2008).


Small Rho GTPases

Small GTPases are molecular switches implicated in the control of a wide variety of cellular processes. They cycle between an active (GTP bound) and inactive (GDP bound) state, with GEF (GTPase Exchange Factors) promoting GTP loading and GAPs (GTPase Activating Proteins) promoting the intrinsic GTPase activity of small GTPases. The largest family of small GTPases is the Ras superfamily which comprises 5 major groups: Ras, Rho, Rab, Arf and Ran. Rho GTPases are more than 20, but the best studied proteins of this family are three: Rac1, RhoA and Cdc42. Over 60 activators (Guanine nucleotide Exchange Factors – GEFs) and 70 inactivators (GTPase Activating Proteins – GAPs) have been described so far for Rho GTPases. In addition, Rho GTPases are regulated also by GDIs (Guanosine Dissociation Inhibitors), which retain the small GTPases in their inactive status. The genes coding for Rho GTPases were first described in 1985 and they were named Ras homologues (Rho) after their similarity to Ras GTPases. This gene family was found to be extremely conserved among the animal kingdom. Microinjection of purified Rho induced dramatic changes in cell morphology and formation of stress fibers, whereas when Rho was inhibited with the C. botulinum toxin C3 the cells rounded up, thus it was proposed that Rho proteins functioned as cytoskeleton regulators. Further studies proofed that and elucidated the mechanisms by which Rac and Rho control the actin cytoskeleton. Injection of constitutively active RhoA into fibroblasts led to the assembly of stress fibers and focal adhesions at their end. Two different patterns were identified: FCS or LPA stimulation rapidly induced actin filaments that assembled into stress fibers. PDGF or insulin, instead, promoted first membrane ruffling and then stress fibers formation. By selectively inhibiting RhoA with the C3 toxin, it was possible to abrogate stress fibers and focal adhesions, but not membrane ruffles. Injection of constitutively active Rac into fibroblasts promoted membrane ruffling and, later, stress fibers. Dominant negative Rac1 (Rac1 T17N) blocked the formation of lamellipodia and the late stress fibers formation. However, FCS or LPA stimulation in Rac1 T17N microinjected cells still resulted in induction of stress fibers, without any lamellipodia. H-Ras is also an inducer of stress fibers and membrane ruffling. When cells were injected with H-Ras plus the toxin C3 to inhibit Rho, Lamellipodia still formed, but stress fibers were completely absent. Conversely, when H-Ras and Rac1 T17N were injected in the same cell, both membrane ruffling and stress fibers were inhibited. Ridley and Hall therefore identified a link between Ras and Rho GTPases and actin cytoskeleton: H-Ras can activate Rac, and Rac1 activation triggers RhoA activation. Each node of this signaling
cascade can also be individually activated by specific stimuli. Nobes and Hall, some years later, observed that injection of active Cdc42 also resulted in focal adhesion assembly and induction of filopodia. Blocking Rho or Rac activation, focal adhesions did not form anymore, but filopodia were still present, meaning that Cdc42 functions upstream of Rac.

Structurally, Rho GTPases are quite similar. They have an effector domain that changes conformation between the GTP and GDP bound status of the proteins. This domain is composed of two regions termed Switch I and Switch II that, upon loading of the GTPase with GTP, undergo a conformational change allowing the interaction with downstream effectors. The Switch I and II region are critical for the interaction of the GTPase with GAPs and GEFs and probably, the angle between Switch I and II is one mechanism that allows GTPases to be specifically activated or inactivated by some regulators but not by others. At the C terminal, Rho GTPases have the so called hypervariable region that ends with the sequence -CAAX. Prenylation of the cysteine residue on the -CAAX sequence is one of the major determinants of the intracellular localization of Rho GTPases. The prenylation consists normally in a geranylgeranylation, but RhoB, RhoD and RhoE are modified with the smaller 15-carbon farnesyl group. After prenylation, proteolysis of the AAX peptide occurs and finally the remaining cysteine is methylated. Prenylation is however not enough for plasma membrane targeting. Targeting to the plasma membrane requires a second signal that consists generally of a polybasic region upstream of the -CAAX motif or another prenylation in the hypervariable region. Such second signal has been well characterized for the different Ras isoforms. H and N-Ras are palmitoylated at the Golgi apparatus and this stabilizes their interaction with membranes. K-Ras is not palmitoylated but instead uses a polybasic signal in its carboxyl-terminus to mediate interaction with negatively charged lipids of the plasma membrane. Palmitoylated H- and N-Ras travel to the plasma membrane via the secretory pathway. Once at the plasma membrane, they are depalmitoylated, resulting in their redistribution to all membranes. At the Golgi, they will be palmitoylated and therefore trapped because of their higher affinity for membranes after palmitoylation. Unlike Ras isoforms, the role of posttranslational modification in regulating the localization of Rho proteins is more complex and much less understood. For instance, Rac1 (but not Rac2 and 3) is also palmytoilated, but it is not accumulated at the Golgi, for a reason that has yet to be determined. This could be due to increased binding to RhoGDI that stabilizes Rac1 in the cytosol even when it is
palmitoylated. On the contrary, Cdc42 is not palmitoylated, but it is found at the Golgi. Finally, RhoA and Rac1 have a polybasic region, but RhoA is actively retained in the cytosol and it is only poorly present at the plasma membrane, probably again because of RhoGDIs.

<table>
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<tr>
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<th>Post-translational modifications</th>
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Abbreviations: PM, plasma membrane; GG, geranylgeranylation; F, farnesylation; P, palmitoylation; PH, phosphorylation.

Table 1 – Localization of small Rho GTPases and their post-translational modifications - From Ridley 2006

For a very long time, only signals originating at the plasma membrane have been studied, but now it is clear that endomembranes can serve as well as signaling platforms. Therefore, the spatial localization of small Rho GTPases is extremely important for their correct function. Golgi localized Cdc42 controls protein sorting from the TGN and COPI vesicle formation on the cis side of the organelle. In addition it was proposed that Golgi localized Cdc42 could be required to establish cell polarity, but a clear mechanism to explain this is still missing. TC10 was also found at the Golgi where it interacts with COPI, thus it is possible that also this GTPase is implicated in the control of Golgi trafficking. RhoG is localized at the Golgi via its interaction with RhoGDI, but the function of this GTPase at the Golgi is still unknown.

Actin regulation by Rho GTPases
Actin is one of the most abundant proteins in the cell. It exists as globular monomers called G-Actin (Globular) that can assemble in filaments called F-Actin (filamentous). Actin filaments
provide a framework that supports the plasma membrane and thus determine cell shape. Two distinct patterns of filaments can be distinguished: bundles, which are composed by parallel, straight filaments, and networks, made by branched filaments. Branched actin filaments are found for example in the lamellipodia, whereas actin bundles sustain fingerlike structures like microvilli. Two major types of proteins are needed in order to assemble actin in bundles or networks. Diaphanous Related Formins (DRFs, a group comprising Dia1, 2 and 3, also called mDia1, 2 and 3) are responsible for nucleating parallel filaments, whereas the Arp2/3 complex leads to formation of a new branch of actin with a 70° angle relative to the original filament, producing branched actin filaments typically referred to as barbed ends 35. Rho GTPases control actin by localizing and activating those actin nucleators. RhoA binds to Diaphanous related formins causing a conformational change that activates them, thus promoting nucleation of parallel filaments of actin that will assemble in bundles to form stress fibers 36. RhoA can also induce stress fiber formation via a cascade involving activation of Rho kinase (ROCK) which activates LIM kinase (LIMK), which in turn phosphorylates and inactivates Cofilin 37. ROCK also phosphorylates MLC (myosin light chain) 38 and at the same time phosphorylates MLC phosphatase 39, thereby increasing myosin contractility, resulting finally in stress fibers formation 40.

RhoA signals to Rac1 via two pathways that antagonize each other. mDia binds to Src, promoting phosphorylation of the kinase Cas that will recruit the Rac GEF Dock180, resulting in activation of Rac 41. At the same time, however, ROCK antagonizes Rac1 activation via mechanism that is not completely clear 41. Rac and Cdc42 also signal to LIM kinase via their shared effector PAK1, thus they promote actin polymerization by inhibiting cofilin 42, 43. In addition, Rac1 controls the Arp2/3 complex by localizing and activating it. It was shown that Rac1 activates the WAVE/Scar complex, a direct activator of Arp2/3, by causing the dissociation of WAVE from the complex that keeps it inactive 44. An important effector of Rac is IRSp53, an SH3 domain-containing scaffold protein that, together with Rac1, forms a ternary complex with The Arp2/3 activator WAVE2. Recruitment of IRSp53 by Rac1-GTP promotes local activation of Arp2/3 and results in actin branching and lamellipodia formation 45 46. However, a study by Krugmann et al. found that IRSp53 interacts mainly with Cdc42 and it is responsible for filopodia, but not lamellipodia formation 47. In this model, the complex Cdc42-IRSp53 recruits MENA, a component of the ENA/Vasp complex. The ENA/Vasp complex promotes actin dynamics by interfering with capping proteins, a class of proteins that blocks
monomer addition at the barbed ends of actin filaments, inhibiting the elongation of such filaments. ENA/Vasp and capping proteins must both be properly regulated at the leading edge in order to promote migration. When ENA/Vasp activity is too high, capping proteins are totally inhibited and as result there will be very long filaments with few branches that will translate into faster speed, but decreased persistence. Conversely, when capping proteins are highly active, the leading edge will be characterized by highly branched actin filaments and migration will be more persistent. With this in mind, it is not surprising that, in addition to recruiting MENA, Cdc42 promotes binding of IRSp53 to the capping protein Esp8 and this protein is also necessary for filopodia formation. IRSp53 could therefore play a role in lamellipodia or in filopodia formation and the data on this appear to be contradictory. Probably, an explanation that reconciles all observations is that there are two pools of IRSp53, one that interacts with Rac and activates WAVE, thus promoting lamellipodia, and another pool that binds to Cdc42, leading to the recruitment of MENA and Esp8 and promotion of filopodia. There is also the possibility that those two pools signal to each other, as it was shown that Esp8 indirectly activates Rac by recruiting the Rac GEF Sos1. Cdc42 can activate the Arp2/3 activators N-WASP and WASP and thus it can activate the Arp2/3 complex itself. Surprisingly, however, depletion of Arp2/3, WAVE and WASP causes loss of lamellipodia, but not of filopodia, indicating that networks of branched actin are not critical for the formation of filopodia. If branched actin is not sustaining filopodia, then it is probably parallel actin fibers that provide the structure to grow filopodia. If this is the case, then Cdc42 should also be able to control DRFs. As a matter of fact, Peng et al. showed that Cdc42 interacts directly with DRF3 (mDia2), this interaction being sufficient for filopodia formation. Filopodia can be induced also by other small Rho GTPases, namely RIF (RhoF), RhoD and WRCH1 and mDia2 was also found downstream of RIF.
Figure 1 – Schematic of the main signaling routes downstream of Cdc42, Rac1 and RhoA. Arrows are of the same color of the GTPase from which they depend and if more GTPases control the same pathway, the overlaid color is used.

**Rho GAPs**

As with all small GTPases, the intrinsic GTPase activity of Rho proteins is very low and the presence of a GAP is required to promote hydrolysis of the GTP to GDP. The human genome is predicted to encode between 59 to 70 GAPs and not all of them have been already characterized. Several RhoGAPs have been crystallized in complex with a small GTPase. The mechanism how GAPs interact with Ras family GTPases is conserved and the key elements are identical between all family members. The switch I and II of the small GTPase stabilize the GTP by coordinating the gamma-phosphate. GAPs use a highly conserved arginine residue (commonly referred to as the arginine finger) to intrude between switch-I&II. This allows for a hydrophilic attack of the negatively charged gamma-phosphate that is pulled out by the positively charged arginine-finger. There are two main prerequisites for this reaction to happen. Firstly, a conserved glutamine residue (Q61 in Cdc42 and Rac Q63 in RhoA) interacts with the gamma-phosphate and allows the hydrophilic attack. Mutation of this glutamine to nonpolar amino acids (most commonly Q to L mutation) results in constitutively GTP-loaded (i.e. active) GTPases. Secondly, the cavity into which the arginine finger intrudes is rather narrow and is lined up by several conserved glycine residues (glycine is the smallest amino acid).
acid). Mutation of glycine to any other residue results in a steric hindrance of the GAP and therefore in a constitutively active GTPase.

An important question that is not completely answered to date is why do cells have so many GAPs. The number of RhoGAPs has consistently increased from yeast to mammals, but in all organisms, RhoGAPs outnumber Rho GTPases of a factor of 2-3. In addition, most GAPs display activity towards multiple small GTPases (at least in vitro), thus increasing the complexity of the possible signaling cascades. An explanation for this is that each GAP might specifically regulate a small GTPase in the context of a specific signaling pathway. In addition, GAPs are not only terminators of the signal, but the contribution to the modulation of several signaling routes. For example, active Cdc42 and Rac1 can bind to the inactive GAP p85 and their binding stimulates PI3K activity. Almost all RhoGAPs have multiple domains in addition to the GAP domain. Those additional domains can target the protein to specific locations. For example, the RhoGAP RICH1 (RhoGAP interacting with CIP4 Homologues1) has a BAR domain that mediates its recruitment to tight junctions. The BAR domain of RICH1 interacts with AMOT, a binding partner of PATJ and Pals1 that mediates RICH1 recruitment at TJs. Using a FRET based method, RICH1 was found to be selectively active only on Cdc42 in cells. Depletion of RICH1 or overexpression of AMOT result in loss of TJ. The same happens when a GAP-deficient mutant of RICH1 is overexpressed, showing that local control of Cdc42 by RICH1 is necessary for TJ formation. Some GAPs are not only directed to specific subcellular locations by binding to other proteins, but they can also be differently active, depending on the membrane to which they are bound. ARAP1, 2 and 3 are GAPs for Rho and Arf GTPases. ARAP1 and 3 localization is dependent on phosphoinositides. ARAP1 localizes at Golgi, endosomes and plasma membrane. Inhibition of PI3K shifts ARAP1 localization towards the Golgi, whereas inhibition of PI4K redirects it to endosomes, underlining the importance of PI metabolism in the targeting of such GAP. Interestingly, ARAP3 activity is regulated by binding to PIP3. In vitro, ARAP3 is a GAP for Cdc42, Rac1 and Rho, but does not have GAP activity towards Arf proteins. However, when PIP3 is added to the reaction, the Rho GAP activity of ARAP3 is shut down and instead the GAP activity towards Arf6 is drastically increased. The additional domains present on RhoGAPs can therefore serve to localize the GAPs, but also can provide a platform for crosstalk between different signaling cascades, as described for ARAPs, which mediate a bridge between Rho and Arf GTPases. GAPs can also mediate crosstalk between MAPK signaling and Rho GTPases. This is the case for BPGAP1, a GAP specific for RhoA.
BPGAP1 interacts directly with EEN II (endophilin II) and this interaction is necessary to target EEN to the plasma membrane, where both proteins will promote endocytosis of the EGF receptor. Accordingly, overexpression of BPGAP1 increases endocytosis of EGFR and leads to increased and more sustained ERK activation. EGFR is still active on endosomes, thus ERK activation is sustained until the receptor is recycled. Interestingly, a mutant of BPGAP with no GAP activity is not able to promote endocytosis of EGFR. BPGAP1 interacts directly also with MEK2 thus, a mutant of BPGAP1 not able to bind EEN does not affect endocytosis of EGFR, but is still able to increase ERK phosphorylation to some extent. Furthermore, MEK2 promotes the binding of Pin2 (Peptidyl-Prolyl cis/trans Isomerase 1) to BPGAP1. Pin1 dampens ERK signaling by decreasing the phosphorylation levels of MEK2 and at the same time it promotes the RhoGAP activity of BPGAP1. The functional relevance of such interaction is still not clear, but it would be interesting to test whether RhoA can influence ERK signaling via BPGAP1.

Evolution has in some cases privileged the scaffolding function over the GAP activity of RhoGAPs as can be concluded by the discovery of proteins harboring a non-functional GAP domain that maintained only their other function. This is the case of IQGAP1 and p85, the catalytic subunit of PI3K. IQGAP1 localizes at adherent junctions of epithelial cells via binding to E-Cadherin. In front rear polarity it goes to the leading edge, where it interacts with APC and CLIP170, capturing the plus ends of microtubules. IQGAP recruits active Rac and Cdc42 to the leading edge, but does not exert any GAP activity on them. Instead, by promoting local accumulation of the active form of Cdc42 and Rac1, it is responsible for local actin assembly. Therefore, IQGAP1 provides a link between microtubules and actin cytoskeleton. IQGAP1 acts also as a scaffold for the ERK signaling cascade. Interestingly, IQGAP1 is frequently upregulated in tumors. In glioma, it is recruited to the leading edge of tumor cells escaping the primary tumor. This recruitment is mediated by Arf6 and it is required for efficient cell migration. In fact, IQGAP1 recruits active Rac1, promoting actin remodeling at the leading edge. The catalytic subunit of PI3K called p85 (α and β) has a RhoGAP domain which lost its GAP function because it is depleted of the critical arginine.

Rho GEFs
The first mammalian RhoGEF was identified as a transforming gene for diffuse B-cell-lymphoma cells and was named Dbl. Since then, almost 70 distinct GEFs have been identified in humans. Dbl has a region homologous to the Rho GEF Cdc24. This region is the...
prototype GEF region used by all Rho GEFs and it is called Dbl Homology domain (DH). DH domains of different proteins share very little homology with each other, except for 3 short conserved regions (CR1, CR2 and CR3). However, the 3D structure of all GEFs is highly similar: they are all composed by a bundle of α-helices, with the three conserved regions forming the domain core. CR1 and 3 are α-helices, while CR2 is only the C-terminal part of α-helix 6. CR1 and 3 interact mostly with the switch I of the small GTPase and CR3 interacts with the Switch II. Small GTPases have a Mg2+ binding pocket and binding to Mg2+ is required to stabilize the binding with the guanine nucleotides. The DH domain of GEFs removes the Mg2+ from its pocket, thereby destabilizing the GDP–GTPase complex, and at the same time, they stabilize the nucleotide and Mg2+-free reaction intermediate. Because of the high intracellular ratio of GTP/GDP, the released GDP is replaced with GTP, leading to activation. Despite the lack of an evolutionary conserved sequence, the mechanism of action of Rho activators is extremely conserved. The same mechanism of action has been described for the bacterial effector SopE of Salmonella thyphimurium. SopE does not have a DH domain, however it potently activates Cdc42, leading to cytoskeletal rearrangements necessary for the bacteria to enter the cell. The protein Dock180 is the archetype for the second family of GEFs, named DOCK. Dock180 displays GEF activity towards Rac1 in cells, despite its lack of a DH domain. The mechanism by which this protein complex mediates nucleotide exchange on Rac is not totally clear. What is known is that the interaction with ELMO is necessary to confer Dock180 its GEF activity. Dock180 preferentially binds to Rac1 in its nucleotide-free status. Therefore, it is possible that the complex Dock180-ELMO stabilizes Rac1 in a nucleotide-free status, leading to GTP loading, as it is described for the Dbl family GEFs. However, there is evidence that the tertiary structure of the Dock180-ELMO complex is different from the normal structure of GEFs. In addition to the DH domain, the vast majority of GEFs possess a PH domain right at the C terminal of the Dbl homology. In several cases, the PH domain participates in the binding to the small GTPase. This is the case for the PH domain of Dbl that directly contacts Cdc42 and is necessary for GDP to GTP exchange. However, other GEFs do not require the PH domain to activate small GTPases. Furthermore, 4 GEFs completely lack a PH domain. Another possible function of PH domain would be the targeting of the GEF to a specific subcellular location. Interestingly, two of the four GEFs lacking PH domains have instead a transmembrane domain that mediates their recruitment to membranes and TUBA has a BAR domain that functionally replaces a PH domain.
However, the PH domains of several GEFs are poorly specific for phosphoinositides, meaning that it is unlikely that they would target GEFs to specific subcellular regions. Furthermore, there are examples where depletion of the PH domains does not affect localization of the GEF. An appealing possible function of PH domains is their ability to modulate GEF activity via binding to PI3K. Han et al. described that the RhoGEF Vav binds directly to Ptdins. Binding to PI3,4,5P2 strongly inhibits its GEF activity towards Rac, Cdc42 and Rho in vitro. Instead, binding to PI3,4P2 or to PIP3 stimulates the GEF activity. Vav needs to be phosphorylated by Lck or other kinases in order to exert its GEF activity. Binding to PI3,4P2 or PIP3 increases the Lck dependent phosphorylation of Vav1. This could be one of the mechanisms that allows Rac activation downstream of Ras. In fact, Ras stimulates PI3K, which would reduce the levels of the Vav inhibitor Ptdins4,5P2 in favor of the presence of PI3,4P2 or to PIP3.

GEFs are tightly regulated in their activity. Although there is no general mechanism of regulation, some common rules can be delineated: (1) post-translational modification (e.g. phosphorylation), (2) subcellular sequestration, (3) relief on inhibition by N-terminal truncation.

As already described, phosphorylation is critical for Vav activation. However, phosphorylation can also result in inactivation of the GEF, as it happens for RasGRF2. RasGRF2 is a Rho GEF and a Ras GEF at the same time. In fact, it possesses a DH-PH domain and a Cdc25 domain, the latter responsible for Ras activation. In CHO cells, overexpression of RasGRF2 leads to increased Rac1 activation, but when RasGRF2 is phosphorylated on Ser737 by p35/Cdk5, Rac1 activation is inhibited. Intriguingly, RasGRF was shown to bind to and inhibit Cdc42 in melanoma cell lines and we could confirm this finding. Sequestration to specific subcellular locations is a commonly used mechanism to modulate the activity of RhoGEFs. As already described in the previous part of this introduction, the interaction of β-Pix with Scribble is necessary to correctly activate the Rac-JNK pathway that leads to apoptosis in response to myc. In cell migration, Scribble recruits β-Pix to the leading edge, promoting local recruitment of active Rac. Par3 binding to Tiam1 restricts the GEF activity of Tiam1 towards Rac and depletion of Par3 results in aberrant activation of Rac through Tiam1.

