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Brief synopsis: Rab35 is a small GTPase that was recently reported to possess oncogenic activating mutations in human tumors. Conversely, Rab35 depletion inverts apico-basal cell polarity and promotes cell migration. In the present review we describe how Rab35's known functions in membrane trafficking, signaling, cell division and cell migration could explain its role in tumorigenesis.

Abstract
Rab35 is a small GTPase that is involved in many cellular processes, including membrane trafficking, cell polarity, lipid homeostasis, immunity, phagocytosis and cytokinesis. Recent studies showed that activating mutations confer Rab35 with oncogenic properties. Conversely downregulation of Rab35 inverts apico-basal cell polarity and promotes cell migration. Here we review Rab35's known functions in membrane trafficking and signaling, cell division, and cell migration in cancer cells and discuss the importance of Rab35-dependent membrane trafficking in cancer progression.

Introduction

Intracellular membrane trafficking regulates uptake of nutrients and signaling from extracellular molecules by controlling the plasma membrane localization of transporters and receptors, and thus plays a key role in cell differentiation and proliferation. In addition, cells rely on membrane trafficking to maintain cell architecture, cell shape and cell polarity, as well as organ structure and function[1,2]. Cancer cells often have altered membrane trafficking that can lead to the upregulation of growth factor receptors at the cell surface (e.g. epidermal growth factor receptor; EGFR) and abnormal levels of adhesion molecules (e.g. β1-integrin, cadherins), which can promote epithelial-mesenchymal transition (EMT) and an invasive phenotype during cancer progression[3-5].

Rab proteins are evolutionarily-conserved GTP-binding proteins that regulate intracellular membrane trafficking in all eukaryotic cells[6-8]. They act as molecular switches between an inactive GDP and active GTP form, where the latter form interacts with specific intracellular membranes and controls local recruitment of downstream effectors. Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) regulate Rab activation and deactivation, respectively[6-9]. Importantly, Rab proteins control the endocytosis, recycling and lysosomal degradation of growth factor receptors including those overexpressed in many cancers[4], such as the EGFR[10], platelet derived growth factor receptor (PDGFR)[11], vascular endothelial growth factor receptor (VEGFR)[12], c-Met[13] and integrins[14,15]. Manipulating the endocytic trafficking and recycling of these receptors has emerged as an attractive method to counteract cancer cell proliferation and survival. For example, pharmacologically-induced accumulation of ligand-activated EGFR in endosomes activates apoptosis selectively in cancer cells[16,17]. Taken together, this suggests the potential for targeting endocytic trafficking to counteract cancer cell proliferation.

There is increasing evidence to implicate a select few Rab proteins in cancer progression (reviewed in ref.[7]). Here we will focus on Rab35, a GTPase[19] first described as being important for endocytic recycling and cytokinesis[18]. Rab35 is unique in that it contains an evolutionarily-conserved polybasic C-terminal tail and localizes both at the plasma membrane and on endosomes[18,20]. Its polybasic tail facilitates its interaction with PtdIns(4,5)*P2* and PtdIns(3,4,5)*P3* at the plasma membrane[20]. Rab35 has attracted a lot of attention in the past ten years in the endocytic community and beyond, as reviewed recently in ref.[21]. Rab35 has many functions in various cellular processes including membrane trafficking (see below), autophagy[22], cell polarity[23,24], cytokinesis[25,26], membrane lipid homeostasis[25,27], neurite outgrowth[28-31], axonal elongation[32], immunity[33-36], phagocytosis[37,38], pathogen infection[39,40], myoblast fusion[41], exosome secretion[42] and Weibel-Palade bodies secretion[43]. Moreover, the mechanisms controlling Rab35 activation in endocytosis is well understood with the identification of Rab35 GEFs (DENND1A-C/Connecdenn1-3[27,44-47]) and GAPs (TBC1D10A-C/EPI64A-C[33,36,48], TBC1D13[49], TBC1D24[50]), and reviewed in detail in [21] and [51].

A recent study described Rab35 oncogenic somatic mutations in human tumors, which activate Rab35 and have transforming capabilities in mouse fibroblasts *in vitro*[11] (see Rab35 tumor somatic mutations in Table 1). In this context, Rab35 regulates PDGFR trafficking leading to cell transformation through a PI3K/AKT-dependent mechanism[11]. Rab35 was initially linked to cancer by its interaction with the oncogenic nucleophosmin-anaplastic lymphoma fusion kinase (NPM-ALK)[52] and the p53-related protein kinase (PRPK), where it negatively regulates PRPK-mediated p53 transcriptional activity[32,53]. However, the relevance of Rab35 function in lymphoma or p53-dependent transformation remains to be established.

