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Review Article

I told you to stop: obscurin's role in epithelial cell migration

Kamrin D. Shultz, Yasmin F. Al Anbari and Nathan T. Wright

Department of Chemistry and Biochemistry, James Madison University, 901 Carrier Dr., Harrisonburg, VA 22807, U.S.A.

Correspondence: Nathan T. Wright (wrightnt@jmu.edu)

The giant cytoskeletal protein obscurin contains multiple cell signaling domains that influence cell migration. Here, we follow each of these pathways, examine how these pathways modulate epithelial cell migration, and discuss the cross-talk between these pathways. Specifically, obscurin uses its PH domain to inhibit phosphoinositide-3-kinase (PI3K)-dependent migration and its RhoGEF domain to activate RhoA and slow cell migration. While obscurin's effect on the PI3K pathway agrees with the literature, obscurin's effect on the RhoA pathway runs counter to most other RhoA effectors, whose activation tends to lead to enhanced motility. Obscurin also phosphorylates cadherins, and this may also influence cell motility. When taken together, obscurin's ability to modulate three independent cell migration pathways is likely why obscurin knockout cells experience enhanced epithelial to mesenchymal transition, and why obscurin is a frequently mutated gene in several types of cancer.

Introduction

Epithelial cell migration is utilized by the body in such diverse events as organ development, normal tissue regeneration, and wound healing [1–4]. During these processes, epithelia partially revert to less differentiated mesenchymal cells, allowing for more efficient migration [1]. This decision and subsequent action is complex, involving the coordination of dozens of independent pathways, and is tightly regulated by a wide variety of cellular factors [5–7]. While there are many kinds of single cell and collective cell migration, this review focuses on cell migration associated with cells that undergo a specific progression towards cancer called epithelial to mesenchymal transition (EMT) [5,7,8]. For these cells to achieve tumor formation, invasiveness, and metastasis, they hijack and dysregulate normal cellular migration pathways. Stated more precisely, precancerous epithelial cells that reflect reversible changes in behavior due to phenotypic plasticity, rather than simply a loss of differentiation, and also become more motile are described as going through EMT [5,7,8].

Obscurin, cancer, and cell migration

The giant cytoskeletal protein obscurin has recently emerged as a central player in controlling epithelial cell migration [9–13]. This is an unexpected development; obscurin was only discovered in the early 2000s, and initially was thought to primarily be involved in sarcomeric organization [14,15]. Obscurin derived its name due to the early difficulties in working with it — obscurin mRNA requires 100% DMSO to unfold it and the protein was originally extremely difficult to solubilize [16]. In addition, the protein is often expressed in low levels, is large (up to 870 kDa), has multiple isoforms, and initially had poor antibody reactivity [10]. These technical hurdles made it an unappealing candidate for most exploratory works. However in 2006 obscurin was linked to cancer when a meta-analysis of breast and colorectal cancers identified obscurin as the second most-mutated gene, behind p53 [17]. This same study also cataloged multiple obscurin mutations resulting in premature stop codons, thereby truncating the obscurin protein so that only the non-signaling N-terminus is expressed in these cancers. Subsequent studies revealed that adding obscurin back to breast cancer cells lacking

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obscurin suppresses cancer progression in mice [12,13]. The emergence of this promising anti-cancer application underscores the need to answer two fundamental questions about obscurin: what is obscurin's normal role in epithelial-derived cells, and relatedly why does obscurin ablation lead to cancer?

In muscle, the N-terminus of obscurin binds to the giant sarcoplasmic organizer titin and the C-terminus binds to the sarcoplasmic reticulum via an interaction with ankyrin-G, -B, and small ankyrin-1 [15,18–20]. It is the only protein known to link the contractile apparatus to the surrounding membrane system, and it tends to be distributed in a ribbon-like pattern near the M-disk and Z-line [21,22]. Obscurin's tandem repeat architecture allows it to physically link various subcellular structures together, which helps in sarcomeric organization and function [23–26]. As one would expect, disruption of obscurin dysregulates both sarcomeric organization and normal muscle contraction. In nonmuscle tissues obscurin is often expressed at lower levels, and can localize cytosolically, nuclearly, at the membrane, and at distinct intracellular structures, depending on the cell type [10,27]. The reason for this complex localization pattern is not understood; neither titin nor the same isoforms of ankyrins are expressed in epithelial cells, and so it seems unlikely this giant protein organizes the cytoskeleton in the same way it does in myocytes [28,29]. On the other hand the obscurin localization pattern suggests that it must have many epithelial cell targets, however there have been no colocalization studies on the subject [30]. The obscurin C-terminal region contains multiple signaling domains interspersed among the cytoskeletal structural domains (Figure 1) [23,27]. In particular, the obscurin RhoGEF domain activates RhoA, and the PH domain inactivates phosphoinositide-3-kinase (PI3K) [31–33]. Both of these targets are important for epithelial cell homeostasis and movement [34,35]. In addition, the first of the two kinase domains in the obscurin B isoform, denoted SK1, specifically phosphorylates N-cadherin [36,37].