Many GEFs contain an internal inhibitory domain. For several GEFs including Dbl, Vav, Asef, and Ect2, the removal of N-terminal sequences leads to constitutive activation when the protein is expressed in cells. As it is the case for Rho GAPs, Rho GEFs can serve as a bridge.
between Rho proteins signaling and other signaling cascades. Many GEFs possess several other domains, in addition to their DH-PH domains. RasGRF, for instance, contains a Cdc25 domain, which is used to activate Ras. Therefore, RasGRF is a potential link between Rac and Cdc42 on one side and Ras signaling on the other. The activity of RasGRF towards any of the GTPases it regulates is regulated by phosphorylation. p35/Cdk5 mediated phosphorylation of RasGRF was shown to control Rac activation, but did not affect Ras signaling. However, phosphorylation on other residues can also affect Ras activation. Cdc42-GDP decreases RasGRF mediated MAPK activation, thereby providing evidence that Rho GTPases can control Ras signaling.

**Rho GDIs**
Rho GDIs are the third class of regulators for Rho GTPases. They interact with the small GTPase in a 1:1 stoichiometry complex and block nucleotide exchange on the small GTPase. Mammals have three GDIs: the ubiquitously expressed RhoGDI (GDI1), the hematopoietically restricted GDI2 (LyD4GDI) and RhoGDI3 (also called RhoGDIγ). GDI1 and 2 are cytosolic, whereas GDI3 is membrane bound and it is normally found on intracellular vesicles. The crystal structure of RhoGDI in complex with Cdc42 has been resolved, leading to a deeper understanding on the mechanism of action of GDIs. RhoGDI is composed of an N terminal arm that binds to the switch I and II of the small GTPase, blocking nucleotides exchange. The C terminal part of RhoGDI has an immunoglobulin shape and if forms a hydrophilic pocket that is able to host the prenyl motif of the small GTPase, thereby stabilizing the Rho protein in the cytosol. Interestingly, RhoGDI is equally affine for Cdc42-GDP and Cdc42-GTP in the cytosol. However, on membranes, its affinity towards the small GTPase changes: GDI1 is now more affine to Cdc42-GDP. X-rays crystal structure did not show appreciable differences in the position of the switch I and II of soluble Cdc42 bound to GDP or GTP. It is therefore possible to speculate that the association with membranes may help GTP-bound Cdc42 to assume a conformational state that is more readily distinguishable from the GDP-bound GTPase and, thus, more receptive to bind target/effecter proteins. RhoGDI does not actively extract Cdc42 from membranes. In fact, Johnson et al. showed that in vitro Cdc42 can dissociate from lipids at the same rates with or without GDI. Rather than directly promoting dissociation from membranes, GDI1 stabilizes Cdc42 as it spontaneously dissociates from membranes. Given that GDI1 binds preferentially to Cdc42-GDP on membranes, the overall
result will be the accumulation of Cdc42-GTP on membranes and the stabilization of Cdc42-GDP in the cytosol. The same mechanism works also for Rac1, but not for RhoA.

There is evidence for GDIs playing important roles in the modulation of intracellular signaling. Overexpression of GDI1 results in decreased levels of active Cdc42, which in turn inhibit RasGRF capacity to activate Ras. Therefore, GDI1 overexpression results in a reduction of MAPK signaling. However, this observation should be interpreted carefully as GDI bound Cdc42 is not able to interact with RasGRF or with any other GEF, thus it is difficult to understand how the complex GDI-Cdc42 could modulate RasGRF with respect to its activity toward Ras. An alternative explanation would be that binding of Cdc42 to RasGRF positively modulates Ras activation and overexpression of GDIs would remove Cdc42 from RasGRF, thus reducing its GEF activity towards Ras. Binding of GDI to Cdc42 is important for the ability of Cdc42 to induce transformation. A mutant of Cdc42 that undergoes faster GDP/GTP exchange (Cdc42-F28L) was shown to promote transformation in NIH3T3 cells. When a mutation that disrupts the binding between Cdc42 and GDI (R66A) was introduced into Cdc42-F28L, the ability of Cdc42-F28L-R66A to induce transformation was completely lost.

Several important questions remain to be answered to fully understand the role of GDIs. First, how are GDIs dissociated from the small GTPase, in order to reactivate signaling downstream.

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**Figure 2** - Model representing the mechanism of action of GDIs. GDIs bind to Cdc42-GDP on membranes, stabilizing it as soon as it spontaneously leaves the membrane. Therefore, GDI slows down the reassociation of Cdc42-GDP to membranes. In the cytosol, GDIs can interact with the same affinity with both GTP and GDP bound Cdc42. Cdc42-GTP is more affine for membranes than Cdc42-GDP, therefore the net result of all these movements will be the enrichment of Cdc42-GTP on membranes.
of the Rho proteins? Second, are GDIs simply cytosolic anchors, or could they work as chaperons for the small GTPases?

To answer the first question, a possibility is that another class of proteins will lead to rapid dissociation of the GDI from the small GTPase. This is the case for Rab GTPases, where GDF (GDI Dissociation Factors) displace the RabGDI from Rabs, thereby making it possible for a RabGEF to activate the small GTPase. However, to date, no evidence of such proteins has been reported for Rho GTPases. Another possibility is that GDIs are phosphorylated and phosphorylation makes them less affine for the small GTPases. For instance, PAK1 phosphorylates GDI1 on Ser101 and Ser174, leading to decrease in its affinity for Rac. In addition, Src can phosphorylate GDI1 on Tyr156 in vitro and in cells. Phosphorylated GDI1 translocates to membranes and shows reduced affinity towards Rho GTPases. Finally, aPKC has been reported to phosphorylate RhoGDI, resulting in this case in the liberation of Rac. Alternatively to GDIs being phosphorylated, there is evidence that phosphorylation of small GTPases can change their affinity for GDIs. Forget et al. showed that PKA phosphorylation of Cdc42 and RhoA increases the affinity of those two small GTPases towards GDI1.

GDIs can bind also to GTP loaded GTPases, the binding preventing interaction with effectors of the Rho proteins and with GAPs. Once the small GTPase is GDI bound, it is protected by GAPs, meaning that it cannot be inactivated, unless before dissociation of GDI is stimulated. This raises the possibility that GDIs can act as chaperones to translocate active small GTPases between different subcellular locations. In agreement with this idea, it was observed that the Cdc42 mutant incapable of binding GDI1 was localized more prominently at the Golgi than the GDI bound protein. The same different localization upon impaired binding to GDI1 was described also for Rac1. In another study from Boulter et al, depletion of GDI1 resulted in reduced plasma membrane localization of Cdc42, Rac1 and RhoA and concomitant increase of their localization on endomembranes. Boulter et al. observed also that in mammalian cells, the level of RhoGDI1 is roughly equivalent to the total levels of RhoA, Rac1 and Cdc42 combined. Therefore, Rho GTPases compete for the binding to GDI and any condition that alters the level of one GTPase will result in disruption of this balance, affecting the normal on/off cycle of Rho GTPases.

One last function of GDIs is to prevent degradation of small GTPases. Once the Rho protein dissociate from membranes, their geranylgeranilation causes them to unfold and therefore
they are degraded by the proteasome. By stabilizing the prenylation, GDIs prevent degradation of small GTPases. This allows cells to have a constant pool of small GTPases that can be rapidly activated in response to stimuli.
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The secretory pathway

Secretory protein trafficking is an essential and homeostatic function of all eukaryotic cells. Proteins are translated on the cytoplasmic side of the rough ER (RER). There, they are either inserted into the ER membrane, if they are transmembrane proteins or translocated in the ER lumen, if they are soluble proteins. Approximately one third of all eukaryotic proteins are targeted to the secretory pathway and the first compartment they encounter is generally the ER. Proteins exit the ER from distinct domains of the ER termed ER exit sites (ERES) that are located in the ribosomes free regions of the rough endoplasmic reticulum. COPII coat components localize at ERES, where they assemble to coat the vesicles leaving the ER. COPII coated vesicles move towards the ER to Golgi intermediate compartment (ERGIC), a compartment which is biochemically distinct from ER and Golgi that sorts cargo to the Golgi or back to the ER \(^1,^2\). Cargo move from the ERGIC to the Golgi on microtubules, using dynein as motor \(^3\). The Golgi apparatus in mammalian cells is a single copy organelle composed of usually 3-7 flattened cisternae that form the Golgi stack. Several Golgi stacks are connected laterally through membranous tubules and thereby form the Golgi ribbon. Typically, the Golgi occupies a perinuclear region and is closely associated with the centrosome. The cisternae of the Golgi stack are oriented with a defined polarity from cis- (closer to the ER) to trans. Several enzymes (like glycosil transferases and mannosidases) are differentially localized along the Golgi cisternae \(^4\). After the trans-cisterna, a meshwork of tubulo-vesicular structures forms the Trans-Golgi Network (TGN) that is distinct from the trans-Golgi cisterna in terms of enzyme composition and markers. From the TGN, transport intermediates will traffic to their final destination such as the endolysosomal system, the plasma membrane or the extracellular space.

Several vesicles are trafficking between ER and Golgi. COPII coated vesicles traffic from the ER to the Golgi, while COPI coated vesicles traffic backward between Golgi cisternae, from the Golgi to the ERGIC and, finally to the ER. The small GTPase Sar1 is responsible for the formation of COPII vesicles. The ER protein Sec12, the only GEF for Sar1 \(^5\), activates Sar1. Sar1-GTP deforms the membrane and recruits the first proteins that will compose the inner layer of the coat, Sec23 and Sec24. Sec23/24 will stabilize the curvature of the membrane imposed by Sar1. Finally, Sec13 and Sec31, which form a heterotetramer, are recruited to form the
outer layer of the COPII coat and to complete the budding of the vesicle. The final size of the vesicle will be around 50-90nm (dependent on species), similar to the vesicles formed at the other levels of the secretory pathway. The different components of the COPII coat fulfill distinct functions. The Sar1 GTPase initiate the COPII coat assembly, deforms membranes and finally also helps in fission of the COPII vesicle. Sec23 acts as a GAP for Sar1 and its activity is stimulated by 10fold upon recruitment of the Sec13-31 hetero-oligomer. This will result in Sar1 inactivation and serves to limit the reaction and to promote disassembly of the COPII coat. Sec24, of which four isoforms exits (A-D), acts as an adaptor for secretory cargo by interacting with various ER export motifs. Besides these core COPII components, several other proteins help in assembly of the ERES, stimulate COPII budding, or aid in exporting secretory cargo. Sec16 for instance, is a large (250 kDa) protein that does not incorporate into the COPII vesicle, but is nevertheless essential to form ERES and it stimulates the formation of COPII vesicles. Sec16, localizes to ERES in a manner independent on COPII components and it interacts with many COPII proteins. Soluble cargo in the lumen of the ER does not have access to the cytosolic COPII components and is therefore dependent on so called cargo receptors. Three major families of cargo receptors exist: the ERGIC53 family (ERGIC53, ERGL, VIP36 and VIPL that bind to glycoproteins), the p24 family (8-10 members, probably with differential affinities for different cargo) and the ERV family (binding to soluble and transmembrane proteins cargo). Receptors of these three categories cycle between ER, ERGIC and Golgi and can bind to both COPI and COPII vesicles.

The mechanism by which COPI coated vesicles form is similar to the biogenesis of COPII vesicles. The Arf small GTPases recruit COPI vesicles. GBF1 is an Arf GEF restricted to Golgi and ERGIC. There are 6 Arfs in mammals, divided in three classes: Class I (Arf 1, 2 and 3) Class II (Arf4 and 5) and Class III (Arf6). Arf6 is on endosomes and at the plasma membrane, whereas Arf1-5 are mainly distributed throughout the secretory pathway. The COPI coat is composed by seven proteins called coatomers that assemble in a subcomplex of three proteins (α, β’, ε) and another of four proteins (β, γ, δ, ζ). The trimeric complex α, β’, ε has some structural similarities to Sec13/31. α, β’ and γ recognize transport motifs of type I transmembrane proteins. Type II transmembrane proteins are recognized by adaptor proteins such as Vps74 and soluble proteins are recognized by the KDEL receptor. There are several γ and ζ isoforms that show different subcellular localization, probably reflecting differences in the composition of COPI coats forming at the Golgi or at the ERGIC. COPI vesicles that
shuttle between Golgi cisternae are different from COPI vesicles trafficking back to the ER\textsuperscript{17}-\textsuperscript{19}. Using p115 (ER, ERGIC and Golgi localization) as tether, only COPI vesicles enriched in p24 family members are found, whereas with the Golgi localized tether Golgin84 it is possible to identify COPI vesicles rich in Golgi resident enzymes (such as Mannosidase I and II), but poor in p24 family proteins\textsuperscript{17}. ArfGAPs are necessary for COPI vesicle formation. The p24 cargo that recycles from the Golgi to the ER can modulate the activity of ArfGAP1. If there is no cargo, ArfGAP activity is high. As soon as p24 proteins accumulate, they inhibit ArfGAP1, thus stabilizing the COPI coat and promoting their recycling\textsuperscript{18}. Contrary to what happens in COPII vesicles, where the Sar1 GAP is part of the coat, ArfGAPs are only transiently associated with the vesicles. However, even if ArfGAPs are not part of the cytosolic COPI vesicles, they are needed to assemble the COPI coat, thus their function is similar to Sar1 in COPII vesicle formation\textsuperscript{20}. Once vesicles bud, they move on microtubules until they are captured by tethering proteins that bring them in close proximity to their target membrane. Tethers also bind SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) and Rabs, necessary for the fusion of the vesicles\textsuperscript{21, 22}. Tethers can be either long coiled coil proteins that catch the vesicle from a long distance or multisubunits complexes that act mainly on the target membrane\textsuperscript{13}. p115 (Uso1p in yeast) is a tether which is dynamically located at the ERGIC and at the Golgi and it is thought to work together with other tethers such as GM130 and GRASP65 to target vesicles to these compartments. Rab1-GTP binds to p115 and recruits it on COPII vesicles. At the same time, Rab1-GTP binds to GM130, either directly, or indirectly via GRASP65, thereby linking the different tethers and allowing fusion of vesicles with their target membrane\textsuperscript{21}. These data are however, difficult to reconcile with the fact that in mammalian cells, COPII vesicles never reach the Golgi\textsuperscript{23}, and therefore, the mechanism of carrier arrival at the cis-Golgi remains elusive and the role of p115 needs to be studied in greater detail. The intra-Golgi tether Golgin84 also binds Rab1, but it does not bind p115, GM130 or GRASP65\textsuperscript{24}. Instead, it interacts with two other tethers localized exclusively at the Golgi: CASP and COG (Conserved Oligomeric Golgi)\textsuperscript{17, 24}. Golgin84 is required for COPI trafficking and, accordingly, its depletion will cause defects in the distribution of several Golgi proteins\textsuperscript{24}. GRASP55, a medial-Golgi protein, interacts with the coiled coil Golgin45 and this interaction is necessary for anterograde trafficking within the Golgi\textsuperscript{22}. Interestingly, GRASP55, Golgin45 and GM130 interact with Rab2\textsuperscript{22}. This allows to draw a trafficking route within the Golgi, where cargo arrives to the cis-Golgi via p115 and its interacting partners GRASP65 and
GM130, then, possibly via GM130, cargo moves forward to the GRASP55-Golgin45 tether. Several tethers not only interact with Rab, but also work as Rab GEFs. This is the case of the TRAPP complex, which is a COPII tether with GEF activity towards Rab1. TRAPP is a multi-subunit tether and depending on the subunits assembled together, three different TRAPP complexes can be distinguished. The TRAPPII complex binds specifically to COPII vesicles and regulates their homotypic fusion, the TRAPPI complex is a COPI tether and the TRAPPIII complex has a role in autophagy. Interestingly, a subunit of the TRAPPII complex was shown to recruit an Arf GEF, therefore conferring to the complex the ability to control the activation of both Arf1 and Rab1. This finding is of relevance because Rab1 is also important for COPI vesicles transport. Overexpression of a constitutively active Rab1 prevents recycling of Golgi proteins to the ER upon BFA treatment. Conversely, dominant negative Rab1 promotes retrograde traffic to the ER and causes dissociation of β-COP from membranes. Probably Rab1 acts upstream of Arf because overexpression of Arf1 rescues the phenotype caused by dominant negative Rab1.

Finally, SNAREs mediate the fusion of the vesicles to the membranes. SNARE proteins are on the vesicle (v-SNARES) or on the target membrane (t-SNARES). Before fusion, SNARE are in trans- (v and t-SNARES are on two different membranes), then after fusion they assume their cis-conformation. Once vesicles are fused, the SNARE complexes are disassembled in an ATP-dependent process through the action of the N-ethylmaleimide-sensitive factor (NSF), thus restoring the original pool of v- and t-SNARES.

There is no unique model describing how proteins traffic through the Golgi. The two main hypothesis are the cisternal maturation model and the anterograde vesicular transport. Both models are supported by experimental observation, but they all suffer from some weaknesses. According to the anterograde vesicular transport, each compartment of the Golgi possess a unique set of enzymes and cargo moves forward via COPI vesicles. Supporting this model there is the evidence that two populations of COPI vesicles exist, one of which containing anterograde cargo. However, big cargo molecules such as pro-collagen, do not fit in COPI vesicles. Therefore, according to this model, large proteins traffic via megavesicles. The existence of such megavesicles is not widely accepted, and evidence for these is sparse. What also argues for the existence of vesicles is the observation that different types of cargo traverse the Golgi stack at distinct kinetics. The second model predicts that cisternae will move forward from cis- to trans- and change their enzymatic content while moving,
thereby maturating. According to this model, COPII vesicles coming from the ER-ERGIC fuse to form the first cis-Golgi cisterna. The cisterna will then progress until the TGN, with COPI vesicles recycling Golgi resident enzymes at different speeds, thus producing the polarized distribution of the enzymes across the Golgi. The problem with this model is that several Golgi resident enzymes have not been observed in COPI vesicles, therefore it is difficult to explain how would the polarity of such enzymes be maintained. In addition, it is difficult to reconcile this model with the fast kinetics observed for small cargo proteins. However, it was observed that cargo arrival to the Golgi induces the formation of tubular connection between Golgi cisternae. The presence of these tubular structures can readily account for the fast trafficking of small cargo and for recycling of Golgi-resident enzymes.

After crossing all the Golgi cisternae, cargo proteins arrive to the TGN. Contrary to what is observed in the rest of the Golgi, the main coat at the TGN is a clathrin coat. Arf1 is important also at the TGN to recruit clathrin adaptors such as AP1 and AP3. The adaptor recruited will determine the final destination of the cargo. In addition, several transmembrane protein have a signal sequence in their cytosolic tail that functions as a sorting motif. Post translational modifications can also work as sorting signals. For instance, O and N glycosylation function as apical targets in MDCK cells. Finally, lipid microdomains can participate in the segregation of specific cargo and target such cargo to apical or basal membranes in polarized cells. In general, apical signals are less characterized than baso-lateral signals and it seems that baso-lateral signals are dominant, as it is often observed that upon removal of baso-lateral signals, proteins are redirected apically. Membranes at the TGN tubulate in an yet unknown mechanism probably involving BAR proteins and lipids. Cargo will then traffic to its final destination via microtubules, using the motor kinesin. The nature of post-Golgi carriers remained elusive for a very long time, but recent work indicates the existence of different types of carriers with selectivity for cargos. The Adaptor Proteins 1b (AP1b) mediates exit from the TGN of baso-lateral protein in a clathrin-mediated way. AP4 was also described to direct baso-lateral proteins from the TGN, but the mechanism directing this process remains to be elucidated and it is probably not dependent on clathrin since AP4 does not interact with clathrin. Another class of carriers was recently identified at the TGN that transports proteins directly to the cell surface, and it was termed CARTS (Carriers of the TGN to the cell Surface). They form in a PKD dependent manner and are responsible only for the transport of specific proteins. It was shown that...
they exclude collagen and VSVG, but they contain pancreatic adenocarcinoma upregulated factor (PAUF). The discovery of CARTS opens the possibility that many more TGN carriers exist and that the diversity of the carriers could be a mechanism to sort proteins to different destinations. However, several questions need to be answered, such as how those transporters are recruited only to specific cargoes. It is possible that such carriers are regulated by signaling. PKD regulates CARTS, but Cdc42 was shown to modulate exit from the TGN, thus it is tempting to speculate that Cdc42 functions by modulating specific carriers at the TGN. If it is known that Cdc42 plays an important role in sorting proteins from the TGN, still, the mechanism by which the small GTPase exerts its function remains elusive. Overexpression of mutants of Cdc42 defective in hydrolysis or in nucleotide binding resulted in altered exit of baso-lateral and apical proteins from the TGN. In particular, overexpression of the constitutively active Cdc42V12 caused apical proteins to exit faster from the TGN and delayed trafficking of baso-lateral proteins. Concomitantly, intracellular actin was almost completely lost and redistributed to the cell cortex, suggesting that actin filaments at the Golgi might play a role in protein sorting. Since both constitutively active and constitutively inactive Cdc42 cause defects in protein sorting from the TGN, it can be concluded that cycling of the small GTPase is necessary to control trafficking from the TGN, thus Cdc42 GEFs and/or GAPs should be also localized to the TGN. To date, only one GEF specific for Cdc42 has been localized there, namely FGD1. Accordingly to the role of Cdc42 in protein trafficking, knockdown of FGD1 or overexpression of the inactive form of the GEF impaired exit of all proteins from the TGN. Whether FGD1 is the only regulator of Cdc42 at the TGN is a question that still need to be answered.