Here, we review the recent data suggesting a direct role for Rab35-dependent membrane trafficking in multiple and intertwined aspects of cancer progression by controlling cell division, polarity and migration, as well as survival and proliferative intracellular signaling.

Rab35 in membrane trafficking and cell division

Cytokinesis (the last step of cell division leading to the physical separation of the daughter cells) and cell polarity are two key processes deregulated in cancer and Rab35 plays a role in both. An RNAi screen looking for Rabs involved in cytokinesis identified Rab35 and showed that depletion of this Rab generated binucleated cells and perturbed a fast recycling pathway[18]. The first isolated effector of Rab35 was the PtdIns(4,5)P_2 phosphatase Oculo-Cerebro-Renal syndrome of Lowe (OCRL), which is necessary for successful cytokinetic abscission[25]. Of note, OCRL traffics in and out of the cytokinetic bridge on Rab35-positive vesicles[25]. Another Rab35 effector, MICAL1, which belongs to an emerging class of oxidation-reduction enzymes that depolymerize F-actin through selective oxidation[54,55], facilitates ESCRT-III recruitment at cytokinetic bridges and thus abscission[26]. Both OCRL and MICAL1 localize to cytokinetic bridges in a Rab35-dependent manner, and their depletion causes the accumulation of F-actin at this location[25,26]. This suggests a parallel, and perhaps synergistic, mechanism of F-actin removal from cytokinetic bridges, which appears to be essential for successful cytokinesis. Importantly, studies have established that cytokinetic failure leading to tetraploidy promotes tumorigenesis *in vivo*[56], as shown experimentally in primary mouse mammary epithelial cells[57].

In addition to a mechanistic role for Rab35 and its effectors in cytokinesis by clearing F-actin, Rab35 plays a role in connecting cytokinesis with apical polarity initiation in MDCK cells grown in Matrigel to form cysts[23]. In this study Rab35 was found to tether vesicles containing important apical proteins, such as Cdc42, Crumbs3, aPKC, and the lumen promoting factor GP135/Podocalyxin (PODXL) at the cytokinetic abscission site to deliver them to the future apical membrane initiation site (AMIS). Consequently, Rab35 depletion or deactivation prevents AMIS and lumen formation, and leads to a complete inversion of apico-basal polarity[23,24]. As changes in apico-basal polarity are believed to favor cancer development, this suggests that loss of function mutations of Rab35 might promote tumorigenesis. Interestingly, Rab35 is downregulated in several tumors including grade IV gliomas, breast and renal carcinomas[58,59].

In summary, the function of Rab35 in the final step of cell division and in apico-basal polarity establishment might be important to explain a role for this GTPase in cancer initiation.

Rab35 in membrane trafficking and cell signaling

Rab35 plays a central role in intracellular trafficking by promoting endocytic recycling of various cargoes, including the transferrin receptor (TfR)[18,40], yolk receptor[60], Shiga toxin[48],

major histocompatibility complex class-I (MHC-I) and MHC-II[33,34], T-cell receptor[36], Ca^{2+}-activated K^+ channel KCa2.3[61], Megalin[62], GLUT4[49], M- and N-Cadherin[41,58] and β1-integrin[58,63]. Moreover, Rab35 knockdown inhibits trafficking of CI-mannose-6-phosphate receptors from endosomes to the trans Golgi network (TGN)[27].

