

Behind the Scenes: Endo/Exocytosis in the Acquisition of Metastatic Traits

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Abstract

Alterations of endo/exocytic proteins have long been associated with malignant transformation, and genes encoding membrane trafficking proteins have been identified as *bona fide* drivers of tumorigenesis. Focusing on the mechanisms underlying the impact of endo/exocytic proteins in cancer, a scenario emerges in which altered trafficking routes/networks appear to be preferentially involved in the acquisition of prometastatic traits. This involvement in metastasis fre-

quently occurs through the integration of programs leading to migratory/invasive phenotypes, survival and resistance to environmental stresses, epithelial-to-mesenchymal transition, and the emergence of cancer stem cells. These findings might have important implications in the clinical setting for the development of metastasis-specific drugs and for patient stratification to optimize the use of available therapies. *Cancer Res*; 77(8); 1813–7. ©2017 AACR.

Introduction

The modern view of endocytic/exocytic membrane trafficking is that of a complex process embedded in virtually every aspect of cell regulation, including control of signaling at multiple levels (reviewed in ref. 1). This view has led to the idea that subversion of the endomembrane traffic machinery could contribute to cancer, a concept that has received substantial experimental corroboration (reviewed in refs. 1, 2). However, the extent of the impact of "traffic" alterations in "real" cancers is still an open question. For the field to move out of the "proof-of-principle stage" and to acquire value in terms of the development of clinically relevant strategies, a number of issues need to be addressed:

1. Although *in vitro* studies are critical for the elucidation of molecular mechanisms, more extensive analyses in real tumors are needed. Given the pervasive role of endo/exocytic traffic in cell physiology, one should not be too surprised that tampering with it might lead to a transformed phenotype *in vitro* that may, however, have little relevance in the clinical setting.
2. In real tumors, although genetic alterations of membrane trafficking genes are not very common, alterations in expression levels are frequently reported. This situation raises questions on the suitability of whole-tissue analyses that are frequently performed on tumor specimens to assess

expression levels. These analyses face several drawbacks. They overlook sample cellularity, cell of origin of the signal, and intratumoral heterogeneity. Thus, to avoid misleading results, analyses at the single-cell level are important.

3. The correlation between the level of expression of a gene-of-interest in a given tumor type and clinical-pathologic parameters is often interpreted as a good indicator of "some role in cancer." Frequently, however, only univariate analyses are reported in the literature. Although this is not a disqualifying issue, one should bear in mind that only multivariable analysis provides proof of an "independent" predictor. The problem is not minor: In breast cancer, for instance, up to 25% of all genes and up to 90% of random multigene signatures are significant outcome predictors in univariate analysis (3).

With these issues in mind, we revisited the literature on endo/exocytosis and cancer, focusing on studies that provide: (i) convincing evidence of endo/exocytic protein/network alterations in real tumors (i.e., genetic/driver alterations, analysis of over/underexpression in sufficiently sized cohorts, correlation with clinical parameters by multivariable analysis); and (ii) extensive characterization of phenotypes and mechanisms (Table 1). The emerging scenario is that altered endo/exocytic routes and networks appear to be preferentially involved in the acquisition of prometastatic traits, frequently, through the integration of migratory/invasive programs with other aspects of cancer biology. Here, we will review selected examples to illustrate this concept.

Induction of Invasive Phenotypes by Endo/Exocytic Harnessing of Cytoskeleton-Regulatory Circuitries

Cellular motility in physiologic conditions and invasiveness in cancer require the enactment of programs involving polarized rearrangement of the actin and tubulin cytoskeleton (4). Critical to these programs is a subfamily of small GTPases, the Rho-like GTPases, to which Rac1 and Cdc42 belong. Rac1 acts as a master

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Table 1. Examples of trafficking proteins altered in cancer