**Signaling at the secretory pathway**

The secretory pathway is targeted by several signaling cascades which modulate its function in different ways. For example, the ER can adapt to changes in its cargo load. When an acute stress induces more trafficking, ERES fuse homotypically resulting in a reduction in the overall number of ERES, but with bigger size. This adaptation is mediated by PI4KIIIa and by Sec16. When a chronic cargo is imposed to the cells, the number of ERES increases, plus an induction of Sec16 is observed. In this case, PI4KIIIa is dispensable for the effect, while Sec16 knockdown abolishes this adaptation. The latter response was shown to be dependent on the unfolded protein response, which induces Sec16 and several other trafficking components.
ERES also respond to environmental stimuli, such as the presence or absence of growth factors and nutrients, which all seem to converge at the level of Sec16 that is targeted by kinases such as ERK2 and ERK8. Membranes of the secretory pathway undergo dramatic changes during mitosis and hence a wealth of information exists on mitotic signaling targeting several components of endomembranes. During the G2 phase of the cell cycle, the Golgi is fragmented in response to a series of signaling pathways mediated by kinases. CDK1 phosphorylates several proteins localized to the early secretory pathway, like Rab1, GM130 and GRASP65. Once phosphorylated, GRASP65 is not able to form oligomers anymore and this is probably the cause of fragmentation of the Golgi. At the same time, phosphorylated GM130 loses its ability to bind p115, resulting in a defect in anterograde trafficking and subsequent fragmentation of the Golgi. Phosphorylated GRASP65 and Rab1 bind to Polo-Like Kinase 1 (Plk1), a kinase necessary for several aspects of mitotic progression. When Golgi disassembly is prevented by addition of antibodies anti-GRASP65, the cell does not enter mitosis. Treatment with drugs that fragment the Golgi release this mitotic block. Therefore, the Golgi acts as a mitotic check point. Plk1 recruitment by GRASP65 is necessary for the correct progression of mitosis. Several other kinases have been shown to signal at the Golgi during mitosis and induce fragmentation of the organelle. Analysis of the kinome and phosphatome revealed that 8% of the phosphatome and 15% of the kinome signal to the secretory pathway, showing that the secretory pathway is a main target for signal transduction cascades. 38 kinases and phosphatases were identified the knockdown of which results in Golgi fragmentation. Knockdown of some of these proteins resulted in impaired cell migration and inhibition of Golgi orientation in a wound assay. This is in line with previous reports showing the intact Golgi architecture is important for directed cell motility. This observation is a hint that signaling to the secretory pathway can modulate cell polarity.

The Golgi was also shown to be target of apoptotic signaling pathways. Several Golgi proteins are cleaved by caspases during apoptosis, which result in Golgi fragmentation or vesiculation. However, this is not simply an epiphenomenon of apoptosis, as Golgi fragmentation was shown to play an active role in the process of cell death. A mutant of GRASP65 resistant to cleavage by Caspase3 blocks Golgi fragmentation and delays apoptosis induced by Fas or by ER stress stimuli. The C terminal fragment of GRASP65 is targeted to mitochondria and interacts with the pro-apoptotic protein Bcl-XL in response to...
Fas. Thus, cleavage of GRASP65 results in translocation of Bcl-X\(_l\) to mitochondria and amplification of the apoptotic signals \(^{68}\). Caspase3 cleaves also p115, resulting in fragmentation of the Golgi \(^{67}\). Interestingly, expression of the C-terminal caspase cleavage fragment of p115 is sufficient to induce caspase mediated apoptosis \(^{67}\). Based on the observation that the C terminal fragment of p115 translocates to the nucleus, it was proposed that such fragments initiates pro-apoptotic stimuli in the nucleus \(^{67}\). Finally, the cis-Golgi protein Golgin160 is cleaved specifically by Caspase2, a Golgi localized caspase \(^{62,63}\). Similar to what has been documented for GRASP65, overexpression of a mutant of Golgin160 not cleavable by caspases rescues Golgi fragmentation and delays apoptosis induced by ER stress stimuli \(^{62}\). Golgin160 might act by sequestering Caspase2 at the Golgi, thus blocking its function. As it is the case for p115, there is evidence that caspase generated fragments of Golgin160 translocate to the nucleus \(^{69}\).

The case of the Golgi in apoptosis is a further example of how endomembranes are targeted by signaling cascades and since fragments of Golgi proteins were also shown to themselves modulate signaling, it is also an example of how the Golgi can modulate a signaling pathway. However, there are cases where endomembranes are the source of the signaling cascade. A lot of excitement was generated when the small GTPase H-Ras was shown to signal from ER and Golgi \(^{70}\). Even more surprisingly, activation of Ras from different subcellular locations resulted in differential activation of pathways downstream of Ras. Golgi activation of Ras resulted in a more potent activation of Akt than PM signaling. From the ER, instead, Ras activated preferentially the JNK pathway \(^{70}\). Src family kinases were shown to be necessary to activate Ras on endomembranes \(^{70}\). Bivona et al. fully elucidated the mechanism that activates Ras at the Golgi. Src signals to PLC\(_\gamma\)1, that in turn recruits the Ras GEF RasGRP1 to the Golgi \(^{71}\). Importantly, Golgi restricted Ras was still able to mediate differentiation of PC12 cells, demonstrating that this pool is biologically relevant \(^{71}\). In another study, constitutively active Ras was targeted to ER or Golgi and several processes downstream of Ras were analyzed. ER signaling resulted overall in an antiapoptotic effect, while signaling from the Golgi resulted in reduced proliferation and transformation \(^{72}\). After Ras signaling, several other signaling cascades were localized to the secretory pathway, and in particular to the Golgi apparatus. Phosphoinositides have been shown to control the Golgi and also to compartmentalize signals from the Golgi \(^{73,74}\). Another signaling cascade was identified at the TGN, where GOLPH3 was shown to regulate mTOR signaling \(^{75}\), but the mechanism behind this connection remains
elusive. In the context of signaling from the Golgi apparatus, GM130 appears to be a very important protein. In fact, it functions as a scaffold for the Ste20 family kinases YSK1 and MST4, the interaction being of relevance for cell motility. The Golgi has long been linked to cell migration and polarity, but no clear mechanism elucidating its function in those processes had ever been proposed, therefore the link between GM130 and YSK is of particular importance.

**The Golgi Apparatus in cell polarity**

Preisinger et al. identified the Ste20 family kinases MST4 and YSK1 as interaction partners of GM130. GM130 is responsible for the Golgi targeting of those two kinases and for their autophosphorylation that activates them. The binding to GM130 probably favors homodimerization of the kinases, resulting in their activation. Overexpression of unphosphorylatable YSK1, as well as depletion of YSK1 or MST4 results in Golgi fragmentation and abolishment of Golgi reorientation in a wound healing assay. Consistently, cells lose their invasiveness into collagen. Instead, unphosphorylatable MST4 does not alter Golgi structure, neither it affect migration or invasion. Overexpression of MST4 wt has the same effects of unphosphorylatable YSK1, speaking in favor of a model where the two kinases antagonize each other. Even if the two kinases are strictly related, they have different effectors. 14-3-3ζ was found to be an effector of YSK1 that most probably mediates the effect of YSK1 in migration and invasion. YSK1 and MST4 have also been linked to neuronal polarity and apico-basal polarity. In intestinal cells, MST4 is recruited to a complex composed by activated LKB1, STRADα and Mo25, and it is necessary to induce brush border formation downstream of LKB1. In resting cells, MST4 is observed at the Golgi, together with GM130. When LKB1 is activated, MST4 translocates to the apical membrane, where it phosphorylates Ezrin, thus promoting brush border formation. The mechanism behind the decision of MST4 to stay at the Golgi or leave it remains unclear since LKB1 does not activate MST4 directly. GM130 and LKB1 could compete for the binding to MST4 or alternatively, they could cooperate to spatially restrict MST4 signaling. LKB1 promotes the recruitment of phospholipase D1 (PLD1) to the apical membrane, which converts PI_4,5_2 in phosphatic acid, resulting in the recruitment of a GEF for the small GTPase Rap2A. Active Rap2A recruits TNIK, the kinase responsible for the activation of MST4 downstream of LKB1. This model raises several question about the role of GM130 in apico-basal polarity. Is the interaction between
MST4 and GM130 necessary for apical brush border formation? LKB1 depletion impairs the ability of the Golgi to reorient towards a wound in migrating cells. Therefore, it can be speculated that LKB1 signals to GM130 and this signaling drives reorientation of the Golgi towards the apical membrane of polarizing cells, ultimately causing polarized delivery of MST4 to the apical membrane. LKB1 and GM130 might thus signal to each other. In neurons, Ysk1 (also called Stk25) mediates crosstalk between GM130 and LKB1. GM130 acts as a scaffold for Ysk1 at the Golgi. Ysk1 interacts with STRADα, the activator of LKB1 and depletion of Ysk1 results in less active LKB1. Knockdown of either Ysk1, LKB1 or GM130 results in fragmentation of the Golgi and impaired axon formation. Interestingly, expression of both active and inactive Ysk1 rescued axon formation in Ysk1 depleted cells, showing that the scaffolding function of Ysk1 is important in this process. Ysk1 overexpression could also rescue the morphology of the Golgi in LKB1 depleted cells, but not in GM130 depleted cells. However it is not clear from this study if LKB1 signals to GM130 or if conversely, GM130 signals to LKB1 via Ysk1. Reorientation of the Golgi towards the growing axon has been observed long time ago and such phenomenon is not simply a passive event of the neuronal differentiation process. It is clear in fact that the position of the Golgi in differentiating neurons will determine which neurite will become the axon. By orienting towards the nascent axon, the Golgi polarizes traffic and thus sustains the growth of the structure. If trafficking is blocked the axon does not form. However, Golgi reorientation towards the axon is only transient, while in the end the Golgi will position in front of a dendrite that will become the primary dendrite, thus the organelle will determine also dendritic polarity. Reorientation of the Golgi precedes and predicts asymmetrical dendritic growth. If post Golgi traffic is blocked by BFA treatment or by expression of a kinase dead PKD, dendrites do not grow anymore, showing the importance of traffic to sustain dendrite growth. When polarization of the Golgi is prevented by overexpressing GRASP65, condition where post-Golgi traffic remains unaffected, the dendrites grow well, but no apical dendrite forms, meaning that if traffic sustains dendrite growth, it is the positioning of the Golgi that determines the primary dendrite. Golgi outposts are also observed at branching points of dendrites, mainly in the primary dendrite. Those Golgi outposts probably work as intermediate stations of protein traffic and are necessary for the dynamical growth and retraction of the dendrites branches. Finally, the Golgi shapes polarity of immune cells during the formation of the immunological synapse. Recognition of a target by either Cytotoxic T Lymphocytes (CTLs)
or Natural Killer (NK) cells triggers rapid polarization of the MTOC and of the Golgi towards the zone of the plasma membrane that established physical contact with the target, thus establishing the so-called immunological synapse. Reorientation of the Golgi makes it possible for CTLs and NK to kill their targets by secreting specialized granules filled with cytotoxic molecules, a process that has been renamed the kiss of death.

**The Golgi Apparatus in cell migration**

In the context of front-rear polarity, it is known that the Golgi reorients towards the front of a migrating cell, but still it is not clear whether this reorientation is a primary event or if it is instead a passive response of the Golgi to cell migration. It is known that the ability of the Golgi to polarize depends on Cdc42 and on dynein. Src and PI3K are most likely the receptors triggering the activation of Cdc42 at the plasma membrane necessary to reorient the Golgi and the whole secretory machinery towards the leading edge. Despite the widely recognized importance of this polarization, it is not yet clear whether the Golgi is instructed to orient towards the leading edge, and therefore is a passive player of the process, or if it is instead an active participant to cell polarization, meaning that it contributes to the whole process by modulating or initiating signaling.

The work of Preisinger et al. showed that signaling at the Golgi is important for cell migration. This was based on the observation that YSK1 localizes to the Golgi and is important for maintaining its structural integrity. However, a kinase dead mutant of Ysk1 did not affect migration, so it cannot be excluded that the defects observed in cell migration and invasion are due to the fragmentation of the Golgi and not a consequence of altered Ysk1 signaling. In fact, there is plenty of examples in the literature of conditions that fragment the Golgi and impair cell migration. Golgin160 or GMAP210 depletion lead to fragmentation of the Golgi. Even if post Golgi trafficking and Golgi to ER trafficking are not affected by fragmentation, the cell lose their ability to polarize secretion of proteins and lipids to the leading edge, thus resulting in impaired directed migration. Accordingly with the inability of the Golgi to polarize, no acetylated microtubules pointing to the leading edge form and the actin cytoskeleton does not polarize upon knockdown of Golgin160 or GMAP210. The proposed model is that impairment of reorientation of the Golgi will result in impaired polarized trafficking and therefore directed migration will be inhibited. However, to finally answer the
question about the role of the Golgi in cell migration, the ideal condition would be to block the polarization cue, leaving intact the structure of the organelle.

In order to correctly polarize secretion, cells need to have microtubules oriented towards the leading edge. The Golgi can nucleate microtubules and the current understanding of Golgi derived microtubules assumes that microtubules arising at the Golgi are the only polarized microtubules in a migrating cell, whereas centrosomal microtubules will be radially distributed and thus not useful to sustain polarized secretion. Golgi microtubules form independently of the centrosome, but still require the γTubulin Ring Complex (γTuRC). Rivero et al. found that AKAP450, a protein that interacts with γTubulin via p150 Glue, is kept at the Golgi by GM130 and it is necessary to nucleate Golgi microtubules. Knockdown of AKAP450 results in a reduction in the acetylated tubulin normally localized at the Golgi area. In addition, cells migrate less efficiently into a wound, even though their Golgi are oriented in the direction of migration. Overexpression of a fragment of AKAP450 containing the GM130 interacting region, but lacking the region binding to p150glue, resulted in loss of Golgi derived microtubules, fragmentation of the Golgi and impaired exit of cargo from the Golgi, with subsequent impairment of cell migration. While the defect in cargo exit from the Golgi was only mild, no polarized secretion could be achieved in a migrating cell. Overexpression of the AKAP450 fragment displaces the full length endogenous protein from the Golgi, but leaves unaffected the other pools of AKAP450. Accordingly, centrosome derived microtubules were unaffected in this condition. The strong defect observed in polarized secretion could be due to the lack of Golgi derived microtubules, thus supporting the hypothesis that this pool of microtubules is necessary for polarized secretion.

By tracking microtubules dynamics in migrating cells, Efimov et al. identified CLASPs as essential components to nucleate microtubules from the Golgi. CLASP1 and 2 are localized at the TGN via an interaction with the golgin GCC185. CLASPs stabilize microtubules growing from the Golgi and depletion of either CLASPs or GCC185 impairs Golgi microtubules formation. As it was observed for AKAP450, depletion of CLASPs results in a defective persistent migration, but does not affect the reorientation of the MTOC. It is possible to combine those two models to explain how microtubules are nucleated at the Golgi: microtubules seeds might be formed at the cis-side of the Golgi via AKAP450, then they either dissociate from membranes and bind again to the TGN or proceed on Golgi membranes until those membranes reach the TGN, where CLASPs stabilize the nascent microtubules and
promote their growth. Recently, a microtubules crosslinking protein named MTCL1 has been identified at the Golgi, its localization dependent on AKAP450 and CLASPs. This protein was previously shown to be important to stabilize the apico-basal bundle of microtubules in MDCK cells. Thus, it can be speculated that Golgi derived microtubules are also involved in the maintenance of apico-basal polarity.

The mechanism that causes reorientation of the Golgi in response to a migratory stimulus has been studied, but it still not well understood. Cdc42 is activated at the plasma membrane downstream of Src and PI3K. Active Cdc42 probably control microtubules dynamics by locally regulating dynein activity. Dynein was shown to be necessary for Golgi reorientation and it is possible that local dynein activity generates a pulling force that leads to Golgi reorientation. The localized polymerization of actin induced by Cdc42 does not seem to be necessary for Golgi reorientation, however it has been reported that treatment of cells with CytochalasinD (which disrupts the actin cytoskeleton) inhibited Golgi orientation in an electric field. In addition to exerting a pulling force, microtubules serve as roads for vesicle traffic. It has been observed that Cdc42 traffics both backward to the cell center and forward to the leading edge following microtubules. In a model of directed migration in an electric field, it has been observed that the first change in the cell in response to a polarizing stimulus is the formation of a lamellipodium. This is followed by reorientation of the Golgi and, if the Golgi does not reorient, the lamellipodium simply disappears, to form again in another point of the plasma membrane. An intriguing hypothesis linking these findings would be that the Golgi delivers either active Cdc42, or activators of this GTPase to the leading edge, thereby maintaining an active pool of the small GTPase and allowing persistent directed migration. Proving this hypothesis would be of great importance for the field and in addition similar work is needed for other modes of directed motility, other than migration in an electric field.

Phosphorylation of GRASP65 by ERK was shown to be necessary to allow reorientation of the Golgi towards the leading edge. Interestingly, ERK phosphorylates GRASP65 on the same residue that is also phosphorylated in mitosis (Ser277). While this leads to Golgi fragmentation in mitosis, it does not have any appreciable effect on Golgi morphology in migrating cells. This can be probably explained by the fact that in mitosis GRASP65 is phosphorylated on multiple sites, and in addition, other Golgi proteins are phosphorylated. During migration, GRASP65 is the only Golgi protein phosphorylated by ERK. It might
be possible that this phosphorylation makes the Golgi less tight and therefore it is easier for the organelle to polarize, but to proof this imaging at higher resolution such as electron microscopy or super-resolution microscopy would be required. When GRASP65 is mutated on Ser277, the Golgi does not reorient, and neither does the centrosome. However, upon BFA dispersal of the Golgi, the centrosome reorients. A possible interpretation is that, because of its bigger size, the Golgi imposes a physical block to the centrosome and when the Golgi is fragmented, this block is removed. The link between Golgi and the centrosome might be mediated by GMAP210. This Golgi protein binds to the minus end of microtubules and it interact with γTubulin. This could represent a physical connection between the Golgi and the centrosome and as a proof of this hypothesis, when GMAP210 is artificially targeted to the mitochondria, the mitochondria assume a perinuclear localization, close to the centrosome. GM130 could represent another link between Golgi and centrosome. GM130 could control centrosome positioning in two ways. First, it interacts with AKAP450, and this interaction is important to maintain Golgi integrity and for the perinuclear localization of the organelle. Second, GM130 binds to TUBA, a Cdc42 GEF that is also important for centrosome positioning. According to this study, Cdc42 activity is required to correctly localize the centrosome in interphase and mitosis, and GM130 is proposed to regulate the activity of the small GTPase specifically at the Golgi. However, we and others failed to localize TUBA at the Golgi, therefore further investigation is needed in order to validate this hypothesis.

**Golgi and cancer**

Given that the Golgi is implicated in cell migration and polarity, it is not surprising to find that the Golgi is also important for tumorigenesis or for metastatic dissemination of tumor cells. Indeed, the structure of the Golgi is altered in many cancers and some Golgi resident proteins have been implicated in controlling tumor formation. Surprisingly, however, none of these proteins is implicated in tumorigenesis because of a function in cell migration and polarity. The first Golgi protein to be linked with cancer was GOLPH3. GOLPH3 is a component of the TGN that regulates retrograde transport of proteins from endosomes to the TGN. GOLPH3 is often amplified in different tumors. In vitro, GOLPH3 knockdown reduces anchorage independent growth and proliferation, while its overexpression enhances these processes as well as H-Ras driven transformation. GOLPH3 interacts with VPS35, a protein of
VPS35 was shown to regulate mTOR activity in yeast\textsuperscript{114} and accordingly, GOLPH3 overexpression resulted in increased mTOR activity, as assessed by phosphorylation levels of Akt (downstream of mTORC2) and S6K (downstream of mTORC1). Cells overexpressing GOLPH3 gave rise to bigger tumors in mouse xenotransplants. Finally, tumors overexpressing GOLPH3 resulted to be more sensitive to the mTOR inhibitor Rapamycin\textsuperscript{75}. The study by Scott et al. provided for the first time a mechanistic insight by which a Golgi protein can regulate tumor progression. However, more work is necessary to fully understand the role of GOLPH3 in cancer.

GM130 has also been linked to cancer. Reduction of GM130 levels by aerosol delivery of shRNA resulted in reduction of tumor growth in the mouse lung\textsuperscript{115}. The study hints that GM130 loss leads to increased levels of autophagy and reduced proliferation. Even if the proposed mechanism is not completely clear, the finding that a Golgi resident protein is important in cancer is interesting and further studies will be necessary to understand the link between Golgi and cancer.

*Figure 1 – Schematic of some of the signaling routes to and from the secretory pathway described in this introduction.*
References


Introduction

– Secretory Pathway

Aim of the Thesis
Aim of the thesis
Rho GTPases are fundamental for polarity and among them, Cdc42 is considered the key regulator of this process. Despite the fact that Cdc42 is largely localized on endomembranes and, particularly, at the Golgi, the possibility that endomembrane signaling of Cdc42 controls polarity has not been investigated. Therefore, we aimed at understanding whether Golgi localized Cdc42 plays any role in the control of cell polarity. It was previously speculated that GM130 could play a role in regulating Cdc42 at the Golgi, thus we analyzed the mechanism by which GM130 controls the activity of the small GTPase.

If Golgi-Cdc42 is important for polarity, this would indicate that signaling from endomembranes plays a role in the control of cellular processes that were previously investigated only in the context of plasma membrane signals. Loss of polarity is increasingly being viewed as a tumor promoting process, therefore it would be of special interest to determine if endomembrane signaling is altered in pathological conditions such as tumors. To do so, we analyzed GM130 expression in a panel of colon cancer patients. Finally we assessed the role of GM130 in cancer related process by stably depleting the Golgi protein from different breast cancer cell lines. We determined which processes involved in tumorigenesis are affected by depletion of GM130.
Results
Results

Part 1 - Spatial Cdc42 signalling regulates polarity and tumorigenesis


The Golgi pool of Cdc42 controls cell polarity

To study spatial Cdc42 activation patterns we used a Cdc42 Raichu probe. For all our measurements we used the probe containing the C-terminal domain of Cdc42 (Cdc42-EM hereafter). This C-terminal domain targets the FRET reporter to different membranes including the plasma membrane and various cellular endomembranes such as the Golgi or endosomes (Supplementary Fig 1A) localizing as endogenous Cdc42 does. Cdc42-EM appeared in a juxtanuclear pool colocalizing with GM130, indicating its presence at the Golgi complex (Fig. S1B), thus allowing us to monitor Cdc42 activation at this cellular location where Cdc42 is active (Fig. 1A). Measuring FRET outside the Golgi-area or the plasma membrane did not reveal any specific signal, thus supporting the specificity of our FRET measurements (Fig. 1A). Silencing GM130 reduced the total cellular pool of Cdc42-GTP (Fig. S1C), reducing substantially the activity of Cdc42 at the Golgi (Fig. 1B). However, GM130 depletion did not affect the activity of this reporter at the plasma membrane indicating that it did not affect Cdc42 activation therein (Fig. 1C). Silencing GM130 inhibited 2D cell migration (Fig S1E) indicating that alteration of spatial Cdc42 signalling is relevant for directed cell motility. Moreover, the recruitment of aPKC to the leading edge of directionally migrating cells was also reduced (Supplementary Fig. 1F). To test for effects on polarity in a 3D cell culture model, we used Caco-2 cells, which are known to spontaneously polarize when plated in a 3D matrix forming cysts with a single lumen. Previous work showed that alteration of Cdc42 activity (and thus of polarity) leads to the formation of multi-lumen (aberrant) cysts. Depletion of GM130 in Caco-2 cells led to an increase in the number of aberrant cysts, which is indicative of a polarity defect (Fig. 1D). These results suggested that regulation of spatial Cdc42 activity by GM130 was relevant for cell polarization in 2D and 3D cell culture. Contrarily, GM130 depletion did not affect: Golgi morphology (Supplementary Fig 2); ER-Golgi trafficking; Golgi-to-plasma membrane trafficking; and coatamer recruitment.
to the Golgi (Supplementary Fig. 3A-C). Furthermore, the capability of the Golgi to nucleate microtubules was also unaffected (Supplementary Fig. 3D), in agreement with previous findings. Thus, the indirect impact of these processes on polarity could be excluded.