Recent studies showed that the tumor suppressor folliculin (FLCN) interacts with GDP-bound Rab35 *in vitro* and negatively regulates EGFR signaling after EGF stimulation[64,65], possibly acting as a Rab35 GEF[66]. Moreover, FLCN-mediated EGFR degradation in HeLa cells requires Rab35 activation[64]. In these cells, depletion of Rab35 increases AKT activation after EGF stimulation and cell proliferation[64], as previously observed in COS-7 cells[58]. Rab35 is downregulated in some cancers[58,59], which could lead to increased EGFR receptor recycling and signaling, a common hallmark of many malignancies[3]. Surprisingly, a recent study reported that depletion of Rab35 in HeLa cells rather diminished AKT signaling, at least after 10% serum stimulation[11]. The authors attributed cell context and experimental conditions as a reason for this discrepancy (perhaps serum vs EGF stimulation[64]) but this needs to be clarified. Nevertheless, two Rab35 activating mutations (A151T and F161L) found in human cancers (lung, uterus and lymphoid tissues; see Table 1) lead to upregulation of the PI3K/AKT signaling pathway via mTORC2, as well as mouse fibroblast transformation and apoptosis evasion[11]. This associates with increased signaling and trafficking of the PDGFR-α into Lamp2-positive vesicles[11]. This study thus suggests a pivotal role for Rab35 activating mutations in endocytic deregulation and PDGFR-mediated oncogenic signaling in cancer progression.

It is not known, however, how exactly Rab35 controls EGFR and PDGFR trafficking in FLCN-depleted cells or in cells expressing activating mutations of Rab35. Given the number of cargoes whose trafficking is regulated by Rab35 (see above), it is likely that Rab35 plays a fundamental and general role in the endosomal pathway. As during cytokinesis, GTP-bound Rab35 binds to the OCRL lipid phosphatase[27], which promotes PtdIns(4,5)P_2 hydrolysis on newborn endosomes/clathrin coated vesicles recently pinched from the plasma membrane[67]. This promotes the generation and maintenance of PtdIns4*P* on endosomes, an important step for normal cargo sorting and recycling towards the plasma membrane or targeting to the trans-Golgi network[67]. In addition to PtdIns4*P vs.* PtdIns(4,5)*P2* homeostasis, active Rab35 might regulate PtdIns(3,4,5)P_3 levels and thus AKT activation, since Rab35 co-immunoprecipitates with PI3K[11]. Therefore, abnormal levels of GTP-bound Rab35 could affect the lipid composition of endosomes in cancer cells and dictate growth factor receptor fate.

In parallel to phosphoinositide regulation, Rab35 controls recycling of cargoes from ARF6-positive endosomes back to the plasma membrane through the recruitment of two additional effectors: the ARF6-GAP ACAP2/CentaurinB2[28,29] and MICAL-L1[68], a scaffolding protein that, contrary to MICAL1 described above, has no redox activity[54,55]. For detailed reviews on ARF proteins and cancer, please refer to refs.[69,70]. Upon activation, Rab35 inactivates ARF6, which promotes endosomal recycling[28,29,68,71]. In addition, Rab35 recruits MICAL-L1, which facilitates tubular endosomal fission by directly interacting with the dynamin-like protein EHD1[28,29,72]. Finally, MICAL-L1 recruits other small GTPases (Rab8, Rab13 and Rab36) that also promote endosomal recycling[73]. Of note, the TP53 status of cancer cells could also influence recycling in this pathway since p53 regulates MICAL-L1 transcription and tubular endosome biogenesis[74].

Altogether, abnormal levels of Rab35 activation impair endosomal recycling and phosphoinositide homeostasis by several mechanisms, which could explain growth factor receptor and survival signaling observed in cancer situations with perturbed Rab35.

Rab35 in cell migration

Cell migration and dissemination play a critical role in tumor metastasis. Several lines of evidence indicate that Rab35 regulates cell migration, however this depends on the cell type and the influence of external cues. In COS-7 cells, depletion of Rab35 induces a phenotype with hallmarks of EMT *in vitro*[58], with increased cell migration and decreased cell-cell adhesion. This can be explained by the fact that Rab35-dependent recycling is required for maintaining cadherins at the cell surface in COS-7 cells[58], as in myoblasts[41]. In addition, Rab35 depletion increases β1-integrin levels at the cell surface and thus promotes cell migration, since Rab35 inhibits ARF6-mediated β1-integrin recycling through the ARF6-GAP ACAP2[58] (see above). Rab35 depletion in HeLa and MDA-MB-231 cells increases β1-integrin levels, however authors attributed this to impaired internalization rather than increased recycling[63]. In any case, overexpression of miRNA-720 (a marker for colorectal cancer[75] and multiple myeloma[76], and which targets Rab35) also resulted in increased cell migration in HeLa cells[77].