As an overview, epithelial cells migrate by extending lamellipodia (sheet-like projections) and/or filopodia (narrow cellular protrusions) at their leading edge [1]. These extensions are created and supported by a mesh of cross-linked and polymerizing actin filaments [38,39]. As these filaments push the membrane outward, transmembrane molecules such as integrins and cadherins fasten the leading edge of the cell to the surface below [40]. Simultaneously, the adhering molecules at the lagging edge of the cell are removed from the

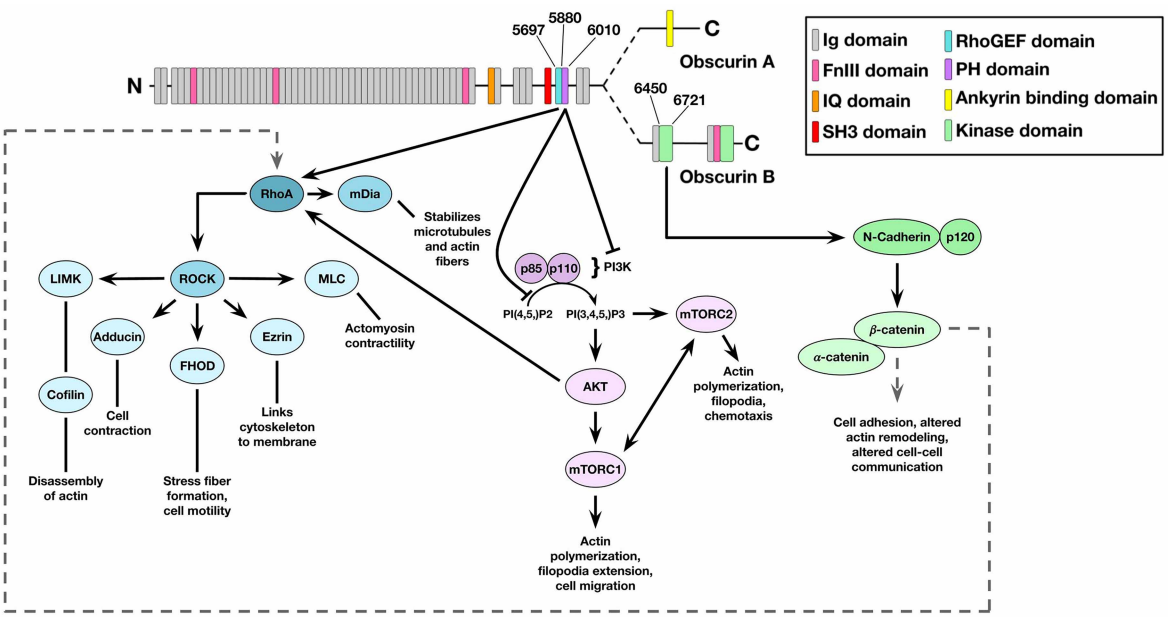

Figure 1. Obscurin signaling inhibits motility-related pathways.

Obscurin modulates RhoA activity through its RhoGEF domain (residues 5697–5880; accession number CAC44768), inhibits PI3K through interaction with the PH domain (residues 5880–6010), and phosphorylates cadherin through its first kinase domain in the Obscurin B isoform (residues 6450–6721; accession number NP_001092093). The obscurin-RhoA pathway leads to decreased cell motility, in contrast with how many other RhoA effectors act. The obscurin inhibition of PI3K also leads to decreased cell migration. The obscurin-cadherin pathway is only fully described in myocytes, and potentially links into RhoA signaling.

membrane through either protease cleavage or internalization, and the lagging edge retracts towards the rest of cell via actomyosin contraction [41,42]. Each of these processes involves input from multiple upstream signals. For instance, RhoA promotes actin stress fibers and actomyosin contraction at the lagging strand, but in lower levels also promotes actin polymerization at the leading strand [43]. In a partially parallel pathway, PI3K also stimulates actin polymerization at the leading edge [44–46].