**Directed trafficking causes asymmetric distribution of Cdc42**

To obtain more direct evidence for the role of the GM130-regulated Golgi pool of active Cdc42, we measured Cdc42 activity at the front and the rear of directionally migrating cells using the Cdc42-EM probe. Strikingly, Cdc42 activity exhibited an asymmetric distribution, being more active at the front compared to the rear of the cell (Fig. 1E, F). Silencing GM130 reduced the amount of activated Cdc42 at the leading edge, thereby breaking the asymmetric distribution of active Cdc42 (Fig. 1E, F). These results were confirmed by immunofluorescence staining against endogenous Cdc42-GTP (Fig. 1G). Sustained accumulation of active Cdc42 at the leading edge could be the consequence of an increased local activation. Alternatively, the Golgi might deliver Cdc42-GTP to the leading edge. If the latter mechanism was true, then blocking trafficking from the Golgi ought to diminish the amount of Cdc42-GTP at the leading edge. To prove this point, directionally migrating cells in a wound closure assay were incubated at 20°C for one hour, which blocks exit from the trans-Golgi. Measuring FRET reporter activity or staining for Cdc42-GTP revealed that blocking post-Golgi trafficking diminished the presence of GTP-Cdc42 at the cell front, thereby abolishing Cdc42-GTP asymmetry (Fig. 1E&F). Importantly, re-incubation of the cells at 37°C for one hour re-established Cdc42-GTP asymmetry with higher levels at the leading edge compared to the rear (Fig. 1F). The loss of Cdc42-GTP asymmetry in GM130 knockdown cells was likely due to an abrogation of directed trafficking. Live cell imaging GFP-VSVG tracks forming from the Golgi showed that while carriers formed readily in GM130-depleted cells, they were non-directed, contrarily to tracks in control cells which mostly trafficked towards the leading edge (Fig. 1H). Of note, only the directionality of post-Golgi trafficking was affected, but not the global rate of post-Golgi trafficking (Fig. S3B). Since, both GDP- and GTP-restricted Cdc42 is known to block post-Golgi trafficking, we conclude that GM130 depletion does not affect the GTP/GDP cycling of Cdc42. Taken together, the Golgi pool of active Cdc42 is relevant for cell polarization.
The GM130-Cdc42 crosstalk does not involve GEFs

Next we asked how the Cdc42 activity at the Golgi was regulated by GM130. One possibility is that GM130 recruited a Cdc42 GEF leading to local activation of this GTPase. If true, then this GEF must interact with GM130 and localize to the Golgi in a GM130-dependent manner (schematic in Fig.2).

To identify GEFs that contribute to activate Cdc42 at the Golgi, we screened a library of 51 siRNAs against Rho family GEFs for effects on the Golgi pool of Cdc42 using the Cdc42-EM FRET probe. We focused primarily on the knockdowns that affected Cdc42 activity at the Golgi in a manner stronger than GM130 depletion. While several GEF knockdowns decreased the FRET values at the Golgi, only three had an effect that was comparable to the effect of GM130, namely ARHGEF9, 11 and 12 (Fig. 3A). Depletion of GEF11 and 12 also resulted in reduced total cellular Cdc42-GTP (Supplementary Fig. 4B) supporting the FRET measurements. Depletion of any of these GEFs also resulted in an inhibition of directed migration as determined in a wound healing assay (Supplementary Fig. 4C). In addition, polarization of the Golgi to the leading edge of migrating cells was also affected (Supplementary Fig. 4D). However, none of these GEFs interacted with GM130 in co-immunoprecipitation experiments, nor did they localize to the Golgi (Fig. 3B, C). We also failed to detect any evidence for an involvement of the GEF Tuba, as it did not localize to the Golgi (Fig. S5A, B), nor did it interact with GM130 (Supplementary Fig. 5C, D) contrarily to previous findings\(^5\), and in agreement with others who failed to detect this GEF at the Golgi\(^12,13\). In addition, nor Tuba depletion (Fig 3A, Fig S5E) neither its overexpression (Supplementary Fig. 5F) changed the activation of Cdc42 at the Golgi. Of note, none of the GEFs tested localizes to the Golgi. Therefore, the GM130-Cdc42 crosstalk was not mediated via recruitment of a GEF for local activation of Cdc42.

The GM130-Cdc42 crosstalk involves RasGRF

Another explanation for the GM130-Cdc42 connection could be that GM130 was preventing the function of some Cdc42 inhibitor (schematic in Fig.2). One possible candidate was the GEF RasGRF2, the RasGRF isoform expressed in epithelial cells, which has been recently shown to act as an inhibitor of Cdc42\(^14\). A marked association of endogenous GM130 and RasGRF2 could be detected in co-immunoprecipitation experiments (Fig. 4A). Moreover, in about 50% of the cells, RasGRF2 co-localized with GM130 at the juxtanuclear region,
consistent with Golgi complex localization (Fig. 4B, C). RasGRF in addition also exhibited a localization pattern reminiscent of the ER, which is in agreement with previous work. Noticeably, upon GM130 depletion, RasGRF2 juxtanuclear localization dropped strongly to less than 10% of the cells (Fig. 4B, C). Moreover we found that the interaction between these proteins was mediated by the C-terminal domain of GM130 and the Cdc25 domain of RasGRF2 (Supplementary Fig. 6A & B). These data suggested that GM130 is involved in localizing RasGRF to the Golgi complex.

Overexpression of RasGRF1 or 2 blocked activation of Cdc42 at the Golgi as determined using the Cdc42-EM probe, in a manner dependent on the GEFs DH domain (Fig. S6C). Importantly, co-knockdown of GM130 and RasGRF2 restored Cdc42 activation levels at the Golgi (Fig. 1A). The involvement of RasGRF in the GM130-Cdc42 crosstalk was further supported by the finding that GM130 depletion did not affect the levels of GTP-Cdc42 in CHL cells which naturally lack all RasGRF isoforms (Fig. 4D). Therefore, we conclude that RasGRF is an essential mediator in the GM130-Cdc42 crosstalk.

Regulation of the GM130-RasGRF complex
We hypothesized that GM130 could sequester RasGRF, thereby preventing it from binding and inhibiting Cdc42. Thus, under conditions that activate Cdc42, GM130 would sequester RasGRF to prevent it from inhibiting Cdc42. To test this hypothesis, we determined whether the GM130-RasGRF complex is affected by serum-starvation, a condition where Cdc42 is not activated. Under these conditions, GM130 did not co-immunoprecipitate with RasGRF (Fig. 5A), contrary to cells in steady-state cultured under complete conditions (Fig. 3). Treatment with 10% FCS for 10 min resulted in re-formation of the GM130-RasGRF complex (Fig. 5A). If RasGRF were free under serum-starved conditions, then we would expect the Golgi pool of Cdc42 to be unresponsive to GM130 depletion. Cdc42 at the Golgi responded to serum treatment in control siRNA transfected cells, while GM130 depleted cells did not respond to this treatment (Fig. 5B). Of note, the FRET values at the Golgi we similar in control and GM130 silenced serum-starved cells, further supporting the notion that this pool becomes GM130-independent under serum-starved conditions (Fig. 5B).

If GM130 acts to protect Cdc42 from inhibition by RasGRF, then depletion of GM130 ought to increase the association between RasGRF and Cdc42. Co-immunoprecipitation
experiments demonstrated that this was the case: the amount of RasGRF in association with Cdc42 increased in GM130 knockdown cells compared to control cells (Fig. 5C). Finally, we tested whether RasGRF is relevant for the GM130-Cdc42 crosstalk in the context of polarity. This was the case, as co-depletion of RasGRF2 and GM130 rescued the polarity defects imposed by GM130 depletion (Fig. 3F&G).

The GM130-RasGRF complex controls Ras activity
Our data indicated that GM130 acts as a repressor of RasGRF. Consistent with this model (Fig. 6A), knockdown of GM130 would release RasGRF, allowing it to inhibit Cdc42. However, RasGRF is also a well-known activator of Ras. Thus, in agreement with our model, loss of GM130 should also result in an increase of Ras activation. To test this prediction, we performed a knockdown of GM130 and measured Ras activation using a pulldown assay. Depletion of GM130 increased the levels of GTP-loaded Ras and co-knockdown of RasGRF abolished this effect (Fig. 6B), indicating that the increase in Ras activity was dependent on RasGRF. We also tested whether signalling downstream of Ras was affected at the level of ERK1/2 response to mitogen treatment. Knockdown of GM130 increased ERK1/2 activation in response to serum stimulation and this effect was again dependent on RasGRF (Supplementary Fig. 7A). To test whether the effect on ERK1/2 signaling is biologically relevant, we depleted GM130 in PC12 cells. These cells are a standard model for ERK-dependent cell fate decisions. The higher and the more sustained ERK1/2 signalling, the higher is the likelihood that PC12 cells shift from proliferation to differentiation (evident by the emergence of neurites). Depletion of GM130 resulted in elevated and sustained ERK1/2 activation (Supplementary Fig. 8A) and accordingly the percentage of cells that underwent differentiation was significantly increased (Supplementary Fig. 8B&C). Thus, the effects of GM130 on Ras signalling are relevant for ERK-dependent cell fate decisions.

Expression of GM130 in colon cancer
Loss of polarity is a frequent feature of epithelial tumours, associated with dysplasia and increased metastatic potential. Our results indicated that GM130 regulates polarity by modulating the Golgi pool of active Cdc42 and, in addition, Ras activity. Therefore, we asked whether GM130 could be associated to the malignant phenotype. To investigate this possible connection, we used two strategies. First, we analysed GM130 expression in a colon
cancer progression TMA comparing normal colon mucosa (29 cases), adenoma (16 cases), and adenocarcinoma (109 cases) (Fig 7A). While the expression of GM130 did only marginally differ in normal colon and adenoma, the expression in carcinoma was reduced markedly (Fig. 7B-D). We next extended this analysis in samples from 16 patients, present on TMA, with matched normal and cancer tissues. We compared the scored intensity of GM130 expression and found that in 14 out of 16 individual patients the expression of GM130 was reduced in tumour compared to the matched normal tissue (Fig. 7E&F). The downregulation of GM130 in cancer was further confirmed by the analysis of 11 matched normal and cancer colon tissues from a different source (Supplementary Fig. 9). In this case, the intensity of the GM130 staining was measured and healthy tissues were compared with the matching cancerous tissues. In agreement with the previous results, GM130 was downregulated in the cancerous tissue of 9 out of 11 patients (Supplementary Fig. 9A-C).

In support for a role of GM130 loss in tumour progression is our finding that knockdown of GM130 in Caco-2 cells led to a loss of E-cadherin expression (Supplementary Fig. 9D), a condition that is often considered to be linked to tumour progression and a loss of epithelial identity and is in line with the notion that loss of polarity is a typical phenomenon in EMT 19. We could show that the effect of GM130 on E-Cadherin was not cell line specific, as it turned out that depletion of GM130 caused loss of E-Cadherin also in another epithelial cell line (Supplementary Fig. 9D).
Figures

Figure 1

A

B

Golgi

C

Plasma Membrane

D

Control

GM130 KD

GM130

Giantin

Actin

E

Control

GM130 Knockdown

F

G

Cdc42-GTP

Giantin

H

% of polarized traffic

Results - 88
Figure 1. GM130 spatially regulates Cdc42 activity to control polarity. (A) One representative HEK293 cell transfected with the Cdc42-EM FRET reporter is shown here. Areas where FRET was measured are marked in red and average FRET values ± standard deviation calculated from 5 independent experiments in control transfected cells are shown. Scale bar = 25 µm. (B, C) HEK293 cells were transfected with the indicated siRNA. After 48h, cells were transfected with the Cdc42-EM FRET reporter. After additional 24 h, cells were fixed and FRET at the Golgi region (B) or at the plasma membrane (C). Averages of at least 3 independent experiments ± standard deviations (at least 15 cells per condition in every experiment) are shown on the graph. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (*p<0.05). (D) Caco-2 cells were transfected with the indicated siRNA. Cells were grown in Geltrex® matrix for four days and cysts were stained for Giantin (red) and GM130 (green) using immunofluorescence and for F-actin (blue) using fluorescent phalloidin. A magnification of a single cyst is displayed in the upper row and an overview of several cysts in shown in the lower row. Scale bar = 7.5 µm for the magnified images (upper images) and 75 µm for the overview images. (E) HeLa cells were transfected with the indicated siRNA. After 48 h, cells were transfected with a plasmid encoding the Cdc42-EM Raichu probe. After 24 h, a wound was generated using a 10 µl pipette tip and cells were allowed to migrate and polarize for approximately 6 h at 37°C followed by fixation. Representative images of cells expressing the Raichu probe which are facing the wound are shown. FRET was measured on the plasma membrane part facing the wound (indicated as “Front”) and on the opposing end of the cell (indicated as “Rear”). Scale bar is 10 µm. (F) Graphic representation of FRET measurements from three independent experiments of HeLa cells expressing the indicated Raichu probe and that that were transfected with the indicated siRNA 72 h prior to the experimental measurements. Above the graph a schematic of the experimental setup: control cells have Cdc42-GTP at the Golgi and deliver it to the leading edge in a polarized way. GM130 knockdown cells have less Cdc42-GTP at the Golgi and lose the ability to polarize their traffic towards the leading edge. Blocking post-Golgi traffic by incubation at 20°C abolishes front-rear asymmetry and re-warming the cells to 37°C re-established asymmetry. White bars represent Front/Rear ratios of FRET values. Results are means ± SD from four independent experiments. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (** p<0.01). (G) HeLa cells were transfected with siRNA. After 72 hours, a
wound was made with a 10 μl tip and the cells were allowed to migrate for 6 hours. Cells were then fixed and immunostained for Giantin and Cdc42-GTP. Intensities of Cdc42-GTP were measured and the Front/Rear ratio is plotted as a bar graph. Results are means ± SD of 3 independent experiments. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (** p<0.01). Scale bar = 25 μm.

(H) HeLa cells were transfected with the indicated siRNA. After 48 h, cells were transfected with the plasmid encoding for VSVG-RUSH-GFP. After further 24 h, a wound was made with a 10 μl tip and cells were allowed to migrate at 37°C for approximately 6 hours followed by live video microscopy. VSVG-RUSH-GFP was released from the ER by addition of biotin and after 25 min VSVG carriers were tracked using ImageJ. Individual tracks are displayed on the representative images. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant differences tested using Student’s T-test (** p<0.01).
Figure 2. Models for the GM130-Cdc42 crosstalk
Schematic representation of two different models. On the left-hand side: GM130 acts as a scaffold for a Cdc42-GEF at the Golgi. In this case, a GEF should be located at the Golgi in a GM130 dependent manner. Upon GM130 depletion, the GEF is lost from the Golgi and subsequently, Cdc42-GTP doesn’t accumulate at the organelle. In the second scenario (right-hand side), GM130 binds to an inhibitor of Cdc42, protecting the small GTPase from inactivation. If GM130 is lost, the inhibitor will bind to Cdc42 in places other than the Golgi, thereby preventing a GEF mediated activation of Cdc42. In this way, Cdc42-GTP will not be accumulated at the Golgi.
Figure 3

A

Cdk2 activation level (difference from control)

B

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C

GM130

Flag

Composite

Flag-ARHGEF9

Flag-ARHGEF11

Flag-ARHGEF12

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Figure 3. Screening for GEFs that regulate the Golgi pool of Cdc42

(A) HEK293 cells were transfected with control or with the indicated siRNA. After 48h, cells were transfected with the plasmid encoding for the Cdc42-EM-FRET probe. After further 24h the cells were fixed and mounted. FRET was measured as described in the Material and Methods section and FRET values are displayed as fold of control and are averages from the measurements of at least 30 cells per condition. Red bars represent CI95 intervals

(B) HeLa cells were transfected with plasmids encoding the indicated GEF. 24 h later, cells were lysed and the lysates were subjected to immunoprecipitation either with a GM130 antibody (+) or with protein G sepharose beads alone. “5% input” indicates lanes where 5% of the total material that was used in the IP is loaded. The immunoprecipitated material was eluted and subjected to SDS-PAGE followed by immunoblotting against the indicated proteins.

(C) HeLa cells were plated on coverslips, then transfected with plasmids encoding the specified GEF. 24 h later, cells were fixed and immunostained for GM130 and Flag. Intensity plot corresponding to the region marked by a white arrow are shown on the right. Scale bar is 10 µm.
Figure 4

A. 5% input versus GM130 IP.

B. RasGRF at the Golgi.

C. Control versus GM130 knockdown with GM130 and RasGRF.

D. GM130 siRNA with Cdc42-GTP and Total Cdc42.

E. GM130 mRNA levels in HeLa and CHL.

F. Percentage of cells with oriented Golgi.

G. Actin and GM130 expression levels.
Figure 4. The effect of GM130 on Cdc42 is dependent on RasGRF

(A) HeLa cells were lysed and the lysate was subjected to immunoprecipitation either with a GM130 antibody (+) or with proteinG sepharose beads alone. “5% input” indicates lanes where 5% of the total material that was used in the IP is loaded. The immunoprecipitated material was eluted and subjected to SDS-PAGE followed by immunoblotting against the indicated protein. (B) Quantification of cells with RasGRF at the Golgi. (C) HeLa cells grown on glass cover slips were fixed followed by immunofluorescence staining of RasGRF and GM130. Images were acquired using a confocal laser scanning microscope. The magnifications correspond to the areas inside the white squares. Scale bar is 25 µm. An intensity plot corresponding to the region marked by a white arrow is shown. (D) CHL cells were depleted of GM130 by siRNA transfection. After 72 h, cells were lysed and GST-PAK1 was added to the lysate followed by pulldown with GSH-sepharose. The eluate was subjected to SDS-PAGE followed by immunoblotting against Cdc42 (Cdc42-GTP). 5% of the original lysate were subjected to SDS-PAGE followed by immunoblotting against Cdc42 (Tot-Cdc42) and against GM130. Bar graph in the right panel represents a quantification of the amount active Cdc42 (GTP-Cdc42) to total Cdc42. Results are means ± SD from three independent experiments. (E) RT-PCR of RasGRF2 comparing HeLa and CHL cells. (F) Cells were transfected with the indicated siRNA. After 72 h, a wound was made using a 10µl pipette tip and cells were allowed to migrate and polarize for approximately 6 h at 37°C followed by fixation and immunofluorescence staining of Giantin to label the Golgi. The Golgi was counted as oriented towards the wound if its major mass was located in a 120° angle facing the wound edge. The red line shows the average orientation of the Golgi immediately after wounding. Results are means ± SD from three independent experiments. Asterisk indicates statistically significant differences using ANOVA with Newman-Keuls multiple comparison test (*p<0.05). (G) Caco-2 cells were transfected with siRNA against GM130, or RasGRF2, or with both siRNA. Cells were grown in Matrigel® for four days to allow cyst formation. Afterwards, cysts were stained for GM130 using immunofluorescence and for F-actin using fluorescent phalloidin as described in “Materials and Methods”. Cysts with a single lumen were counted as normal, while cysts with multiple or no lumen were counted as aberrant. The percentage of normal and aberrant cysts in each condition was counted from three independent experiments which are displayed in the bar graph in the lower panel. Results are means ± SD. Asterisk
indicates statistically significant differences using ANOVA with Newman-Keuls multiple comparison test (*p<0.05; **p<0.01). Scale bar is 7.5 μm.