Conversely, Rab35 activation after prolonged EGF stimulation reduces the interaction between the G protein-coupled receptor kinase interacting ARFGAP 2 (GIT2) and its binding partner RUSC2 and consequently reduces directional cell migration in non-small cell lung cancer (NSCLC)[78]. However, contrary to the studies mentioned above, Rab35 activation downstream of Wnt5 signaling promotes cell migration in breast cancer MCF-7 cells via a Dvl2/Rab35/Rac1 signaling pathway[79], perhaps due to additional dominant effects downstream of Wnt5.

Finally, EGF-mediated Rab35 activation leads to the activation of the enzyme MICAL1, and this associates with reactive oxygen species (ROS) production, AKT phosphorylation, and cell invasion in a Matrigel Transwell assay in breast cancer cells[80]. Since GTP-Rab35 binding to MICAL1 releases an inhibitory intramolecular folding, activates the redox activity of MICAL1 and selectively depolymerizes F-actin[26], it would be interesting to characterize whether Rab35 remodels cortical actin during invasion.

These seemingly contradictory results suggest that depending on the migration assay (with or without extracellular matrix), the cell type and on the means by which Rab35 is activated or inhibited, Rab35 plays different roles in migration vs. invasion.

Concluding remarks

Abnormal activation of Rab35 modifies actin dynamics, cell division and polarity. In addition, Rab35 regulates the endocytic recycling of key cargoes involved in cell migration, such as β1-integrin as well as growth factor receptor recycling and signaling through PDGFR-α, EGFR and AKT (Figure 1). Based on the *in vitro* studies reviewed above, modification of Rab35 activation observed in several cancers might influence EMT and cancer cell migration/metastasis. However, additional work is needed to address experimentally the consequences of Rab35 upregulation or downregulation on tumorigenesis *in vivo*.

Rab proteins have gained interest as targets for the development of treatments for diseases[81,82]. As Rab35 is known to play an important role in endocytic recycling, an interesting approach would be to target the Rab35-specific effectors responsible for this function instead of the Rab35 molecule itself, which is involved beyond, and thus specifically

affecting the endocytic trafficking of cancer-involved growth factor receptors. Perhaps pharmacologically targeting the Rab35 effector OCRL could be an attractive possibility. Interestingly, a bistable switch between Rab35 and ARF6 has been postulated[21], since GTP-Rab35 interacts with ACAP2/CentaurinB2 (an ARF6 GAP)[28,29], while GTP-ARF6 interacts with TBC1D10/EPI64 (a Rab35 GAP)[33]. Thus, activating ARF6 in the endocytic pathway may also prove interesting for inactivating Rab35-dependent trafficking of growth factor receptors.

While both Rab35 activating mutations[11] and Rab35 downregulation[58,59] are described in cancer, these phenomena could play complementary roles at different stages of cancer development through a process known as clonal evolution[83]. One can indeed speculate that during initial tumorigenic events, activating mutations described to upregulate PDGFR trafficking and AKT activation would lead to cell survival and transformation. In contrast, Rab35 downregulation leads to a migratory or invasive EMT phenotype and could be the consequence of gene silencing at a later stage, after cell transformation.

Taken together, Rab35 emerges as a key player in cancer and future studies regarding Rab35 should shed light on how this GTPase contributes to oncogenesis, linking the importance of membrane trafficking to tumorigenesis.