Gene	Function	Alteration	Tumor type
<i>RAB5A</i>	Rab GTPase	O	Breast
<i>DYN2</i> (Dynamin 2)	GTPase (fission)	O	Pancreas
<i>SYNJ2</i> (Synaptojanin 2)	Phosphoinositide phosphatase	A	Breast
<i>CIP4</i>	Cdc42 effector	O	Breast, lung
<i>RAB25</i>	Rab GTPase	A	Breast, ovary
<i>RCP/RAB11FIP1</i>	Rab effector	A	Breast
<i>RAB1A</i>	Rab GTPase	O	Colon
<i>RAB2A</i>	Rab GTPase	A	Breast
<i>NUMB</i>	Adapter	U	Breast, lung
<i>CLIC3</i>	Rab25 effector	O	Ovary
<i>SH3GL1</i> (Endophilin A2)	Adapter	O, M(T)	Breast, leukemia
<i>RAB35</i>	Rab GTPase	M	Lymphoma, lung, uterus
<i>GOLPH3</i>	Golgi protein	A	Multiple
<i>CBL</i>	E3 ligase	M	Leukemia
<i>CAV1</i> (caveolin)	Coat protein	U, M, O	Multiple
<i>DAB2</i>	Adapter	U	Multiple
<i>HIP1</i>	Adapter	O, M(T)	Multiple
<i>NDRG1</i>	Rab4 effector	U	Prostate, breast, lung
<i>CLTC</i> (Clathrin)	Coat protein	M(T)	Lymphomas, kidney

NOTE: The Table displays examples of endocytic/exocytic proteins altered in human cancers. Proteins reviewed in detail are shaded. The list is not comprehensive and additional hits (and detailed referencing to the proteins not covered in this review) can be found in refs. 1, 2, and 48.

Abbreviations: A, amplified; M, mutated (point mutations); M(T), mutated (translocation); O, overexpressed (in the absence of reported gene amplification); U, underexpressed.

regulator of cytoskeletal organization and polarized signaling connected with cellular motility. Physiologically, trafficking processes are central to Rac1 regulation, ensuring its continuous relocalization to membrane sites where the execution of polarized functions takes place (5). Accordingly, cancer-specific alterations of trafficking proteins hijack Rac1-based circuitries, leading to aberrant migratory/invasive phenotypes. Some examples are provided below.

A major circuitry regulating Rac1 involves the Rab5A/Rab4 axis. These proteins belong to the family of Rab GTPases that behave as critical molecular switches in the regulation of membrane dynamics, including vesicle formation, movement, maturation, and connection with the actin and tubulin cytoskeleton (6). Rab5A is a master regulator of endosomal dynamics, whereas Rab4 is involved in recycling to the plasma membrane (PM; ref. 6). Rab5A is overexpressed in breast cancers and is an independent predictor of unfavorable outcome (7). Phenotypically, Rab5A overexpression can be correlated with increased invasiveness *in vitro* and *in vivo*, extension of invadosomes (actin protrusions involved in the coupled processes of extracellular matrix degradation and cell motility), and importantly, conversion of *in situ* carcinomas to invasive carcinomas *in vivo* (7). The molecular action of Rab5A in this context is 2-fold. First, it promotes the encounter on early endosomes of Rac1 with a specific activator, the guanine exchange factor (GEF) Tiam1, after which activated Rac1 is recycled to restricted regions of the PM, where it controls polarized protrusion of lamellipodia and directed migration (5). Second, Rab5A controls the trafficking of metalloproteases and integrins (7). In this latter circuitry, Rab4, which is also overexpressed in breast cancer, acts downstream of Rab5A by recycling metalloproteases and integrins to regions of the PM where invadosomes will form (7).

A different pathway relying on another Rac1-GEF, Vav1, is subverted in pancreatic cancer. In this case, the protein involved is Dynamin 2 (Dyn2), whose major function is to control fission of endocytic vesicles (reviewed in ref. 8). Dyn2 is overexpressed in pancreatic ductal carcinomas, and its overexpression promotes a migratory/invasive phenotype *in vitro* and *in vivo* (9). Mechanistically, this phenotype is not due to the canonical role of Dyn2 in

vesicle fission, but rather to its direct interaction with Vav1 (10). Dyn2 stabilizes Vav1 by protecting it from proteasomal degradation, which presumably leads to Rac1 activation (10). Interestingly, Vav1 is also overexpressed in pancreatic cancer due to demethylation of its gene promoter and is an independent predictor of survival (11).

In addition to the upstream regulation of Rac1, endocytic proteins also participate in Rac1 effector function. This is the case of Synaptojanin2 (SYNJ2), a phosphoinositide phosphatase, which binds to activated Rac1 and mediates effects of the latter on cell motility and endocytosis (12). The *SYNJ2* gene is part of the 6q25 breast cancer amplicon, and its overexpression predicts poor prognosis in some breast cancer subtypes (13). Overexpression of *SYNJ2* increases cell-migratory/invasive phenotypes *in vitro* and *in vivo*, and its silencing leads to the impairment of protrusive/invasive structures (13), compatible with its role as a Rac1 effector.