Figure 5

A

![Image of protein blots for RasGRF and GM130 IP](image1)

![Image of protein blots for RasGRF and GM130 5% Input](image2)

B

Cdc42 FRET at the Golgi

![Graph showing FRET efficiency](image3)

C

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![Image of protein blots for Cdc42 and RasGRF](image4)
Figure 5. The GM130-RasGRF complex is regulated by serum (A) MCF7 cells were grown to confluency. Then, cells were serum-starved for 2 h followed by stimulation with 10% FCS for the indicated time points. Cell lysates were subjected to immunoprecipitation against GM130. The immunoprecipitate was subjected to SDS-PAGE followed by immunoblotting against the indicated proteins. (B) HeLa cells were transfected with control or GM130 siRNA 72h prior to the experiment. After 48 h, cells were transfected with the plasmid encoding for the Cdc42 Raichu probe. Cells were serum-starved for 2 h followed by stimulation with 10% FCS for 0, 1 and 5 min and FRET was measured as indicated in “Materials and Methods. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (*p<0.05). (C) HeLa cells were transfected with the indicated siRNA. After 48 h, cells were transfected with empty vector or with a plasmid encoding HA-tagged RasGRF1. After 24 h, cells were serum-starved for 2 h followed by stimulation with 10% FCS for the indicated time points. Cell lysates were subjected to immunoprecipitation against HA. The immunoprecipitate was subjected to SDS-PAGE followed by immunoblotting against the indicated proteins.
Figure 6. GM130 depletion increases Ras activity

(A) Working model: GM130 acts as suppressor of RasGRF activity, thereby modulating the activities of Ras and Cdc42. According to this model, the knockdown of GM130 will result in increased Ras activation and decreased Cdc42 activation. (B) HepG2 cells were transfected with the indicated siRNAs. After 72 h, cells were lysed and Ras pull-down was performed using GST-RBD. The eluate form the beads was subjected to SDS-PAGE followed by immunoblotting with a pan-Ras antibody (Ras-GTP). 5% of the original lysate was subjected to SDS-PAGE followed by immunoblotting against tubulin and GM130. Bar graph represents a quantification of the amount active Ras (Ras-GTP) normalized to tubulin. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (*p<0.05; **p<0.01).
Figure 7. Dysregulation of GM130 in human colon cancer.
GM130 expression was measured by IHC on a Colon Progression TMA. (A) Graphic representation of the neoplastic evolution of colon carcinoma from the normal colon mucosa through the adenoma. (B) Bar graphs depicting the average expression level (± SD) of GM130 in Normal Colon Mucosa, Colon Tubulovillous Adenomas and Colon Adenocarcinomas or (C) the percentage of low and high GM130-expressing samples when using a cut off score value of 1.5 (differences are significant as assessed by Pearson χ2 analysis P < 0.001). (D) Representative images of immunohistochemical staining for GM130. (E) Bar graphs depict the expression score of GM130 in 16 patients for which matched normal and tumor tissue were available on the TMAs. GM130 is downregulated in colon cancer with respect to the normal colon mucosa in 14 out of 16 patients. (F) Representative core images of normal and tumor tissue from patient 14 and 19. Scale bar, 100 μm
Suppl. Fig. 1

A

[Diagram showing molecular interactions: activation and inactivation of Cdc42-GTP]

B

[Imaging of RAICHU probe, GM130, and Merge]

C

[Graph showing % of Cdc42-GTP for GM130 KD #1 and GM130 KD #2]

D

[Graph showing relative levels of RaGFR2 mRNA]

E

[Graph showing migrated distance (% of control) for GM130 KD #1 and GM130 KD #2]

F

[Imaging of Control and GM130 knockdown, showing % of cells with aPKC at the leading edge]
Supplementary Figure 1. Effect of GM130 on Cdc42 activity and on polarity proteins.
(A) Schematic representation of the Raichu FRET probes. Starting from the N-Term we find CFP, the CRIB domain of PAK1 (effector of Cdc42), Cdc42 deprived of its C-Term, YFP and a C-terminal motif which is either the K-Ras C-Term sequence (PM-Probe) or the Cdc42 C-Term sequence (EM-Probe). Once Cdc42 is activated, the probe binds and CFP and YFP are brought close to each other, resulting in FRET. (B) HeLa cells were plated on coverslips. 24 h later, cells were transfected with the plasmid encoding for the Cdc42-EM FRET probe. 48 h later, cells were fixed and processed for immunofluorescence staining of GM130. (C) Caco-2 (left panel) or HeLa (right panel) cells were transfected with the indicated siRNA (#1 = Dharmacon, #2= Qiagen). After 72 h, cells were lysed and GST-PAK1 was added to the lysate followed by pulldown with GSH-sepharose. The eluate was subjected to SDS-PAGE followed by immunoblotting against Cdc42 (GTP-Cdc42). 5% of the original lysate was subjected to SDS-PAGE followed by immunoblotting against Cdc42 (Total Cdc42) and against GM130. The amount of active Cdc42 (GTP-Cdc42) was quantified and normalized to total Cdc42. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant difference (p<0.01) tested using one-side Student’s t-test.
(D) HeLa cells were transfected with the indicated siRNA (QIAGEN SI04329941). After 72 h, cells were lysed and total RNA was extracted with the Qiagen RNeasy kit. The RNA was then retrotranscribed to cDNA by using the high capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturers’ instructions. Q-PCR was performed by using Fast SYBR green PCR MasterMix (Applied Biosystems) and primers specific for the cDNA of interest. (E) HeLa cells were transfected with the indicated siRNA (#1 = Dharmacon, #2= Qiagen). After 48h, cells were plated into IBIDI chambers and after additional 24 h, the plastic support was removed to allow cells to migrate for 19 h. After 19 h of migration, cells were fixed and immunostained with concanavalin-488. The length of the remaining wound was then measured with ImageJ. The distance migrated by control cells was set as 1 and the other conditions were normalized to it. On the graph, averages of at least 4 independent experiments are shown ± standard deviation. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (**p<0.01; *** p<0.001). (F) HeLa cells were transfected with the indicated siRNA. After 72 h, a wound was made and cells were allowed to polarize for 1 h at 37°C followed by fixation and staining.
for aPKC and GM130. The position of the wound is indicated by the white lines. Cells facing the wound showing an enrichment of aPKC at the leading edge were counted in control and GM130 knockdown cells. Results are displayed as bar graphs in the right panel. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant differences at p<0.01 (Student’s t-test).
Suppl. Fig. 2

A

B

Control siRNA

GM130 siRNA

% of pre-bleach fluorescence

0 20 40 60 80

Seconds

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Supplementary Figure 2. GM130 depletion does not affect Golgi integrity. 

(A) HeLa cells were transfected with the indicated siRNA. After 72 h, cells were fixed and processed for immunofluorescence staining of GM130 and Giantin. In case the cells exhibit more than three fragments of the Golgi, they were termed “fragmented”. The percentage of cells exhibiting a fragmented Golgi was counted and the results are displayed as a bar graph in the lower panel. Results are means ± SD from 3 independent experiments where at least 80 cells were counted per condition per experiment. The statistical difference was tested using a Student’s t-test and found not be significant. White bars correspond to 25 µm. (B) HeLa cells were transfected with control siRNA or GM130 siRNA. 48h later, cells were transfected with a plasmid encoding a fluorescent protein targeted to the Golgi (pmTurquoise-Golgi). After another 24 h, cells were analysed at the microscope. A region corresponding to approximately 1/3 of the Golgi was bleached and the fluorescence recovery was monitored. Results are average of 3 different experiments ± SD. No significative differences were observed between control siRNA and GM130 siRNA. White bars correspond to 10 µm.
Suppl. Fig. 3

A

Control (0 min)  GM130 KD (0 min)

Control (30 min)  GM130 KD (30 min)

% of cells with ManII at the Golgi after 30 min

- control
- GM130 KD

B

control  GM130 KD

GM130

VSVG-GFP

GFP

GFP in the cytosol

- control
- GM130 KD

C

β-COP at the Golgi

- control
- GM130 KD

β-COP

Giantin

D

Control

TGN46  GM130  Tubulin

GM130 KD

TGN46  GM130  Tubulin

% of cells with Golgi MTs

- Control
- GM130 KD

Average nr of Golgi MTs/cell

- Control
- GM130 KD
Supplementary Figure 3. GM130 depletion does not affect trafficking, Golgi structure or microtubule regrowth. 

(A) HeLa cells stably expressing the RUSH-ManII-GFP construct were transfected with the indicated siRNA. After 72 h, cells were either fixed directly (0 min) or treated with biotin followed by fixation at 30 min after treatment (30 min). The amount of cells displaying a Golgi pattern at 30 min after treatment was evaluated and is shown in the left panel. Results are means ± SD from 3 independent experiments. No significant difference was detected (Student’s t-test). White bars correspond to 25 µm. 

(B) HeLa cells were transfected with the indicated siRNA. After 48 h, cells were transfected with a plasmid encoding the temperature sensitive GFP-tagged VSVG-ts045 (VSVG-GFP). Cells were incubated overnight at 40°C to accumulate the protein in the ER. On the next day, cells were either fixed directly (0 h) or were incubated at 37°C for 1 h followed by fixation. Cells were stained by immunofluorescence for GM130. The amount of VSVG-GFP at the plasma membrane was measured using ImageJ and normalized to the GFP-fluorescence inside the cell. Results are displayed as a bar graph in the right panel. Results are means ± SD from 3 independent experiments where at least 15 cells per condition were evaluated per experiment. The statistical difference was tested using a Student’s t-test and found not be significant. White bars correspond to 25 µm. 

(C) HeLa cells were transfected with the indicated siRNA. After 72 h, cells were fixed followed by immunofluorescence staining of β-COP and Giantin. The intensity of β-COP staining at the Golgi was normalized to the intensity β-COP in the cytosol. The results are displayed as a bar graph in left part of the panel. Results are means ± SD from 3 independent experiments. No significant difference was detected (Student’s t-test). White bars correspond to 25 µm. 

(D) RPE-1 cells were transfected with control or GM130 siRNA. After 72 h, cells were processed to stain nucleating microtubules, as described in the “Materials and Methods” section. With bars correspond to 10 µm. Cells showing microtubules nucleating from the Golgi were counted. The result is shown in the upper bar graph. In addition, the average number of Golgi derived microtubules was calculated and the result is shown in the lower bar graph. Results are averages of at least 3 independent experiments ± SD. No significant difference was detected (Student’s t-test).
Supplementary Figure 4. Effect of ARHGEF 9, 11 &12 on Cdc42 activity, cell migration and Golgi orientation.

(A) RT-PCR quantification of the expression of ARHGEF9, 11 & 12 in cells transfected control siRNA or with siRNA against the given ARHGEF. 
(B) HeLa cells stably expressing Venus-Cdc42 were transfected with the indicated siRNA. After 72 h, cells were lysed and GST-PAK1 was
added to the lysate followed by pulldown with GSH-sepharose. The eluate was subjected to SDS-PAGE followed by immunoblotting against Cdc42 (Cdc42-GTP). 5% of the original lysate was subjected to SDS-PAGE followed by immunoblotting against Cdc42 (Total Cdc42). (C) HeLa cells were transfected with the indicated siRNA. After 48h, cells were plated into IBiDi chambers and after additional 24 h, the plastic support was removed to allow cells to migrate for 19 h. After 19 h of migration, cells were fixed and immunostained with concanavalin-488. The length of the remaining wound was then measured with ImageJ. The distance migrated by control cells was set as 1 and the other conditions were normalized to it. On the graph, averages of three independent experiments are shown ± standard deviation (left image). (D) Cells were transfected with the indicated siRNA. After 72 h, a wound was made using a 10µl pipette tip and cells were allowed to migrate and polarize for approximately 6 h at 37°C followed by fixation and immunofluorescence staining of Giantin to label the Golgi. The Golgi was counted as oriented towards the wound if its major mass was located mainly in a 120° angle facing the wound edge. The red line shows the average orientation of the Golgi immediately after wounding (45%). Results are means ± SD from three independent experiments. The red line represents the average % of oriented Golgi in cells fixed immediately after wounding. Asterisks indicates statistically significant differences using ANOVA with Newman-Keuls multiple comparison test (*p<0.05).
Suppl. Fig. 5

A

B

C

D

E

F

G

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Supplementary Figure 5. TUBA is not involved in mediating the GM130 effect on Cdc42. 
(A&B) HeLa cells were transfected with a plasmid encoding HA tagged Tuba. After 24 h, cells were fixed and stained for HA and GM130 or Giantin. An intensity plot corresponding to the region marked by an arrow is shown. (C) HeLa cells were transfected with an empty vector (-) or a plasmid encoding HA tagged Tuba (+). After 24 h, cells were lysed and an IP against HA was performed. “5% input” indicates lanes where 5% of the total material that was used in the IP is loaded. The immunoprecipitate was then subjected to SDS-PAGE followed by immunoblotting against the indicated proteins. (D) HeLa cells were transfected with an empty vector or a plasmid encoding HA tagged Tuba. After 24 h, cells were lysed and IP against GM130 was performed either with a GM130 antibody (+) or with proteinG-sepharose beads alone (-).5% input indicates lanes where 5% of the total material that was used in the IP is loaded. The immunoprecipitate was then subjected to SDS-PAGE followed by immunoblotting against the indicated proteins. (E) HeLa cells were transfected with control or TUBA siRNA (QIAGEN - nr SI04220237) 72h prior to the experiment. FRET was measured as indicated in “Materials and Methods. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant differences tested using one-side Student’s T-Test (*p<0.05). (F) HEK293 cells were transfected with a plasmid encoding for the Cdc42 Raichu probe plus another plasmid encoding for HA-TUBA or for HA alone. After 24 h, cells were fixed. FRET was measured at the Golgi as described in the Materials and Methods section. Results are averages of 3 independent experiments. No significative difference was observed between control or TUBA KD cells (Student T-Test). (G) HEK293 cells were transfected with siRNA against TUBA (QIAGEN nr. SI04220237 or siRNA from the GEF siRNA library - Dharmacon). After 72 h, cells were lysed and the expression of TUBA was quantified using RT-PCR.
Suppl. Fig. 6

A

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**RasGRF**

150

50

**Myc**

B

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**Flag**

150

100

75

**GM130**

150

C

![FRET efficiency graph]

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Supplementary Figure 6. Characterizing the GM130-RasGRF complex.

(A) HeLa cells were transfected with plasmids encoding HA-tagged RasGRF1 (RasGRF1-HA) and myc-tagged GM130 lacking its first 690 amino acids (GM130-Δ690-myc). After 24 h, cells were lysed and the lysate was subjected to immunoprecipitation with an anti-myc antibody followed by SDS-PAGE and immunoblotting against the indicated proteins. (B) HeLa cells were transfected with plasmids encoding Flag tagged wild type RasGRF2 (RasGRF2-Flag) or Flag-tagged RasGRF2 where the DH domain has been deleted (RasGRF2ΔDH-Flag) or with Flag-tagged RasGRF2 where the Cdc25 domain has been replaced by GFP (Flag-ΔCdc25). After 24 h, cells were lysed and the lysate was subjected to immunoprecipitation with an anti-GM130 antibody followed by SDS-PAGE and immunoblotting against the indicated proteins. (C) HeLa cells were transfected with control or GM130 siRNA 72 h prior to the experiment. RasGRF1 or RasGRF2 indicate conditions where control HeLa cells were expressing Flag-tagged RasGRF2 or HA-tagged RasGRF1 were co-expressed together with the Raichu probe. RasGRF2ΔDH indicates a condition where RasGRF2 lacking the DH domain was co-expressed together with the Raichu probe. FRET was measured as indicated in “Materials and Methods”. Results are means ± SD from three independent experiments. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (*p<0.05; ** p<0.01).
Supplementary Figure 7. Effect of GM130 depletion of ERK activation. (A) HEK293 cells were transfected with the indicated siRNA. After 72h, cells were serum starved for 2h and then stimulated with 10% FCS for the indicated time points. Cells were subsequently lysed and the lysates were subjected to SDS-PAGE followed by immunoblotting against the indicated proteins. Results are means ± SD from five independent experiments. Asterisks indicate statistically significant differences tested using ANOVA with Newman-Keuls multiple comparison test (*p<0.05).
Supplementary Figure 8. Effect of GM130 on PC12 cell differentiation

(A) PC12 cells were transfected with plasmids encoding either non-targeting shRNA constructs (control; blue line) or shRNA against GM130 (red line). Cells were treated with

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NGF followed by fixation at the indicated time points and staining of dually phosphorylated ERK1/2 (p-ERK). *(B&C)* PC12 cells were transfected with plasmids encoding either non-targeting shRNA constructs (control) or different shRNAs against GM130 (shRNA 1-4). Cells were treated with differentiation medium for 24 h and cells were fixed and the amount of differentiated cells was scored. A cell was termed differentiated if it had a process that is longer than 1 cell diameter.
Supplementary Figure 9. Downregulation of GM130 in colorectal cancer.

(A) Two examples of normal colonic mucosa (left) and the matching cancerous tissue (right) stained for GM130 (brown) and DNA (nuclei, blue). (B) Median value of the GM130 staining from 11 patients. Asterisks indicate statistically significant differences at **p<0.01 (Student’s t-test).

(C) Quantification of the GM130 staining (normalized to the number of nuclei) in 11 patients measured with ImageJ. (D) The indicated cell lines were transduced with lentiviruses encoding either control or GM130 shRNA. After 4-6 passages in culture, cells were grown in culture dishes to 90% density, lysed and the cell lysates were subjected to SDS-PAGE followed by immunoblotting against the indicated proteins.
HeLa cells grown to confluency were serum starved and restimuated with 10% FCS for the indicated time points or alternatively they were treated for 24 h with either ionomycin or PMA. Cells were then lysed and the lysate was subjected to immunoprecipitation either with a GM130 antibody or with protein G sepharose beads alone. ‘5% input’ indicates lanes where 5% of the total material that was used in the immunoprecipitation is loaded.
Part 2 - Regulation of spatial Cdc42 signaling by GM130 and its effects on cell migration, invasion and polarity

Baschieri F, Uetz Von-Allmen E, Legler DF, Farhan H. Regulation of spatial Cdc42 signaling by GM130 and its effects on cell migration, invasion and polarity – Cell Cycle – Accepted on January 8th, 2015.

GM130 expression in breast cancer

We showed previously that GM130 expression is reduced in colorectal cancer 20. Searching the Oncomine database (https://www.oncomine.org), we found several studies that reported lower GM130 mRNA levels in breast cancer compared to normal mammary tissue and two examples are displayed in Figure 1B. Thus, the defect in polarity imposed by GM130 depletion potentially represents a selective advantage to breast cancer, which is in line with findings of others who showed that loss of polarity genes promotes tumor progression 17, 21, 22. We compared the levels of GM130 mRNA and protein expression in a panel of breast cancer cells (five basal and four luminal cell lines). There was no significant difference between the two subtypes with respect to GM130 levels (Fig. 2A&B). Interestingly, while most cell lines had relatively comparable amounts of GM130 mRNA (Fig.2B), the variability in the levels of GM130 protein was more pronounced (Fig.2A) and did not correlate with the differences observed at the mRNA level. For instance while BT20 cells had higher GM130 mRNA levels than BT549 cells, the situation was opposite at the protein level.

Golgi morphology in breast cancer cells

Since cancer cells were shown to often display altered Golgi structure 23, we determined the Golgi compactness index as described previously 24. Some cell lines displayed strongly fragmented Golgi phenotypes, while others exhibited the Golgi as a single copy organelle located in the center of the cell (Fig. 2C&Fig.3). Overall, there was a weak correlation ($R^2=0.42$) between the expression levels of GM130 and the degree of Golgi compactness (Fig. 2D). However, when the two subtypes were analyzed separately we observed that luminal cell lines have a stronger correlation between Golgi compactness and GM130 levels, while basal cell lines displayed a very weak correlation.
GM130 depletion does not affect proliferation, apoptosis or adhesion

Based on our previous work 20, we speculate that the effect of GM130 loss is that it deregulates cell polarity and thereby promotes tumorigenesis. However, we did not consider the possibility of effects on other cancer hallmarks such as proliferation, apoptosis or adhesion 25, which could arise from pathways independent on polarity or from the defect on polarity itself 26, 27. We therefore stably depleted GM130 in two luminal (MCF7 and T47D) and two basal (MDA-MB231 and BT549) cell lines (Fig. 4A). In none of the cells could we detect an appreciable effect on adhesion or proliferation (Fig. 4B&C). In the case of apoptosis, we compared the basal level of apoptosis as well as cell death induced by the cytostatic drug Doxorubicin. In all cell lines, we observed a significant increase in apoptosis after treatment with the cytostatic agent (Fig. 4D). We next compared the sensitivity towards Doxorubicin treatment in mock-depleted and GM130-depleted conditions. In T47D cells, GM130 knockdown led to a reduced sensitivity towards Doxorubicin treatment, although the result was statistically significant, it was very weak. In MDA-MB231 cells, depletion of GM130 increased sensitivity and this effect was not only statistically significant, but was also strong.

Effect of GM130 depletion on cell motility

We finally tested the effects of GM130 depletion on cell migration. A classic is to perform wound-scratch assays and as shown previously for HeLa cells 20, directed motility of four breast cancer cell lines was inhibited by GM130 depletion (Fig. 5A). This is consistent with the defect in cell polarization imposed by GM130 depletion. Furthermore, we performed an invasion assay wherein cells had to invade through Matrigel towards a gradient (serum). In agreement with a polarization defect, GM130 knockdown reduced the invasive capacity of cells (Fig. 5B). However, loss of polarity genes was previously shown to promote metastasis 17, 21, and GM130, which we identified as a polarity regulator, also appears to be downregulated in breast (Fig. 1B) and colonic 20 cancer. It is difficult to reconcile a protumorigenic effect with an inhibition of invasion. However, we stress that the wound scratch assay and invasion assays towards a chemoattractant are strictly dependent on polarity and therefore are of limited use to evaluate the role of polarity proteins in cancer. Using intravital microscopy, it was shown recently that tumors exhibit two populations of cells, one that is fast moving and one that is slow 28. There, it was hypothesizes that the fast moving cells are those who cover large distances until they reach the proximity of a vessel, where they
become slower. Another report showed that cancer cells that move faster are those which are more likely to metastasize, as they give rise to more circulating tumor cells \(^{29}\). We therefore tested the effect of GM130 depletion on random motility of two breast cancer cells, BT549 and MDA-MB231, both of which are of the basal subtype, which is known to give rise to more metastasis than luminal cells. In BT549 cells, we observed a robust increase in a random motility assay (Fig. 5C). The magnitude of the effect of GM130 depletion is in the range of what has been observed for other genes of relevance to cell motility \(^{30}\). Of note, persistency of cell movement was reduced by GM130 knockdown (Fig. 5C). A similar observation has been made for HeLa cells migrating on collagen (not shown). In MDA-MB231 cells, velocity tended to increase by GM130 depletion, but the effect did not reach statistical significance and was weaker compared to BT549 cells (Fig. 5C). This is potentially due to the fact that MDA-MB231 cells express a constitutively active K-Ras oncogene (table 1). Hyperactive Ras signaling will over-activate ERK1/2, which is a positive driver of cell movement. In addition, GM130 depletion itself hyperactivates ERK1/2 \(^{20}\). Therefore, it might not be possible to detect the contribution of GM130 towards motility in cells with an already highly active Ras-ERK pathway. Finally, we wanted to determine the effect of GM130 depletion on invasion using an experimental setting that does not rely on a gradient. Strikingly, GM130 depletion in BT549 cells strongly increased the invasive capacity (Fig. 5D). On the contrary, MDA-MB231 cells, which did not respond in the random migration assay, did not show the same phenotype (Fig. 5D). Luminal cells are less invasive than basal cells. Nevertheless, T47D were shown to be slightly invasive \(^{31}\) and when we tested the effect of GM130 depletion in this cell line, we found that the invading cells doubled. However, the total number of invading cells remained markedly below what we observed for the two basal cell lines (data not shown).
Table 1

<table>
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<tr>
<th>Cell line</th>
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<th>Mutations</th>
<th>Ref.</th>
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<td>-</td>
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<td>+</td>
<td>TP53</td>
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<td>+</td>
<td>+</td>
<td>PI3KCA</td>
<td>31, 32, 34</td>
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<td>-</td>
<td>-</td>
<td>CDKN2A, PI3KCA, TP53</td>
<td>31, 32</td>
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<tr>
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<td>Basal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PTEN, RB1, TP53</td>
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<td>-</td>
<td>BRAF, CDKN2A, KRAS, NF2, TP53, PDGFRA</td>
<td>31, 32, 36</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>PTEN, RB1, SMAD4, TP53</td>
<td>31, 32, 36</td>
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Table 1 – Main characteristics of the cell lines analyzed
Figures

Figure 1

A

Polarization competent cell

Direction of migration

Outcome

Balance between Cdc42 and Ras signaling

Normal conditions

GM130 lost

Polarization incompetent cell

No directed migration

Outcome

Altered balance between Cdc42 and Ras signaling

B

Finak Breast Statistics

p-value 1.68E-25

Curtis Breast Statistics

p-value 4.47E-4

Breast (6)
Invasive Breast Carcinoma (53)

Breast (144)
Breast Carcinoma (14)
Figure 1
(A) Schematic representing the mechanism of action of GM130: GM130 binds to RasGRF and blocks its function. Once Cdc42 is activated, it accumulates on membranes and the Golgi sends Cdc42 in a polarized fashion to the Leading Edge of the migrating cell, thereby conferring persistence to the migration. GM130 will therefore contribute to maintain the balance between Cdc42 and Ras signaling. When GM130 is lost, the cell cannot migrate persistently and there is an imbalance between Cdc42 and Ras signaling. (B) Box Plots of two studies 37, 38 comparing the mRNA levels of GM130 in normal tissues and in breast cancer tissues (obtained from Oncomine).