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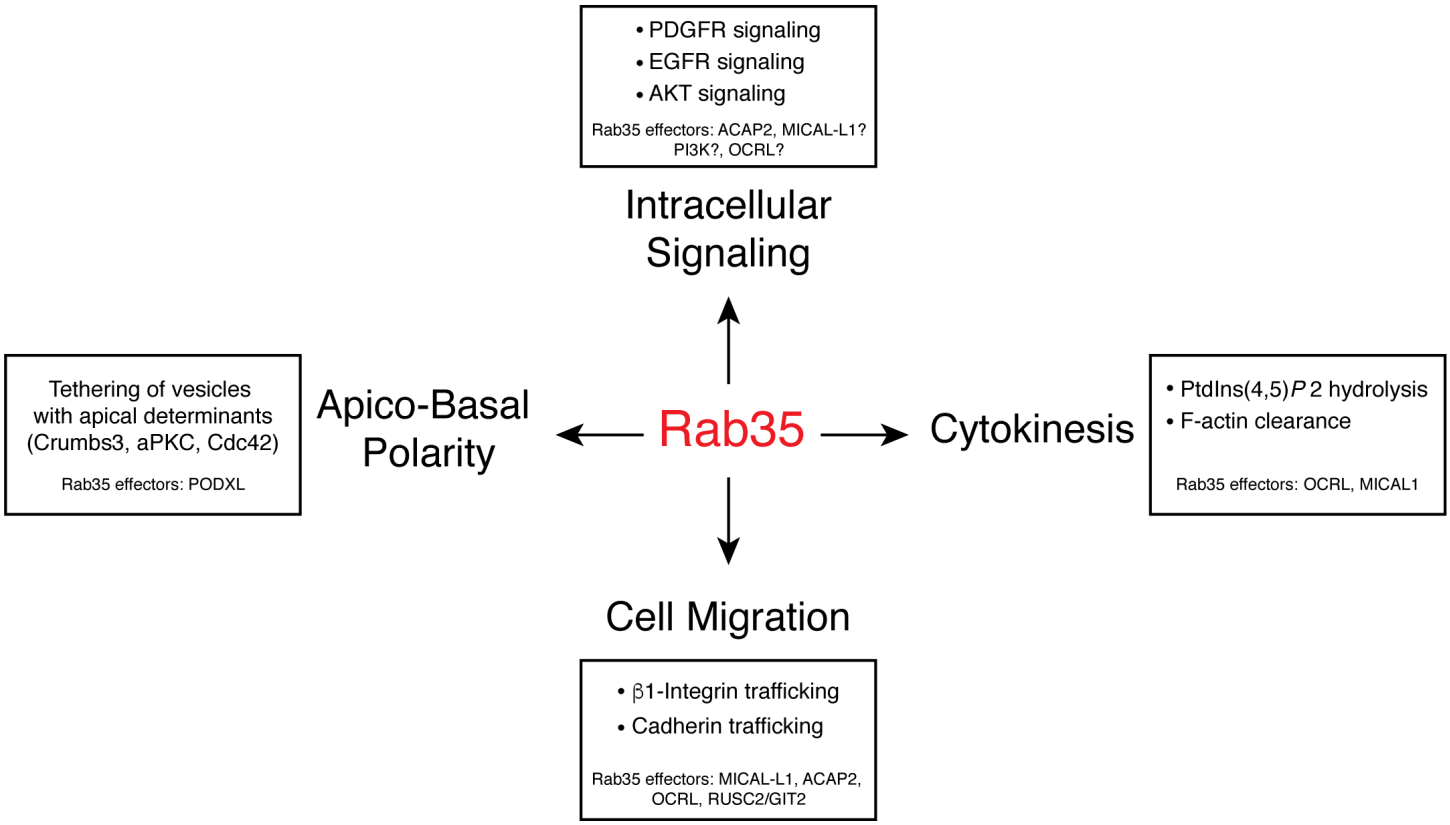

Figure 1. Rab35 functions in intracellular signaling, apico-basal polarity, cytokinesis and cell migration. Rab35's functions in these processes are described. Moreover, known and possible effectors involved in each process are indicated.

Table 1. Pathogenic Rab35 somatic mutations found in human tumor samples according to the Catalogue of Somatic Mutations in Cancer (COSMIC) database. We indicated synonymous mutations as they can be sometimes pathogenic.

Tissue	Amino acid change	Mutation
Bone	V187V	Synonymous
Brain	V90I	Missense
Breast	A29V	Missense
	F45L	Missense
	G18V	Missense
Cervix	F45F	Synonymous
Colon	A139V	Missense
	E94K	Missense
	F33F	Synonymous
	G80E	Missense
	G83R	Missense
	G140R	Missense
	R27C	Missense
	R71C	Missense
	R79Q	Missense
	R127Q	Missense
	R196Q	Missense
	S150S	Synonymous
	V188M	Missense
	-	Intronic substitution
Endometrium	A151T	Missense
	S22N	Missense
Esophagus	I14I	Synonymous
	V97V	Synonymous
Hematopoietic and lymphoid	A151T	Missense
Liver	A65V	Missense
Lung	A29V	Missense
	F70F	Synonymous
	F161L	Missense
	R101Q	Missense
Pancreas	G80R	Missense
Prostate	N156S	Missense
Stomach	R27H	Missense
	R196*	Nonsense
Thyroid	G18S	Missense
Upper aerodigestive tract	E159Q	Missense
	T191M	Missense