A final example of a cancer-altered endocytic protein functioning as a Rho-subfamily GTPase effector is CIP4 (CDC42-interacting protein 4). CIP4 belongs to the superfamily of BAR (Bin/Amphiphysin/Rvs) domain proteins that couple curvature of the membrane with reorganization of the membrane-associated actin cytoskeleton (14). CIP4 is an effector of Cdc42, another Rho-like GTPase critical to actin dynamics, migration, and endocytosis that is also frequently altered in cancer. CIP4 overexpression in breast cancer represents an independent predictor of disease outcome, being associated with poorer prognosis and metastasis (15, 16). Mechanistically, this is due to increased E-cadherin internalization leading to cell scattering and improved actomyosin contractility (16).

Rab-Centered Circuitries Selected in Cancer Integrate Motility/Invasiveness with Survival/Resistance to Environmental Stresses

The ability to metastasize is linked to the acquisition of a number of characteristics, including increased motility and

resistance to anoikis and environmental stresses, such as low nutrient availability. Cancer-detected alterations of Rab25- and Rab1A-dependent signaling exemplify how the acquisition of these characteristics might happen.

RAB25, belonging to the Rab11 subfamily of Rabs involved in endosomal recycling, is the driver gene of amplicon 1q22 in breast and ovarian cancers, and its overexpression has been linked to resistance to apoptosis and anoikis (17). Evidence that Rab25 is involved in cancer cell dissemination derives from its role in the trafficking of the adhesive receptor integrins. Overexpression of Rab25 stimulates an invasive mode of migration based on the formation of long pseudopods. At the tip of the pseudopods, Rab25-positive vesicles promote $\alpha 5\beta 1$ integrin treadmill that, in turn, favors persistent and directed cell migration (18). Interestingly, Rab25 overexpression is not sufficient to transform immortalized/nontumorigenic cells (17, 19, 20); rather, it stimulates the acquisition of invasive properties in already transformed cells.

Rab25 integrates invasiveness with other prometastatic programs that help invasive cells elude environmental stresses. In low nutrient conditions, inhibition of mTORC1 results in the accumulation of $\alpha 5\beta 1$ integrin on late endosomes/lysosomes, and in tumors characterized by a potentiated Rab25 circuitry, this might promote integrin recycling and cell invasion (21). Under similar conditions, Rab25 stimulates glucose uptake, through direct binding to and activation of AKT, resulting in higher glycogen synthesis and elevated ATP levels, thereby providing cancer cells with an alternative energy source (20).

Depending on the context, Rab25 might also behave as a tumor suppressor (19). In a cohort of human colorectal cancers, Rab25 levels decrease in a stage-dependent manner and correlate with reduced survival in univariate analysis (22). In addition, while Rab25-KO mice do not exhibit spontaneous tumors, they display increased incidence of colonic neoplasia when crossed with an APC^{Min/+} genetic background. In these tumors, localization of $\beta 1$ integrin to the lateral membrane of intestinal cells is severely reduced (22). As mislocalization of $\beta 1$ causes intestinal hyperplasia (23), loss of Rab25-mediated $\beta 1$ trafficking in intestinal epithelial cells might contribute to tumor development by affecting cell polarity (22).

Notably, downstream effectors of Rab25 are also altered in cancer and are associated with the acquisition of prometastatic phenotypes. This is the case of the Rab11 family effector, Rab coupling protein (RCP), CLIC3 and Tensin1 (21, 24, 25). The case of RCP is particularly interesting. RCP is the driver of amplicon 8p11 in breast cancer, and its overexpression predicts metastatic recurrence (26). In the recycling pathway, RCP acts as a scaffold for $\alpha 5\beta 1$ integrin and EGFR, allowing their coordinated relocalization to the elongating pseudopods that characterize $\beta 1$ -dependent 3D invasive migration (25). Interestingly, RCP appears to represent a point of convergence of different cancer pathways, as mutant, gain-of-function, p53 proteins can drive cancer cell invasion by exploiting RCP (27). These mutant p53 proteins promote the binding of RCP to $\alpha 5\beta 1$, by as yet unclear mechanisms, thus stimulating the coordinated recycling/relocalization of EGFR and $\alpha 5\beta 1$ integrin to the PM (27).