Figure 2
(A) Histogram representing the quantification of GM130 levels compared to actin, obtained from 3 independent western blots. Results are showed as averages ± SE. Below, a
representative western blot. (B) mRNA levels of GM130 normalized to GAPDH mRNA. Results are shown as $1/(C_{GM130} - C_{GAPDH})$. Bar graphs are averages of 3 independent experiments ± SE. GAPDH was amplified at cycle $15.8687 ± 0.3265$, confirming that it can be considered an housekeeping gene also when comparing different cell lines. (C) The Golgi Compactness Index (GCI) was calculated as described in the materials and methods. More than 20 cells per experiment in three independent experiment were scored for each cell line. Results are shown as averages ± SE. (D) GCI was plotted on the x axis, the average of the protein levels of GM130 was plotted on the y axis. The linear correlation between these two parameters was assessed for all data points (black), only for luminal cell lines (blue) or only for basal cell lines (red).
Figure 3

A

**Luminal cell lines**

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<tr>
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<th>MDA-MB-361</th>
<th>SKBR3</th>
<th>T47D</th>
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<td>![Image](Giantin(T47D.png)</td>
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</table>

B

**Basal cell lines**

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<th>CAL51</th>
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</tr>
<tr>
<td>Giantin</td>
<td><img src="Giantin_BT20.png" alt="Image" /></td>
<td><img src="Giantin_BT549.png" alt="Image" /></td>
<td><img src="Giantin_CAL51.png" alt="Image" /></td>
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<td><img src="Giantin_MDA-MB-468.png" alt="Image" /></td>
</tr>
</tbody>
</table>

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**Figure 3**
Cells were plated on coverslips and processed for immunofluorescence staining using antibodies against GM130 (green) and Giantin (red) to visualize the Golgi, and DAPI to stain the nuclei. Scale bars, 10µm. **(A)** Representative images of Golgi in luminal cell lines. **(B)** Representative images of Golgi in basal cell lines.
Figure 4

A  MCF7  T47D  BT549  MDA-MB-231

RHYTHM  SHRNA  RHYTHM  SHRNA  RHYTHM  SHRNA  RHYTHM  SHRNA

GM130  GM130  GM130  GM130
Actin  Actin  Actin  Actin

B  Adhesion

MCF7  T47D

Ratio Area/After/Before

pLVTHM  GM130 shRNA  pLVTHM  GM130 shRNA

BT549  MDA-MB-231

Ratio Area/After/Before

pLVTHM  GM130 shRNA  pLVTHM  GM130 shRNA

C  Proliferation

MCF7  T47D

KRG positive cells

pLVTHM  GM130 shRNA  pLVTHM  GM130 shRNA

BT549  MDA-MB-231

KRG positive cells

pLVTHM  GM130 shRNA  pLVTHM  GM130 shRNA

D  Apoptosis and response to Doxorubicin

MCF7  T47D

Mean APE (Annexin-V)

pLVTHM  pLVTHM + DOX 5 nM  GM130 shRNA  GM130 shRNA + DOX 5 nM

BT549

Mean APE (Annexin-V)

pLVTHM  pLVTHM + DOX 5 nM  GM130 shRNA  GM130 shRNA + DOX 5 nM

MDA-MB-231

Mean APE (Annexin-V)

pLVTHM (starv.)  pLVTHM (starv.) + DOX 5 nM  GM130 shRNA (starv.)  GM130 shRNA (starv.) + DOX 5 nM
Figure 4

(A) The indicated cell lines were stably transduced with either a control plasmid (pLVTHM) or a plasmid encoding a shRNA against GM130 (GM130 shRNA). The cells were lysed and processed for western blot with antibodies against GM130 and actin to verify the efficiency of the shRNA against GM130. (B) The capacity of the indicated cells to adhere to a soft substrate (Collagen type IV) were assessed as described in the materials and methods. Results are shown as averages of three independent experiments ± SE. No significant difference was observed using the Student T test. (C) Cells were plated on coverslips, grown to subconfluency and processed for immunostaining against the proliferative marker Ki67 and DAPI. The percentage of cells positive for Ki67 was calculated counting at least 300 cells per experiment in three independent experiments. Results are shown as averages ± SE. No significant difference was observed using the student T test. (D) 200,000 cells were plated on 6-well plates. The next day, cells were either left untreated or treated with the indicated concentration of doxorubicin overnight. MDA-MB231 were also left overnight in the absence of FCS, in addition to the addition of doxorubicin. Cells were then processed for Annexin-V-APC as described in materials and methods. The mean fluorescence of the Annexin-V staining was measured in at least three independent experiments for every condition. Results are shown as averages ± SE. Asterisks indicate statistically significant differences calculated with ANOVA using the Newman-Keuls correction for multiple comparisons (*P<0.05; **P<0.01, ***P<0.001).
Figure 5

A  Wound Healing

- MCF7
  - pLVTHM
  - GM130 shRNA

- T47D
  - pLVTHM
  - GM130 shRNA

B  Gradient invasion

- BT549
- MDA-MB-231

- 0.5% FCS
- 10% FCS

C  Random Migration

- BT549
- MDA-MB-231

D  Random invasion

- GTex
- 10% FCS
Figure 5

(A) Wound healing assays were performed as described in materials and methods. The area migrated by the cells was measured. Results are expressed as percentage of migration compared to control conditions. Results are shown as averages of at least three independent experiments ± SE. Asterisks indicate statistically significant differences calculated using Student T test (*P<0.05; **P<0.01). (B) Cells were plated on top of Geltrex covered membranes with 8 µm pores and let invade in the presence of a chemotactic gradient (FCS) for 24 h. The number of cells which invaded through Geltrex was then counted. Results are shown as averages of at least three independent experiments ± SE. Asterisks indicate statistically significant differences calculated using Student T test (*P<0.05). Below the bar-graph, schematic of the experimental settings. (C) Cells were plated on glass bottom slides and imaged overnight. Velocity and persistence were calculated as described in the materials and methods. Results are shown as averages of three independent experiments ± SE. Asterisks indicate statistically significant differences calculated using Student T test (*P<0.05). (D) Cells were plated on top of Geltrex covered membranes with 8 µm pores and let invade in the absence of a chemotactic gradient for 24 h. The number of cells which invaded through Geltrex was then counted. Results are shown as averages of at least three independent experiments ± SE. Asterisks indicate statistically significant differences calculated using Student T test (*P<0.05). Below the bar-graph, schematic of the experimental settings.
References

Materials and Methods
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Cell Culture and transfection

HeLa, HEK293, HepG2, MCF7, MDA-MB-361, T47D cells were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. RPE-1 cells were grown in DMEM/F12 supplemented with 10% FCS and 100U/ml penicillin/streptomycin. MDA-MB-468, SKBR3, CAL51 were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 20% FCS and 100 U/ml penicillin/streptomycin. BT20 were cultured in Advanced MEM supplemented with 10% FCS, 2mM ultraglutamine, and 100 U/ml penicillin/streptomycin. MDA-MB-231 and BT549 were cultured in RPMI supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. PC12 cells were cultured in RPMI supplemented with 10% HS, 5% FCS and 100U/ml penicillin/streptomycin. Caco-2 cells were cultured in minimal essential medium supplemented with 20% FCS and 100 U/ml penicillin/streptomycin. To generate stable cell lines, cells were transduced with lentiviruses (produced according to the protocols of Trono’s lab) and transduced cells were either selected with antibiotics or sorted via FACS (using a BD FACS Aria™ cell sorter). Transfection of siRNA was performed using HiPerFect (Qiagen) or Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. The siRNAs used for the GEF screening were purchased from Thermo Fisher Scientific. Control siRNA was purchased from Thermo Fisher Scientific (ON_TARGETplus Control pool – Cat. Nr. D-001810-10-20). The siRNAs against GM130 were from Thermo Fisher Scientific (ON-TARGETplus smart pool – Cat. Nr. L-017282-00 – indicated in the text as siRNA1) and from QIAGEN (SI04235147 indicated in the text as siRNA2 – and SI00429191, indicated in the text as siRNA3). The siRNA against RasGRF2 was from Qiagen (SI04329941). The siRNA against TUBA was from QIAGEN (SI04220237 – used in Fig 1B). Plasmid transfection was performed using Fugene6 (Promega) according to the manufacturer’s instructions. Cells were typically transfected 24 h prior to the experiment. PC12 cells were transfected with FugeneHD according to the manufacturer’s instructions.
Plasmids
Cdc42-GFP-wt (nr. 12599), Cdc42-GFP-TN (nr. 12601), Cdc42-GFP-QL (nr. 12600) were from Addgene (Klaus Hahn’s Laboratory). RasGRF1-HA, RasGRF2-FLAG, RasGRF1-ΔPH-DH, RasGRF1ΔCdc25, RasGRF2-ΔDH and RasGRF2-ΔCdc25 were described previously. myc-GM130-ΔN690 was cloned using pcDNA 3.1 myc-His as backbone pLV-Venus-Cdc42 was cloned using a modified version of pLVTHM as backbone. RAICHU-Cdc42-EM and RAICHU-Cdc42-PM were kind gifts from Dr. Michiyuki Matsuda (Kyoto University, Japan). The RUSH-MannII-GFP expressing HeLa cells, as well as the VSVG-GFP-RUSH plasmid were a kind gift from Dr. Franck Perez. Temperature sensitive VSVGts045-GFP was kind gift from Dr. Kai Simons. Flag tag ARHGEF9, 11 and 12 were a gift from Dr. Oliver Rocks. Lentiviral packaging plasmids were bought from Addgene (psPAX2 nr. 12260, pMD2.G nr. 12259 and pLVTHM nr. 12247, from Didier Trono’s laboratory). GM130 GIPZ shRNA was bought from Thermo Scientific (Clone ID: V3LHS_313361). GM130 rat shRNAs (RSH050521 nr 1 to 4) and Control shRNAs (MSH029723-CH1) were bought from Genecopoeia. pmTurquoise2-Golgi was from Addgene (nr. 36205).

Pull-down experiments
To pull down active Cdc42 cells were lysed in buffer (25mM HEPES pH7.5, 150mM NaCl, 1% Nonidet-P-40, 10mM MgCl₂). Purified GST-tagged effector domain of PAK1 immobilized on GSH-sepharose beads was added to the lysate that was pre-cleared by centrifugation at 21’000xg for 5 min at 4°C. After 1 h incubation at 4 °C, beads were washed twice followed by boiling in sample buffer to elute bound material.
To pulldown active Ras, the same procedure was used with the exception the purified GST-tagged Raf1-RBD immobilized to GSH-sepharose beads were added to the lysate.

ERK1/2 activation assays
250,000 HeLa or HEK293 cells were plated in 35mm dishes and transfected with siRNA at the same time (HiPerFect from Qiagen). After 72h cells were serum-starved for at
least 2 h. Cells were subsequently stimulated with 10% FCS followed by lysis in ice cold MAPK buffer (150mM NaCl 20mM TRIS-HCl pH7.5, 1% Triton X-100) supplemented with protease and phosphatase inhibitors. The amount of phosphorylated ERK1/2 was determined by immunoblotting.

**PC12 differentiation**
PC12 cells were plated at low density on Collagen coated coverslips. 24 h later, cells were transfected with plasmids encoding either control shRNA or GM130 shRNA. Cells were then starved overnight. Cells were restimulated with 10% NGF and 1% HS for 48h, then the coverslips were stained and fixed. Cells with neurites as long as the body length were counted as differentiated. Due to the presence of a GFP in the plasmid encoding for the shRNAs, we were able to count only cells effectively transfected. To measure P-ERK intensity, cells were stimulated as described above for 0, 30 or 90 minutes and then stained.

**Antibodies and immunofluorescent labels**
Rabbit monoclonal anti-Cdc42 (dilution 1:300 – Cat. Nr. 2466), rabbit polyclonal ERK1/2 (dilution 1:1000 – Cat. Nr. 4695) and rabbit polyclonal against phospho-ERK1/2 (dilution 1:1000 – Cat. Nr. 9101) were purchased from Cell Signalling. Mouse monoclonal GTP-Cdc42 was purchased from NewEast Biosciences (dilution 1:50 - Cat. Nr. 26905). To stain for GTP-Cdc42, Image-iT, FX Signal Enhancer from Invitrogen was used according to manufacturer’s instructions. Mouse monoclonal anti-GM130 antibody was from BD-Biosciences (IF dilution: 1:1000 – WB dilution 1:250 – Cat. Nr. 610823). Rabbit monoclonal anti-GM130 was from cell signalling (dilution 1:500 – Cat. Nr. 12480). Rabbit monoclonal anti-RasGRF (WB dilution 1:200 – IF dilution 1:50 – Cat. Nr. Sc-863) and Mouse monoclonal E-Cadherin antibody (dilution 1:500 – Cat. Nr. 21791) were purchased from Santa-Cruz. Rabbit polyclonal anti-Giantin antibody was from Covance (dilution 1:1000 – Cat. Nr. PRB-114C). Monoclonal ANTI-FLAG® M2-Peroxidase (HRP) was from Sigma (dilution 1:2000 – Cat. Nr. A8592). Mouse monoclonal anti-HA 12CA5 antibody was from Roche (WB dilution 1:4000 – IF dilution
Materials and Methods

1:500 – Cat. Nr. 11583816001). Mouse monoclonal anti-γ-tubulin (dilution 1:1000 – Cat. Nr. Ab11317), Rabbit polyclonal anti TGN46 (dilution 1:1000 – Cat. Nr. Ab50595) were from Abcam, Mouse monoclonal anti-GFP (dilution 1:1000 – Cat. Nr. 11814460001) was from Roche. AlexaFluor647 Phalloidin (Cat. Nr. A22287) and AlexaFluor488 concanavalin (Cat. Nr. C11252) and AlexaFluor647HPA (Cat. Nr. L32454) were from Invitrogen. Rabbit polyclonal anti-Ki67 antibody was from Abcam (IF dilution 1:1000 in PBS-BSA 3% – Cat nr. 15580). For immunofluorescence staining cells were grown on glass coverslips. To stain for RasGRF, cells were fixed on ice with 4% formaldehyde for 10 min followed by permeabilization with PBS containing 0.05% triton X100 for 3 min at RT. To stain for β-COP cells were fixed with methanol-acetone (50/50) for 2 min on ice. For all other stainings, cells were fixed with 4% paraformaldehyde for 15 min at RT followed by permeabilization with PBS containing 3% BSA and 0.2% triton X100. DAPI Fluoromount G (Southern biotech – Cat nr. 0100-20) was used in most cases to mount the coverslips.

**Microtubule nucleation assay**

RPE-1 cells were plated on coverslips and transfected with siRNAs using HiPerFect. After 72 h, 100μM HEPES pH 7.35 was added to the cells and the cells were then transferred on ice for 40 minutes. Then cells were put back at room temperature and fixed after 0, 3 and 4 minutes at room temperature in fixation/permeabilization buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0, PFA 2% in buffer CB, 0,025% glutaraldheide, 0,2% Tx-100). Immunostaining was then performed using antibodies against GM130, TGN46 and Tubulin.

**Caco2 Cysts**

Caco-2 cells were plated in 35mm dishes. 24 h later they were transfected with siRNAs using Lipofectamine 2000. After additional 24 h cells were trypsinized and an additional siRNA transfection using Lipofectamine 2000 was performed. Cells (6x10⁴) were re-suspended in 60% DMEM + 40% Matrigel (previously thawed overnight on
ice). 100μl of this solution was plated into 8-wells chambers (BD-biosciences), incubated at 37°C for 30 minutes and, once solid, Matrigel was covered with 400μl of DMEM. Media was changed every other day. On the 4th day of culture cysts were fixed with 300μl of paraformaldehyde (4%) 30 min at RT, washed with PBS, permeabilized with 0.5% triton X-100 for 10 min and washed with PBSG (PBS containing 7.5mg/ml glycine). Non-specific staining was blocked with 300μl of blocking buffer (7.7mM NaN₃, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween20) and afterwards the 8-wells chambers were incubated overnight with the primary antibodies diluted in PBS containing 3% BSA. On the next day, cells were washed with blocking buffer and secondary antibodies + fluorescent phalloidin were added for 2 h at RT. Cells were washed again with blocking buffer and the plastic support was removed. A glass coverslip was put on the top of the matrigel and the samples were imaged.

Confocal microscopy image analysis and FRET

Microscopy was performed on a LeicaSP5 confocal laser scanning microscope equipped with a Ludin chamber. Most imaging was performed using the 63X objective (1.4 NA). Alternatively, a Zeiss Axiovert 200M with 37°C incubation chamber was used. A 10X objective (NA 0.45) was used for live images with the Zeiss system. Fluorescent images were acquired on the Zeiss Axiovert 200M using 20X objective (NA 0.4) or a 100X oil immersion objective (NA 1.4). Images were converted into TIFF files and analysed using ImageJ. FRET was performed using the donor-recovery after acceptor photobleaching method using the FRET-AB wizard of the Leica software. Acceptor (YFP) fluorescence was bleached at 100% laser intensity assuring a minimal extent of bleaching by 75%. As for FRET at the Golgi, we chose a region in the juxtanuclear region where this reporter accumulated. To test whether we could recognize the Golgi correctly when looking at the reporter, we initially performed experiments where we colocalized the reporter with Golgi markers and found that we could always recognize the Golgi correctly. To measure FRET at the plasma membrane, we chose random regions at the plasma membrane of cells grown up to 80% confluency. Briefly, cells were divided into 4 regions (quadrants of a Cartesian Coordinates Plane) and FRET
was measured every time in one different region (the first cell was measured in the first quadrant, the next cell in the second quadrant, and so on). The only limitation we imposed was to avoid regions overlapping with neighbor transfected cells. ImageJ was used to process the images. Briefly, the process “subtract background” was used on every image. If needed to improve visibility, brightness and contrast of the images were corrected (linearly) using the “adjust” menu of ImageJ. Images coming from the same experiment were all modified the same way to leave unaltered the information. Merged images were created with the appropriate function of ImageJ, after correcting the single channel images as described above. The Golgi compactness index was calculated using the formula \( GCI = \frac{4\pi \times \text{Area}}{\Sigma \text{Perimeter}^2} \) as described in Bard et al.\(^5\), with GM130 as Golgi marker.

**GEF screening**

FRET measurements at the Golgi were performed in HEK293 cells expressing the Cdc42-EM probe. We calculated the CI95 of GM130 knockdown vs. control cells according to:

\[
\text{CI95} = (\text{FRET value of control} - \text{FRET value of GM130 knockdown}) \pm 2.\text{SE}
\]

Any value below the CI95 (red line in Fig. 2) was considered a hit. Our previous measurements of control and GM130 knockdown cells were used to quantitate the CI95 with the formula described above. 30-45 cells were measured for each condition from two-three independent experiments. The rationale for measuring at least 30 cells was based on our calculation that 18 data points (i.e. cells) are necessary to distinguish a population that differs from the control by 80% of the GM130 effect.

**FRET of directional migrating cells**

HeLa cells were plated on glass coverslips and transfected with siRNA (HIPerfect). After 48 h, cells were transfected with plasmids encoding for the Cdc42-EM or Cdc42-PM FRET probe (Fugene6). After 72 h, a wound was made using a 200µl pipette tip and cells were allowed to migrate at 37°C for 6 hours. Then cells were either fixed or incubated for 1 h at 20°C and subsequently fixed. To measure FRET, we selected the
region of the cells directly facing the wound and named it “front”. For regions designated as “rear” we chose regions from the side of the cell opposing the wound. Up to 3 different regions were measured in the “rear” and averaged. We did not detect any dependency on where we measured as long as the plasma membrane was opposing the wound. We avoided to measure FRET in regions overlapping with neighbor transfected cells.

**Video acquisition of VSVG-GFP vesicles**
HeLa cells were plated on glass coverslips and transfected with siRNA (HiPerfect). After 48 h, cells were transfected with plasmids encoding for the VSVG-GFP-RUSH (Fugene6). After 72 h, a wound was made using a 200µl pipette tip and cells were allowed to migrate at 37°C for 4 hours. Then imaging was started and biotin added to release the VSVG construct from the ER. Images were acquired every 1.5 sec.

**Golgi orientation, cell migration and aPKC recruitment assay**
For Golgi orientation assays, cells were plated on glass coverslips. After 72 h, a wound was made manually using a 200µl pipette tip. Cells were stained with antibodies anti γ-tubulin and anti-giantin immediately after wounding and after 6 hours. For the assays measuring aPKC recruitment to the leading edge, cells were fixed already 1 h after wounding.

For migration assays, cells were transfected with siRNA, and trypsinized after 48 h and plated into Ibidi® chambers placed on glass cover slips. This insert consists of two chambers separated by a 600 μm wall. Removal of the insert generates a wound with a defined width of 600 μm. 12000 cells were plated into each chamber. After 6h, the IBiDi insert was removed and cells were allowed to migrate at 37°C for 24 h. Then, cells were fixed and stained with concanavalin-alexa 488.

**Wound Assays**
Cells were grown on glass coverslips, or on 8 wells glass bottom chambers (BD Falcon Cat Nr. 354108), or on 4 well glass bottom chambers (Lab Tek Cat Nr. 177399). Once
confluency was reached, a wound was made using a 10 µm pipette tip. HEPES at a final concentration of 100mM was added to the cells before starting the overnight imaging. One image every 5 minutes was taken using transmitted light for at least 8 hours. Alternatively, wound scratches were imaged immediately after scratching and after overnight migration. The area covered by the cell sheets during migration was measured. The area migrated by control cells was set as 100% and the other values were calculated subsequently. At least three matched experiments (control and GM130 shRNA cells) per conditions were performed.