Rab1A-dependent signaling represents another example of how invasive phenotypes and sensitivity to nutrients can be coordinately derailed in cancer. Rab1A, a GTPase involved in endoplasmic reticulum (ER)-to-Golgi transport, is overexpressed in colon cancer and predicts poor outcome and cancer invasive-

ness (28). By sensing amino acid levels, Rab1A becomes activated and binds to mTORC1, thereby promoting proliferation and cellular transformation. Interestingly, the circuitry requires a Rab1A-dependent physical association with the Golgi to be functional, confirming the link between endomembrane dynamics and nutrient sensing. It is tempting to speculate that the potentiation of the Rab1A/mTORC1 axis, observable in colon cancers, augments sensitivity to amino acids, thereby reducing the effective concentration of these nutrients needed to sustain cell proliferation. This advantage might however become an "Achilles' heel" for the tumor, as it has been shown that colon cancer cell lines overexpressing Rab1A become addicted to amino acids (28). We will discuss this issue further below.

Membrane Traffic, Epithelial-to-Mesenchymal Transition and Cancer Stem Cells

Epithelial-to-mesenchymal transition (EMT) is a complex transcriptional program enacted by cells that undergo a switch from an epithelial to a mesenchymal/migratory state. In physiology, EMT is critical for embryonic development and tissue repair; however, its aberrant activation is linked to pathologic conditions, first and foremost cancer (29). Recent work, pioneered in Weinberg's laboratory, has highlighted a major role of EMT in the emergence of cancer stem cells (CSC). These cells are not only the initiators of cancer, by definition, but also represent the subpopulation of cells most likely responsible for metastasis and resistance to therapy (reviewed in ref. 30). Although these findings blur the traditional boundaries between cancer initiation and progression, they offer an additional angle to rationalize the impact of endo/exocytosis in cancer. Trafficking networks are critical in regulating the activity of signaling pathways leading to EMT, such as those activated by TGF β and WNT, or in modulating the dynamics of adhesion molecules involved in the maintenance of epithelial polarity, such as E-cadherin (reviewed in ref. 31). Here, we will analyze the case of Numb and of Rab2A.

Numb sits at the intersection of multiple functions, including cell fate decisions, maintenance of stem cell compartments, regulation of cell polarity, adhesion, and migration (reviewed in ref. 32). At the molecular level, Numb is involved in the formation of endocytic vesicles and in their recycling to the PM (32). In breast cancer, Numb is a tumor suppressor whose underexpression represents an independent predictor of unfavorable prognosis (33). Loss of Numb expression leads to EMT and to the emergence of CSCs (34), effects that can be mechanistically linked to at least three "points of action." First, by controlling endocytosis/recycling of E-cadherin and the proper localization of the Par3 complex, Numb regulates epithelial polarity, adherens junctions, and tight junctions (35). Second, Numb antagonizes the signaling receptor Notch by controlling its endocytosis and trafficking (36). Loss of Numb leads to unchecked Notch activity, which in cooperation with other pathways may lead to EMT (reviewed in ref. 37). Finally, Numb stabilizes p53 (33). This latter activity is particularly interesting: Numb binds to and inhibits the ubiquitin ligase Mdm2, which in turn, is responsible for p53 ubiquitination and proteasomal degradation. Thus, loss of Numb results in decreased levels and activity of p53 (33). This effect of Numb on p53 stability is likely to be highly relevant to cancer, as p53 ablation in *in vivo*

model systems leads to expansion of the mammary stem cell compartment accompanied with the emergence of CSCs (38). Indeed, the effects of loss of Numb on CSCs and EMT are mediated by loss of p53 protein (34). It is, however, unclear whether the control of Numb over p53 is linked to its function in membrane trafficking, a possibility that warrants investigation especially in light of the connection between p53 subversion and the RCP-mediated endocytic/trafficking pathways in cancer.