**Random Migration**

2E4 cells were plated per well in an 8-well glass bottom chamber slide (BD Falcon Cat Nr. 354108) or alternatively on glass bottom dishes (MatTek corp. P35G-1.5-20-C). Imaging was started after an appropriate time to allow cells to adhere (at least 4 h after plating). HEPES at a final concentration of 100mM was added to the cells before starting the imaging. 1 image every 5 minutes was acquired using transmitted light for at least 8 hours. Individual cells were manually tracked with the ImageJ plugin MTrackJ. Average velocity is automatically given by MTrackJ. Persistency was calculated using the formula: D2S/LEN. D2S = linear distance between first and last position, LEN = total length of the track. At least 5 cells/experiment were tracked in every condition, in at least 3 different experiments.

**Coimmunoprecipitations**

1x10^6 cells were plated in 10cm² cell culture dishes. After 24 h, cells were transfected. After additional 24 h, cells were lysed in IP buffer (20mM HEPES pH 7.3, 200mM KCl, 0,5% Triton X-100), then centrifuged for 15 min at 20’000xg. The supernatant was incubated with protein G sepharose beads coupled to the appropriate antibodies. For anti-HA immunoprecipitation commercially available anti-HA beads (Sigma) were used. After 2 h incubation at 4°C, beads were recovered by centrifugation, washed and the bound material was eluted by boiling in sample buffer.
ER-Golgi trafficking
The RUSH assay was performed as described previously. Briefly, 250’000 HeLa cells stably expressing RUSH-ManII-GFP were plated in 35mm cell culture dishes and transfected with siRNA (using HiPerFect from Qiagen). After 72 h, cells were either fixed directly or were treated with 40 μM of biotin for 1 h followed by fixation in 4% paraformaldehyde at RT.

To study Golgi to plasma membrane trafficking, ts045VSVG-GFP was used. HeLa cells were plated on glass coverslips. 24 h later, cells were transfected with siRNA (using Lipofectamine 2000®). Another 24 h later, cells were transfected with a plasmid encoding for temperature sensitive VSVG-GFP (ts045VSVG-GFP). 8 h after transfection, HEPES was added to the medium to a final concentration of 100mM, plates were sealed with parafilm and incubated overnight at 40°C to accumulate VSVG-GFP in the ER. Then, coverslips were either fixed (T0) or incubated at 37°C for a hour to allow trafficking of VSVG-GFP to the plasma membrane and then fixed.

RT-PCR
Total RNA was isolated from cells by using the Qiagen RNeasy kit. cDNA was prepared from 100ng total RNA by using the high capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturers' instructions. Q-PCR was performed by using Fast SYBR green PCR MasterMix (Applied Biosystems). RT-PCR were run on 7900HT Fast RT-PCR system (Applied Biosystems). Expression of each gene was normalized to the expression of ubiquitin and β-2-microglobulin. Specific primers for RasGRF2 were described in Jacinto et al. Primers for TUBA, ARHGEF9, ARHGEF11 and ARHGEF12 were designed using PrimerExpress®. Specific primers for GM130 were designed with the online software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Specific primers for GAPDH were previously described. Sequences are provided below:

TUBA: F: 5' TGCGCTGCTTGAAATCTACG 3' - R: 5' ATTTGTGCATCCCCATTCGT 3' –
RasGRF2: F: 5' CTACTTCGAGGGCGAGCA 3' - R: 5' TCCAGTGGCTTCTGACCTTC 3' -
**Materials and Methods**

**GAPDH**: F: 5' CGCTCTCTGCTCCTCCTGTT 3' - R: 5' CCATGGTGTCGAGCGATGT 3'

**GM130**: F: 5' TGCCAGTCTCAGCATGG 3' - R: 5' TGACACATGTCGCAGACTCA 3'

**Immunohistochemistry**
Three-µm slides cut from three different tissue microarray block containing a total of 229 different formalin-fixed, paraffin-embedded samples from colorectal cancers (n=133), tubulovillous adenomas (n=27) and normal colonic mucosa (n=69) were used (duplicate cores for each samples). The project was approved by the ethical committee of the IEO and consent from patients was obtained. Samples were rehydrated through xylene and graded alcohols. Antigen retrieval was accomplished using 1 mM EDTA, 0.05% Tween. After peroxidase block with 3% H$_2$O$_2$ for 5 min, then blocked with 2% goat serum in PBS for 1 h, samples were incubated with primary antibodies (Mouse monoclonal anti-GM130 antibody, BD-Biosciences) in a 1:50 dilution for 60' min at RT in 2% goat serum. Immunocomplexes were visualized by the EnVision™+ HRP Rabbit (DAKO, K4011), and acquired with the Aperio ScanScope system. Duplicate cores for every sample were evaluated and then the scores were averaged and considered as weak expression (average score 0.5 - 1), moderate expression (average score 1.5 - 2), strong expression (average score 2.5 - 3), or as negative if they scored 0. IHC data for GM130 expression were available only for 29 normal colonic mucosa, 16 tubulovillous adenoma and 109 colorectal cancer samples, since in some cases, individual cores detached from the slides during the manipulations.

11 matched colon cancer samples were analysed at the University of Regensburg according to the following procedure: two-µm slides cut from a tissue microarray block containing 60 different formalin-fixed, paraffin-embedded samples from colorectal cancers (n=20) and matched normal tissues (n=40) were used. Immunohistochemistry of GM130 was carried out in a BenchMark ULTRA (VENTANA) autostainer according to the manufacturer’s instructions. Briefly, retrieval was done using a cictrate buffer, pH6.0 for 44 min. The anti-GM130 antibody was used in a 1:100 dilution and incubated for 32 min. The slides were detected using the ULTRA View.
DAB detection kit (VENTANA). Negative controls without primary antibody were included in each experiment.

**Adhesion assay**
The adhesion assay was performed as described previously. Briefly, glass coverslips or glass bottom dishes were coated with tail vein rat collagen type IV at a concentration of 2mg/ml (Roche Cat nr. 11 179 179 001). Surfaces were covered with collagen and air dried followed by washing with PBS and storage at +4°C. Cells were trypsinized and counted. Imaging was performed on a LeicaSP5 confocal laser scanning microscope using a 10X objective (NA 0.3) at 37°C. Imaging was started immediately after plating cells onto the glass bottom dish. The time point when cells settled on the bottom of the coverslip was used as time point 0. One image was acquired every 10 minutes until cells started to adhere on the substrate. The final time point was chosen according on when the morphological changes of control transduced cells became evident. This corresponded to 80 minutes after plating for MCF7 and BT549, 50 minutes for MDA-MB-231 and 460 minutes for T47D. The total area occupied by the cells present in one field of view was measured with ImageJ at the time point 0 and at the final time point. The ratio between the area at the final time point and the area at time point 0 was then calculated (Area After/Before). At least 3 matched experiments (control and GM130 shRNA cells) per condition were performed.

**Apoptosis assay**
3E5 cells were plated on 6 well plates. 24 h later, Doxorubicin was added to the cells at the minimum concentration required to induce apoptosis (determined experimentally). Cells were stained with Annexin V-APC (BD Pharmingen – Cat nr. 550474) according to manufacturer’s instructions. 24 h later, cells were trypsinized, resuspended in 1ml of medium, washed with ice cold PBS, resuspended in binding buffer (0.1 M Hepes (pH 7.4) 1.4 M NaCl, 25 mM CaCl2) at a density of 1E6 cells/ml. 1E5 cells were then mixed with 5 µl of Annexin-V-APC (BD Pharmingen – Cat nr.
and incubated at RT for 15 minutes. Then, flow cytometry analysis was performed on a BD LSR II. At least 3 matched experiments per conditions were performed.

**Proliferation assay**

1.5E5 cells were plated on glass coverslips. 48 h later, cells were fixed with PFA 3% for 10 minutes and subsequently stained with an anti Ki67 antibody and DAPI. Images were acquired with the Zeiss Axiovert 200m using a 20x objective. 3 to 4 images per condition were taken. Cells positive for Ki67 were counted manually in ImageJ with the "cell counter" plugin and compared with the total number of cells (counting of DAPI stainings). At least 300 cells per experiment were counted, in at least 3 matched experiments.

**Invasion assay**

Fluoroblok™ inserts with 8 µm membrane pores, 24 well plate format (BD Falcon – Ref. 351152) were coated with 50 µl Geltrex (Gibco – Life Technologies – Cat. Nr. A14132-02) diluted in RPMI or DMEM with 0.5% FCS (gradient) or with 10% FCS (random) to a final concentration of 1.2-1.8 mg/ml. Then, coated inserts were stored at 37°C for 1 h, to allow Geltrex to polymerize. In the lower chamber of the invasion well, 600 µl RPMI or DMEM supplemented with 10% FCS were added. 1E5 cells were resuspended in a volume of 200 µl of medium with 0.5% FCS (gradient) or 10% FCS (random) and subsequently were plated in the upper chamber of the invasion well. Plates were then stored at 37°C and allowed to invade for 24 h. Afterwards, the upper chamber was cleaned with a cotton swab and the inserts were analyzed on a Zeiss Axiovert 200M with a 10x objective. 4 images/well, corresponding to ¼ of the total area of the invasion insert, were analyzed in every condition. Cells were counted in every image with the “cell counter” plugin of ImageJ and each condition was compared to control cells.
Statistical analysis
The data were analysed using Student T Test, Chi Square test or ANOVA test for multiple comparison in GraphPad Prism. All IF images are representative of at least 3 independent experiments. Western blots evaluations and IF evaluations are averages of at least 3 independent experiments. For TMA analysis differences between experimental groups were examined for statistical significance using the nonparametric comparisons for each pair using Wilcoxon Method (Figure 7B – Results Part 1) or Pearson Chi Square test (Figure 7C – Results Part 1). Where applicable, data are expressed as average ± SD. TMA data analysis were performed using JMP 10.0 statistical software (SAS Institute, Inc).
References

Discussion
Discussion
The results of our work clearly indicate that cell polarity is subject to regulation by an endomembrane pool of Cdc42 that is regulated by a GM130-RasGRF complex that we identified. This finding is of interest for cell biology as it provides the first evidence for a biological role of an endomembrane pool of Cdc42. In addition, our results have further implications for biomedicine as we observed that GM130 is down-regulated in colon cancer and we found evidence in databases that the expression of this Golgi matrix protein is also reduced in breast cancer. In the Introduction, I gave an overview of the link between polarity and cancer, and in light of this, the downregulation of GM130 could be interpreted as further supportive evidence for a role of polarity in cancer. In the following sections, I will critically discuss whether this conjecture is true, which also necessitates a critical discussion on what is already known about the role of polarity in cancer.

Cdc42 signaling from endomembranes
An active role for the Golgi in controlling cell polarity had already been proposed in previous researches, but the evidence supporting this claim was weak. Preisinger et al. showed that GM130 anchors the kinase YSK1 at the Golgi and promotes autophosphorylation and activation of the kinase. YSK1 will promote then migration and invasion via its effector 14-3-3-ζ. However, such model suffers of two major problems: (i) a kinase dead version of YSK1 (ATP binding site mutant YSK1K49R) is still able to support migration and (ii) unphosphorylatable YSK1 fragments the Golgi. Therefore an alternative conclusion is that the kinase activity of YSK1 is not needed for migration and that YSK1 plays instead a structural role at the Golgi apparatus. 14-3-3 proteins play also a role in ER to Golgi traffic by competing away βCOP from cargo proteins, thus allowing the release of such proteins from the ER. Thus, it is reasonable to think that if 14-3-3-ζ is not recruited at the Golgi, a defect in ER to Golgi traffic will lead to fragmentation of the organelle that will cause the defects in migration and invasion and it is possible that altered YSK1 signaling will lead to mispositioning of 14-3-3. Yadav et al. found that when GMAP210 and Golgin160 are depleted, cell migration in a wound assay is impaired. Knockdown of these two Golgi proteins leads to fragmentation of the Golgi, but secretion still works. When cells polarize, trafficking of proteins is also polarized mostly to the leading edge and if GMAP210 or Golgin160 are depleted, the secretory traffic does not reorient, thus causing the observed defect in migration. In order to polarize post-
Golgi traffic, the Golgi itself has to orient towards the leading edge, but when the Golgi Apparatus is fragmented, no orientation is possible. Therefore, also the model proposed by Yadav does not provide strong evidence for an active role of the Golgi in the establishment of front-rear polarity. It is evident that to conclude that to conclude that signaling originating at the Golgi supports cell migration, it is necessary to find a condition where Golgi structure is preserved, but cell migration is impaired. Kodani et al. found that GM130 somehow controls Cdc42 activation and they propose that such regulation happens at the Golgi apparatus. They propose that GM130 recruits the Cdc42 GEF TUBA at the Golgi, but some key experiments are missing in their report. If TUBA is the link between GM130 and Cdc42, then TUBA should colocalizes with GM130 and in order to conclude this, confocal imaging is required. Contradicting this possibility, several papers where the localization of the GEF was investigated did not find trace of TUBA at the Golgi. We also found no evidence of TUBA interacting with GM130 either in co-immunoprecipitation experiments or in immunofluorescence, where we also excluded the possibility that TUBA could colocalize with a second Golgi marker Giantin. If in the absence of GM130, TUBA is artificially targeted to the Golgi, then Cdc42 activation should be rescued, but this was not tested. Finally, it is not possible to conclude that Cdc42 is activated at the Golgi by performing biochemical pull-down assays. By using FRET reporters we overcame this problem and we were able not only to confirm the finding that GM130 is implicated in the control of Cdc42, but we could also expand such statement by showing that GM130 specifically controls Cdc42 at the Golgi, without exerting any effect on plasma membrane pool of the small GTPase. Knockdown of GM130 impairs the ability of the Golgi to reorient towards the LE of a migrating cell, but does not affect the secretory function of the Golgi. What is affected is polarized secretion, as in the absence of GM130, secretion remains unpolarized. The difference with the previously proposed models is that GM130 knockdown did not fragment the Golgi in our experimental settings, thus we have a model where a structurally intact Golgi is not able to reorient and, subsequently, trafficking does not reorient too. The fact that GM130 knockdown does not affect Golgi structure is in contradiction with some publications, but in agreement with others. Technical differences in the experimental setting could provide a possible reason for this contradiction. While we use 10 pmol of siRNA (5 nM), in other publications between 4 and 6 folds more siRNA were used to knockdown GM130. In addition, we use HiPerFect for siRNA transfections, while Puthenveedu et al and Marra et al used Oligofectamine. 

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HiPerFect is known to be less toxic for the cells than other transfection reagents. Finally, Golgi matrix proteins are to a certain level redundant in their function, so it is possible that depending on the cell line, the function of GM130 could be taken over by other Golgins.\textsuperscript{11, 12}

Our FRAP data show that there is still continuity between Golgi membranes when GM130 is depleted, but electron microscopy would be required to assess if minor changes occur when the Golgi protein is depleted.

We could show that Cdc42 is active at the Golgi and that different regulatory mechanisms exist to regulate Golgi-Cdc42 and plasma membrane-Cdc42. We provide evidence that the Golgi supplies the leading edge with active Cdc42, therefore sustaining migration over a long period. Such flux of active Cdc42 is necessary for two reasons. First, once it arrives at the plasma membrane, Cdc42 is not physically restricted to the LE and it will quickly diffuse away. Second, the plasma membrane contains several GEFs and GAPs for Cdc42, thus once the small GTPase reaches the LE, not only it will diffuse away, but it will also be inactivated. Instead, the Golgi is poor in both Rho GEFs and GAPs\textsuperscript{13} (personal communication from Dr. Oliver Rocks), therefore when Cdc42 reaches the Golgi, its activation status is preserved. So, the polarized flux of Cdc42 from the Golgi provides a mechanism to localize Cdc42 and its activity at the LE during migration. Another possible interpretation of our data is that the Golgi supplies the leading edge with a Cdc42 GEF. However, such model would not fit with our finding that co-knockdown of GM130 and RasGRF restores normal activation levels of Cdc42 at the Golgi and would require a GEF to be localized at the Golgi, while with our current knowledge we have no evidence for a GEF localizing at the Golgi.

An open question in our model regards Cdc42 activation. We showed that the Golgi preserves the activation status of Cdc42, but we didn’t find evidence of Cdc42 being activated locally at the Golgi. Cdc42-GTP is more membrane bound than the inactive protein. Therefore it is possible that once Cdc42 is activated, it will bind to any membrane in the cell. This is supported by the fact that Cdc42 was localized at Golgi, ER and plasma membrane\textsuperscript{14}. Being the Golgi poor in Cdc42 GAPs, Cdc42 will remain active at the Golgi for a longer period than on other membranes, thus resulting in the trapping of the small GTPase at the organelle. However, such model would predict that any generic condition that decreases Cdc42 activity would lead to decreased Cdc42 activity at the Golgi and this is not the case. In fact, we found that knockdown of only 3 out of 51 Rho GEFs affected Cdc42 at the Golgi, hinting for the existence of a more specific model to locally regulate Cdc42. The three GEFs (ARHGEF9, 11, 12).
and 12) responsible for activating the Golgi pool of Cdc42 appear to be cytosolic, thus future experiments should try to resolve the spatial regulation of Cdc42 by these GEFs.

Based on our model, where Golgi localized Cdc42 is important for directed cell migration, we would expect that tumor cells exhibit abnormal Cdc42 activity at the Golgi. Interestingly, changes in the expression level or in the GEF activity of ARHGEF9, 11 and 12 have been reported in cancer. High levels of ARHGEF9 were found in hepatocellular carcinoma, and it was shown that high levels of this Cdc42 GEF are responsible for increased invasiveness and metastatic ability of cells in mice. Alternative splicing of ARHGEF11 was observed mostly in cells at the end of EMT. The result of this alternative splicing is a protein missing its C-terminal domain that exhibits more GEF activity, especially towards RhoA. High levels of ARHGEF11 were also reported in the invasive areas of human breast cancers. In line with our findings, depletion of ARHGEF11 resulted in impaired directed migration and polarization of cells. Knockdown of ARHGEF11 resulted also in enhanced adherent junctions and in the loss of a pool of actin from the center of the cell. Considering that Cdc42 was implicated in the control of post-Golgi trafficking, it would be important to check whether this actin pool is situated at the Golgi and whether ARHGEF11, by regulating Cdc42, plays any role in post-Golgi trafficking, that would explain the alteration of adherent junction observed upon depletion of the GEF. Finally, ARHGEF12 was shown to support migration and invasion in different types of cancers. The involvements of those GEFs in cancer speaks in favor of an important role for endomembrane signaling in tumorigenesis. Future work should aim to better elucidate the role of such GEFs in endomembrane signaling and in cell polarity.

Golgi Orientation
An indirect conclusion that can be drawn out of our data is that the Golgi pool of Cdc42 is necessary for Golgi orientation. In fact, conditions where the activity of Cdc42 is reduced specifically at the Golgi, but not at the plasma membrane (depletion of GM130, ARHGEF9, 11 or 12) impair the ability of the Golgi to reorient towards the leading edge. How the Golgi reorients is still unclear. Golgi microtubules are captured and stabilized at the leading edge by several proteins (CLASPs, APC, DLG, Par3). Furthermore, the Golgi can capture centrosomal microtubules via the recently described protein MTCL1. Because of the localization of the Golgi, these microtubules will grow in direction of the leading edge and will be stabilized by LE proteins. Therefore the simplest way to explain Golgi reorientation would be via
microtubules that pull the organelle. However, when Golgi microtubules are disrupted or when MTCL1 is knocked down, no defect in Golgi orientation is observed \(^\text{23}\). Vesicular trafficking, which is known to be dependent on MTs, is severely affected in these conditions, thus resulting in impaired migration of cells despite the presence of oriented Golgi and MTOC. CLASPs \(^\text{24}\), AKAP45 \(^\text{25}\) and MTCL1 \(^\text{23}\) have all been implicated in the nucleation of Golgi microtubules and/or in the ability of the Golgi to capture centrosomal microtubules. It is possible that those proteins are redundant in their function and that in order to assess if Golgi derived microtubules are necessary for Golgi reorientation, all these proteins have to be depleted at the same time. In addition, based on our data that active Cdc42 at the Golgi is necessary for Golgi polarization, it would be interesting to test whether reorientation of the Golgi is made possible by an effector of Cdc42 localized at the Golgi. Such effector could be a motor protein like dynein, which is present at the Golgi and necessary for trafficking.

**RasGRF regulation by GM130 and local regulation of Cdc42**

The interaction we describe between RasGRF and GM130 is most likely dynamic and thus it is possible that by regulating this interaction, a fine tuning of Cdc42-GTP at the Golgi could be achieved. Indeed, we found that growth signal deprivation and Ca\(^{2+}\) increase abolish the interaction between GM130 and RasGRF, which is then reestablished upon re-addition of FCS to the medium. Switching from a low to high Ca\(^{2+}\) medium induces TJ formation in epithelial cells \(^\text{26}\) and concomitantly, an increase in the intracellular levels of Ca\(^{2+}\) is observed \(^\text{27}\). RasGRF1/2 have IQ domains that directly respond to calcium \(^\text{28, 29}\), therefore it can be speculated that Ca\(^{2+}\) induces a conformational change in the structure of RasGRF that causes its release from GM130, thus activating the protein towards Ras and Rho GTPases. The reason why intracellular calcium increases is not clear, but intracellular calcium is proposed to be as important as extracellular calcium in the establishment of polarity \(^\text{27}\). In order to form TJs, cell needs to locally activate Rac or Cdc42. Therefore, it would be expected that an inhibitor of Cdc42 has to remain sequestered at the Golgi. However, since RasGRF has been reported to be an active GEF for Rac, we can speculate that upon release from the Golgi, RasGRF is recruited to primordial junctions where it locally activates Rac. An alternative and less speculative hypothesis is that increased Ca\(^{2+}\) will modulate the interaction between RasGRF and GM130, thus resulting in precise regulation of Cdc42 activity at the Golgi, which could be important for sorting of TJ proteins from the TGN. In neurons, RasGRF1 activates Ras in
response to an increase in intracellular Ca\(^{2+}\). The activity of RasGRFs can be modulated by phosphorylation. In particular, signals from G-protein coupled receptors, as well as PKA signaling cause an increase in the GEF activity of RasGRF1 towards Ras by phosphorylating the protein. Src signaling leads to phosphorylation of RasGRF1 (probably on multiple sites), thus triggering its GEF activity towards Rac1, but leaving unaltered its activity on Ras. In this study, RasGRF1 was found to have GEF activity only on Rac, but not on Cdc42 or RhoA. RasGRF2 activity can also be modulated via signaling. The complex p35/Cdk5 was found to be responsible for phosphorylating the protein on Ser737 and phosphorylation of this residue led to downregulation of Rac1-GTP. Thus, the GEF activity of RasGRF can be drastically affected by signaling. RasGRF1/2 was reported to be an inactive GEF for Cdc42, but the importance of signaling in the specificity of RasGRF1/2 towards Cdc42 has not been investigated. We can speculate that GM130 works as a scaffold between RasGRF and a kinase that phosphorylates and thus inactivates RasGRF. Indeed, GM130 directly interacts with MST4 and YSK1, kinases implicated in polarity processes. In order to establish front rear polarity, RasGRF has to be kept inactive towards Cdc42. This could be the mechanism by which YSK1 regulates migration. Further work will be required to assess whether GM130 prevents RasGRF from acting on Cdc42 simply via sequestration, or if some signaling events are also involved in this regulation.