Rab2A is amplified and overexpressed in breast cancer (39, 40), and it represents an independent predictor of metastasis (40). Rab2A controls ER-to-Golgi transport (41); however, it is also present on late endosomes, where it interacts with VSP39 (40, 42), a component of the homotypic fusion and vacuole protein sorting complex (HOPS) that is responsible for fusion of late endosomes with lysosomes (43). Not surprisingly, therefore, hyperactivation of Rab2A in breast cancer cells affects more than one trafficking step. On late endosomes, it stimulates VSP39-dependent postendocytic recycling of the metalloprotease MT1-MMP to the PM, promoting matrix degradation. At the Golgi, it delays E-cadherin trafficking to the cell surface, reducing junctional stability and cell compaction. The resulting combined effect is increased cellular invasiveness (40). The acquisition of an invasive phenotype by Rab2A-overexpressing cells might be, however, part of a more complex program, connected with EMT. Indeed, increased Rab2A expression leads to the acquisition of EMT traits and to the expansion of the CSC compartment in mammary model systems (39). This function is mediated by prolongation of signaling by ERK1/2, to which Rab2A binds and protects from deactivation, and is controlled by the prolyl isomerase Pin1 that increases the transcription of Rab2 through yet unknown mechanisms. Interestingly, Pin1 is itself overexpressed in breast cancer and controls the normal and neoplastic stem cell compartment (44). Finally, Numb- and Rab2A-controlled pathways might converge in the fine-tuning of cellular responses leading to EMT, as it has been shown that Pin1 and Notch are involved in a feed-forward loop that potentiates the level and activity of both proteins (45).

Outlook: An Endo/Exocytosis-Centered Strategy toward a Metastasis-Specific Therapy

In this review, we focused on trafficking proteins and pathways altered with reasonable frequency and with a reasonable degree of confidence in human tumors. The goal was to distill from a vast amount of literature paradigmatic examples of clinical interest, especially in the perspective of targeted therapies.

At the biological level, as discussed, cancer-altered endo/exocytic proteins seem to be preferentially associated with prometastatic phenotypes, most notably increased migration/aggressiveness, rather than with "canonical" hyperproliferative phenotypes. Mechanistically, the prometastatic phenotypes are mainly caused by alterations in the delivery of critical effectors to the PM, including adhesion molecules (integrins and cadherins), metalloproteases, and regulators of actin dynamics (such as Rac1). These observations uphold the emerging concept of endocytosis/exocytosis cycles acting to maintain homeostatic levels of PM proteins and, when needed, to rapidly deliver cargoes to regions of the PM where polarized functions must occur (1). At the same time, the appearance of invasive properties is frequently accom-

panied by other phenotypes related to survival and resistance to environmental stresses, which can also be considered part of the "metastatic toolkit."

One important question is whether endo/exocytic proteins represent viable targets for the development of molecular therapies. This possibility could be actualized through two major strategies. On one hand, endo/exocytic proteins directly involved in cancer might be targetable in themselves as suggested by the successful identification of specific inhibitors of the 5'-phosphatase activity of SYNJ2 that prevent *in vitro* cell invasion (13). On the other hand, the trafficking "signature" of some cancers could be used to stratify patients and optimize therapies based on the unique characteristics conferred by endo/exocytic alterations to cancer cells. The proof of principle of this approach is represented by the drug responsiveness of colorectal cancer cells overexpressing Rab1A. As discussed, these cells are addicted to amino acid-induced mTORC1 activity (28). As a result, xenografts established from Rab1A-high colorectal cancer cells were highly sensitive to the mTORC1 inhibitor rapamycin, whereas Rab1A-low colorectal cancer cells were not (28).

Finally, the emerging connection between alteration of endo/exocytosis and induction of EMT/emergence of CSCs also harbors therapeutic implications. In recent years, considerable effort has been directed toward high-throughput profiling of human tumors. One of the most unexpected findings that emerged from these studies is that some tumors are metastatically "imprinted" *ab initio* (46), an idea that contrasted the "canonical" view of tumor progression in which the emergence of metastatic subpopulations represents a late event in the natural history of the tumor. Nevertheless, these results received substantial molecular confirmation from studies showing how EMT can give rise to CSCs, which fuel both tumor growth (due to their self-renewal ability) and metastasis (due to their migratory/invasive ability; ref. 30). An intimate link between migration and tumor growth was proposed recently on the basis of theoretical modeling showing that cellular dispersal and turnover can account for potent selective advantages within a tumor mass (47). The combined analysis of these findings argues for a paramount role of migration in the natural history of the tumor, both in its primary location and in its metastatic ramifications. In this framework, endo/exocytic proteins, because of their ability to network circuitries controlling migration, polarity, proliferation, and survival, appear to be uniquely posited to represent interesting targets for anti-CSC and anti-metastatic therapies.

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