**Polarity and cancer**

The role of polarity in cancer represents an expanding research area. Loss of polarity has been shown to promote tumor growth and metastasis. However, the mechanism by which polarity loss promotes tumorigenesis is to date not completely clear and some hypothesis may even appear to be contradicting existing data. The effect of polarity on cancer has to be analyzed at two different levels: initiation and local growth of the tumor and metastatic dissemination. Classically, a cancer-initiating condition is considered if an alteration of cell homeostasis confers growth advantage and immortality to cells, which is achieved by activation of pro-proliferative pathways and by overcoming senescence. Once initiated, it is the current consensus that additional mutations foster the initial events. For instance, it is very important for many tumor types to lose contact-inhibition. Too rapid growth of the tumor results in the inner parts of the tumor rapidly developing hypoxia. Therefore, tumors need to promote angiogenesis to maintain a constant supply of oxygen. Furthermore, the
rapid growth of tumors necessitates the remodeling of cellular metabolism. While most normal cells generate energy by relatively low levels of glycolysis followed by oxidation of pyruvate, tumor cells satisfy their higher energetic demand by high levels of glycolysis (>100 times higher than in normal cells) followed by lactic acid fermentation. This phenomenon is known as the Warburg effect. Finally, some tumors develop a hyper-migratory phenotype and invade locally, break into vessels and disseminate systemically, thereby forming metastasis. In epithelial cancers, this is often accompanied by a loss of epithelial characteristics and the gain of mesenchymal features, a process called epithelial-mesenchymal transition (EMT). The connection of cell polarity to each of the aforementioned processes must be discussed carefully.

![Diagram of polarity pathways](image)

**Figure 1 – Polarity pathways possibly involved in tumor initiation and promotion of metastasis. Polarity proteins are colored in orange.**

**Initiation of tumors by polarity proteins**

In order to acquire a growth advantage, cells can either acquire mutations that will render them hyperproliferative, or alternatively, they can inhibit growth suppression. Unlimited replicative potential is a characteristic of stem cells and it is possible that differentiated cells revert to an undifferentiated state, thus gaining back their stemness. Those new stem cells could be responsible for starting a tumor and they are called Cancer Stem Cells (CSC) \(^\text{41}\). Whether polarity can initiate cancer is currently unclear. Loss of polarity is one of the first hallmarks of cancer, but whether this is cause or consequence of cancer is not clear. Almost all core polarity protein have been implicated in regulation of proliferation. Depletion of Par3
Discussion

in normal mammary epithelial cells leads to increased rates of apoptosis \(^{42,43}\), indicating that loss of polarity per se is not sufficient to increase proliferation. Based on this, loss of polarity cannot be considered an initiator of cancer. This work is important as it is one of the few examples where loss of polarity has been introduced into normal cells, which are neither transformed, nor immortalized. Nevertheless, it has to be stressed that this conclusion is not safe. The experiments in the work cited above were performed in primary mammary epithelial cells that were transplanted into mammary fat pads. The conclusion on apoptosis was based on the observation that Par3-depleted cells are less efficient in generating a new mammary gland. Whether loss of Par3 per se induced apoptosis cannot be concluded as the result could also be interpreted such that Par3-depleted cells more sensitive to the stress imposed by cell transplantation. Notably, if an oncogenic signal is used to overcome apoptosis, an increase in proliferation is detected upon depletion of the polarity protein \(^{36,44}\). Therefore, loss of polarity seems to promote tumorigenesis, rather than initiating it. Par6b overexpression resulted in hyperactive ERK and increased proliferation in breast cancer cell lines \(^{45}\). Since ERK1/2 is a major growth-promoting pathway, this result tempts to conclude that loss of polarity induces hyperproliferation. However, since the work relied on the use of transformed breast cancer cells, it is not correct to consider polarity loss as an initiating event because initiation has already occurred in transformed cell lines. At best, this finding can be interpreted such that loss of polarity promotes growth of an already initiated tumor. Interestingly, Par3 was also shown to control ERK1/2 signaling \(^{37}\) and the interaction between Par6 and Par3 was not required to control ERK1/2, thus implying that Par3 and Par6 can regulate this MAP kinase pathway via two independent pathways. Finally, the Par complex can activate Rac and Cdc42, which was proposed to lead to malignant transformation of the cells. Par3 regulates the activity of the Rac GEF Tiam1 \(^{46}\). When Par3 is depleted, Tiam1 is hyperactive and mislocalized and this will result in increased Rac signaling \(^{39}\). Downstream of Rac1, the kinase PAK will phosphorylate MEK and ERK, thus resulting in increased ERK signaling and increased proliferation of MCF10A cells. Par6, Par3 and PKCζ interact also with another Rho GEF, named Ect2 \(^{47}\), which is a known oncogene in many cancers \(^{48}\). Disruption of the Par complex could result in mislocalization and uncontrolled activation of Ect2 \(^{49}\), leading to increased Rac signaling. Ect2 is on the same locus of aPKCζ, and these two proteins are often amplified together in cancer \(^{48}\). Furthermore, Ect2 promotes aPKCζ activation \(^{47}\). One of the substrates of aPKC is the senescence inducer p21. Hyperactive aPKC will inhibit p21,
Thus suppressing senescence signaling. This function of aPKC appears to be restricted to cancer cells as in non-transformed cells like MCF10A, depletion of aPKC did not result in induction of senescence, which was instead the case in breast cancer and glioblastoma cells. Therefore, the same set of proteins promotes hyperphosphorylation of ERK and hyperproliferation, and in addition suppress senescence. If this is of relevance in cancer, it would be expected that tumors lose Par3, while upregulate Par6 and/or aPKC. This is indeed the case. Par3 depletion is often observed in tumors, though it is unlikely that this will be the driving mutation of the cancer because of the pro-apoptotic effect generated by loss of the polarity protein. Par6 and aPKC on the contrary, are often overexpressed in tumors. Those two proteins could collaborate to induce hyperproliferation in the tumors: Par6 would increase ERK signaling, whereas aPKC would inhibit senescence, which could be triggered by ERK. Overexpression of a protein can also result in its mislocalization and disruption of the Par complex will lead to hyperactivation of Rac that in turn can drive transformation. Two other Par proteins could be involved in the generation of CSC. LKB1 (Par4) is a critical factor needed to establish cell polarity. Loss of this protein causes the Peutz-Jeghers syndrome, also known as hereditary intestinal polyposis syndrome, where the patient spontaneously develops benign polyps in the intestine. LKB1 is often inactivated or lost in lung and breast cancer and in MCF10A cells, loss of LKB1 cooperates with the oncogene myc to induce hyperproliferation. The effect of LKB1 loss on proliferation could be due to hyperactivation of the mTOR pathway. In fact, LKB1 inactivates mTOR through its downstream effector AMPK that in turn phosphorylates the mTOR inhibitor TSC2. LKB1 loss could also lead to evade contact mediated inhibition. The kinase negatively regulates the Hippo pathway. LKB1 phosphorylates MARKs that in turn phosphorylate MST1/2. The signal converges to the kinase LATS1/2 that phosphorylates YAP/TAZ. Once the transcription factors are phosphorylated, they are excluded from the nucleus and therefore they are inactive. YAP/TAZ translocation to the nucleus is sufficient to confer stemness to a cell and this system is exploited in cancer to generate CSC. This puts LKB1 at the center of the stage among all Par proteins because it is the only protein that controls both proliferation and replicative potential of cells, the two characteristics needed to initiate a cancer.

In Drosophila it was demonstrated that perturbing asymmetric division in the larval neuroblast led to hyperproliferation and neoplastic transformation. Even though a similar evidence in mammal is still missing, it can be proposed that impaired segregation of cell fate
determinants will result in promotion of proliferation of undifferentiated cells. Asymmetric cell division is typical of stem cells and interestingly, a recent paper showed that there is a strong correlation between the number of stem cell divisions in a tissue and the risk of developing cancer in that tissue. Based on this observation, it is tempting to speculate that alterations in the control of stem cell division is crucial for the development of cancer also in mammals. Considering that the Par proteins were identified in C.elegans for their ability to segregate cell fate determinants in the daughter cells, Par complex proteins could be implicated in asymmetric cell division and thus in stem cell renewal also in mammals. However, very little is known about the role of Par proteins in asymmetric division in mammals. Only recently it was shown that Par3L, a protein similar to Par3, but lacking the interaction sites for Par6 or aPKC, is responsible for the maintenance of a pool of stem cells in the epithelium of the mouse breast. Surprisingly, Par3L functions by inhibiting LKB1. It would be interesting to assess whether Par3L is upregulated in cancers and if this upregulation leads to the formation of CSC by activation of YAP/TAZ.

The Hippo pathway is a point of convergence of all the polarity complexes. In fact it can be regulated also by Scribble and Crumbs. If cells are plated very sparse, YAP/TAZ are active, whereas, as soon as the cells establish contact with other cells, YAP/TAZ are inactivated and contact inhibition of growth is achieved. The Crumbs complex plays an important role in the process as all its components interact with YAP/TAZ retaining them at TJs. Depletion of Crb3 disrupts the Crumbs complex and results in translocation of YAP/TAZ to the nucleus, that renders the cells insensitive to contact inhibition and causes hyperproliferation. Scribble acts as a scaffold for MST1/2, LATS1/2 and TAZ. The close proximity of the transcription factor to the kinases responsible for its inactivation cause the silencing of the hippo pathway. However, when Scribble is mislocalized or lost, its scaffolding function is lost and YAP/TAZ translocates to the nucleus. Interestingly, Scribble is very often mislocalized in breast cancer and lost in several other types of tumors. The possibility that loss of mislocalization of Scribble might generate cancer stem cell is currently not well investigated, but it appears a likely scenario. Conversely, the role of Scribble on cell proliferation is well studied. Mislocalization or loss of the polarity protein inhibit the apoptotic response generated by the oncogene Myc, thus potentiating the pro-proliferatory ability of Myc. Several proteins related to the ERK pathway directly bind to Scribble and loss of Scribble results in hyperactivation of ERK and promotion of cell proliferation. Scribble bind directly to 2
antagonists of Akt signaling, PTEN $^{38}$ and PHLPP1 $^{62}$. It was possible to show in vivo that mislocalization of Scribble resulted in hyperactivation of Akt and subsequent hyperproliferation $^{38}$. Transgenic mice expressing a variant of Scribble that does not correctly localize showed hyperproliferation of the mammary gland epithelial cells and the hyperproliferation eventually progressed to tumors in the aging mice $^{38}$. However, the tumors were very heterogeneous, meaning that probably they harbored several other mutations in addition to mislocalization of Scribble that could have initiated the process. Together with LKB1, Scribble is the only polarity gene that could control proliferation and stemness. However, it was recently observed that cells depleted of Scribble undergo apoptosis induced by contact with the surrounding cells expressing Scribble and the hyperactivation of the Hippo pathway was observed only in tissue composed entirely by cells lacking the polarity protein $^{63, 64}$. Thus it appears difficult to imagine how loss of Scribble could initiate a tumor without further mutations.

**Golgi and tumor initiation**

GM130 depletion results in increased ERK signaling and promotion of differentiation of PC12 cells in response to NGF. However, we did not find any relevant effect of GM130 depletion on cell proliferation or apoptosis in transformed breast cancer cell lines, thus we have no evidence to speculate that loss of GM130 could be sufficient to initiate a tumor.

The Golgi has a role in controlling entry into mitosis. GRASP65 phosphorylation was shown to be a key event in this process and preventing this event resulted in block of the cell cycle $^{65}$. However, hyperphosphorylation of GRASP65 does not lead to a faster entry into mitosis and thus it does not cause hyperproliferation. Several Golgi proteins are targeted by caspases during apoptosis and the proteolytic fragments of these proteins are important to sustain progression of the apoptotic process $^{66-69}$. Whether alterations preventing the cleavage of such Golgi proteins are present in tumors is not investigated and it is possible to speculate that mutations in the Golgi proteins that control apoptosis could result in increased survival of tumor cells.

The recent discovery that GOLPH3 regulates mTOR could confer the Golgi a role in tumor initiation. GOLPH3 overexpression increases the speed of the cell cycle by modulating mTOR signaling and this results in hyperproliferation $^{70}$. In addition, GOLPH3 is implicated in the response to DNA damage and high levels of GOLPH3 prevent apoptosis induced by DNA damage $^{71}$. When DNA is damaged, the kinase DNA-PK is activated and phosphorylates
GOLPH3, promoting the interaction between GOLPH3 and the unconventional myosin MYO18A. This gives rise to an increased tensile force of the actin filaments that impairs post-Golgi trafficking and eventually leads to Golgi fragmentation. Golgi fragmentation requires DNA-PK, GOLPH3, MYO18A and F-actin and if this pathway is blocked at any step (by inhibition of the kinase, depletion of GOLPH3 or MYO18A or expression of unphosphorylatable GOLPH3), the Golgi remains compact 71. Interestingly, when GOLPH3 or MYO18A are depleted, the Golgi remains intact and cells are sensitized to apoptosis induced by DNA damage. On the contrary, when GOLPH3 is overexpressed, cells become more resistant to apoptosis, meaning that GOLPH3 plays a role in apoptotic pathways downstream of DNA damage 71. Tumor cells have often alterations in the DNA damage response machinery and chemotherapies and radiotherapies take advantage of this by inducing DNA damage that will specifically kill cancerous cells 72. However, if GOLPH3 is overexpressed, this approach could fail. Therefore, high levels of GOLPH3 activate mTOR, which results in hyperproliferation, and at the same time, protect cancer cells from apoptosis in response to DNA damage, thus further increasing the hyperproliferative effect due to mTOR. This confers to GOLPH3 an important role in tumor formation and progression that doubtless needs to be studied carefully in the future. Finally, a role for the Golgi in asymmetric cell division has been proposed. ACBD3 (also called GCP60) localizes to the Golgi by an interaction with Giantin. During mitosis the Golgi fragments and ACBD3 is released in the cytosol, where it modulates the activity of the cell fate determinant Numb 73. The mechanism causing trapping of ACBD3 at the Golgi and its release during mitosis is not known yet, so it is not possible to conclude whether Golgi localized proteins could function as oncogenes by deregulating asymmetric cell division and thus affecting stem cell renewal.

**Promotion of tumor growth by polarity proteins**

Loss of polarity profoundly affects the migratory capacities of the cells. If the polarity machinery is not intact, directed migration is inhibited and the ability of cells to invade through matrigel following a chemoattractant is strongly impaired. However, there is strong evidence that loss of polarity increases the metastatic power of a tumor 36, 39. For a process known to increase metastasis, this is at first sight counterintuitive. How can a cell that cannot migrate or invade towards a gradient produce more metastasis? Perturbation of polarity reduces directionality of cells, but increases the speed of cells migrating in 2D 74, 75. In addition,
Par3 depletion cooperates with oncogenic Ras, NICD and HerB to increase invasion through ECM\textsuperscript{36,39}. This could be unique to Par3 depletion because it was shown that loss of this polarity protein leads to increased STAT3 signaling and release of MMP7, that will degrade the ECM and thus promote invasion\textsuperscript{36}. Protease dependent movement was thought to be important for tumor dissemination, but clinical trials with MMP inhibitors did not have any impact on patient survival\textsuperscript{76}. The reason lies probably in the high plasticity of the cells. When proteases are inhibited, cells compensate by moving both in an amoeboid and mesenchymal way. Thus, using the holes present in the ECM, cells can move without the need of actively carving their own road. We could show that depletion of GM130 resulted in increased invasiveness of breast cancer cells in an assay not dependent on polarity. We could not find evidence of upregulation of any MMP, as it was the case for Par3 depletion, thus we speculate that the increased invasion is due to the fact that cells depleted of GM130 move faster in 2D. We can speculate that when the polarity machinery is broken, cells become less organized and switch between amoeboid and mesenchymal movement, resulting in increased speed. More polarity related genes should be tested to understand if this is a general mechanism by which polarity loss promotes metastatic spread. However, this hypothesis is based on the assumption that metastatic spread is not driven by any gradient and it is just the result of cells moving randomly until they encounter a blood vessel. To date, no clear evidence has been provided for the existence of such a gradient, even though directed motility of cancer cells towards newly generated blood vessels has been documented\textsuperscript{77}. EGF might drive metastatic cells towards the blood vessels as it was observed that metastatic cells have more EGF receptor than the non-metastatic cells from which they derive\textsuperscript{78}. Tumor associated macrophages could provide a paracrine gradient of EGF and accordingly, it was observed that macrophages enhance the motility of cancer cells\textsuperscript{79}. In a recent report, two patterns of migration were described for cancer cells escaping the primary tumor, single cells moving randomly and streams of cells (not connected between each other), with streaming cells moving faster than single cells. The presence of macrophages was found to be correlated with the streaming pattern, where cells move faster. Inhibition of macrophages resulted in an abolishment of this type of movement and in a striking decrease of circulating tumor cells. Therefore, it was proposed that the fast moving cells are the ones that will give rise to metastasis\textsuperscript{80}. Still, the fact that streaming depends on macrophages points to the possibility that a gradient might be driving the migration of streaming cells. In another study, several
parameters known to be associated with tumor cell motility were taken in exam (vasculature, ECM, and macrophages). Again, faster and slower cells were identified, but the speed of the cells did not correlate with any of the parameters considered. Anyway, a correlation between appearance of protrusions and distance from the blood vessels was found, thus the authors proposed that blood vessels could be responsible for the establishment of a gradient driving cell migration. Melanoma cells metastasize by following self-generated gradients. This observation could partially reconcile the contradiction that polarity loss increases metastasis, but decreases the ability of cells to follow a gradient. Muinonen-Martin et al. showed that melanoma cells break down LPA faster than normal cells because they express more LPAR1 on their surface. Once reached a critical mass, the gradient becomes robust enough to force polarization and migration of the cells away from the primary tumor. Thus, cells follow a gradient to escape from the primary tumor, but they are not directed towards a blood vessel. Once cells are too far from the tumor and do not sense the gradient anymore, polarity defective cells will move faster than the cells where the polarity machinery is still intact, thus they will explore a wider area and will have more probabilities of encountering a blood vessel.

**Golgi and tumor progression**

Given the widely recognized connection between Golgi and cell migration, it is somehow surprising that studies on Golgi and tumor progression are still very scarce. It is known that most highly invasive tumors have elevated levels of the O-GalNAc glycan Tn on the cell surface. The presence of glycans at the plasma membrane is known to promote adhesion of cells and could also be involved in the recognition of tumor cells by immune cells. Interestingly, tumors with high Tn levels at the plasma membrane show a relocalization of N-acetylgalactosamine-transferases (GalNAc-Ts) from the Golgi to the ER and this causes cells to form more invadopodia and to migrate better. Relocalization of several glycosyltransferases was described to happen as a consequence of Golgi fragmentation in prostate cancer. Tumors cells often display a fragmented Golgi and we could speculate that Golgi fragmentation is induced to promote cell motility and invasion. However, Golgi fragmentation results in most of the cases in impaired migration of cells in a wound assay, meaning that cells with a fragmented Golgi have a polarity defect. It should be tested whether Golgi fragmentation increases the speed of randomly migrating cells, as it is the case for other conditions where polarity is disrupted. Petrosyan et al. showed that Rab6a associates with
NMIIA (non-muscle myosin IIA) and NMIIA prevents Rab6 association with Giantin, necessary to maintain Golgi structure. If NMIIA is depleted, the Golgi becomes compact again, mislocalized glycosyltransferases relocate to the Golgi and cancer cells become more susceptible to galectin-1 induced apoptosis. Our data confer the Golgi protein GM130 a main role in controlling cell polarity and migration. We showed that GM130 is progressively lost in colon cancer and often downregulated in breast cancer and thus we speculate an involvement of the Golgi in metastatic spreading. GM130 depleted cells retain a compact Golgi, but are defective in directional migration. Despite being not directional, GM130 depleted cells move faster than control cells and become more invasive. GM130 depletion alters two pathways, Cdc42 and Ras. Cdc42 and Ras are both important in cell migration and we could show that inhibition of Ras together with GM130 depletion resulted in a stronger decrease in the ability of the cells to close a wound. This implies that Cdc42 and Ras affect migration via two different pathways. MDA-MB-231 cells, which have a constitutively active Ras, didn’t migrate faster and didn’t become more invasive after GM130 depletion, whereas cell lines with no mutation in the Ras pathway became faster and more motile upon GM130 depletion. We speculate that a constitutively active Ras might mask the effect of GM130 depletion on cell motility.

How does GM130 control cell velocity? Depletion of the polarity protein Scribble gives phenotypes very similar to the ones observed upon depletion of GM130, thus it is tempting to speculate that GM130 might control Scribble. Scribble connects β-catenin and E-Cadherin and it was shown that the increase in cell velocity caused by loss of Scribble could be dampened by rescuing the interaction between β-catenin and E-Cadherin. GM130 could control Scribble expression levels or alternatively, it could control its localization. It was reported a role for Cdc42 in sorting proteins from the TGN to apical or basolateral membranes, therefore we could speculate that by regulating Cdc42 activity at the Golgi, GM130 regulates Scribble positioning. However, it is not known if the same happens when Scribble is mislocalized or if instead it is necessary to lose Scribble to increase cell velocity.
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