Since RhoA, PI3K, and cadherin are all involved in cell adhesion and migration signaling, obscurin is potentially involved in three separate pathways that control cell motility [47–49]. Obscurin is not known to interact with any other oncoproteins, and thus this connection to various migration pathways is an attractive mechanism to explain how obscurin links to cancer. In the following sections we will delineate each of these pathways with an emphasis on examining obscurin's role in controlling cell migration.

RhoA

The obscurin Rho-GEF domain activates the RhoA/ROCK pathway in skeletal muscle [33]. In epithelial cells, knockdown of obscurin decreases RhoA and ROCK activity, and this leads to increased cell migration [12,31,50]. This relationship is different from almost all other RhoA signaling pathways; normally a decrease in RhoA activation leads to decreased epithelial cell migration [39,51–53]. Given this surprising and paradoxical relationship, it is worthwhile examining the normal RhoA pathway, obscurin's relationship to this pathway, and why obscurin may act the way it does.

Like other small GTPases, RhoA only binds to and activates its downstream targets while also bound to GTP [54–57]. While RhoA possess the ability to slowly hydrolyze GTP into GDP [58], the predominant RhoA GTP hydrolysis mechanism and subsequence GDP replacement for a new GTP molecule involves the use of GTPase-Activating Proteins (GAPs) and guanine nucleotide exchange factors (GEFs) [59,60]. Select GEFs bind to RhoA and facilitate the exchange of GDP for GTP, leading to a conformational change that allows direct RhoA binding to its downstream effectors [61]. GAPs bind to RhoA and stimulate more efficient GTP hydrolysis into GDP, thus returning RhoA to its original conformation and deactivating the molecule [62,63]. There are over 20 GEFs and GAPs that regulate RhoA activity, and many are associated with cell motility [60]. RhoA activity can also be modulated by phosphorylation at Ser-26 and Ser-188 and ubiquitination at Lys-6 and Lys-162 [64–66]. Both of these posttranslational modifications inhibit RhoA activity.

RhoA's ability to affect cell migration is nuanced. Moderate early RhoA activation near the leading edge of a migrating cell promotes membrane ruffling and lamellae formation (Figure 2) [43,67–70]. Higher RhoA activation later in migration at the trailing edge of the cell promotes actin stress fiber formation and enhances actin/myosin contraction [71–73]. Both processes are vital for migration, and changing the amount, timing, and location of RhoA activation in migrating epithelia can dramatically slow down migrating cells; both constitutively active RhoA and complete inhibition of RhoA through ROCK-inhibiting drugs leads to decreased epithelial cell migration [74,75]. In the former case, the lack of migration is caused by too many stable and immovable stress fibers, a loss of cell polarization, and increased focal adhesions, all of which act to freeze the migrating cell

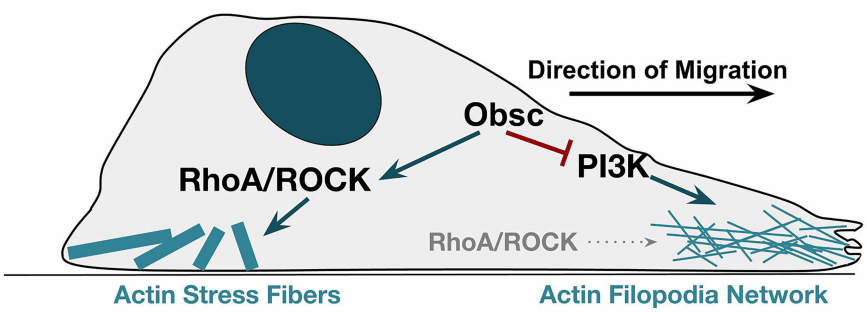

Figure 2. Spatiotemporal effects of obscurin targets on epithelial cell migration.

Increased RhoA/ROCK at the lagging cell edge leads to stress fiber formation and actomyosin contraction, while more moderate and earlier RhoA/ROCK activation proximal to the leading edge leads to actin filament stabilization, membrane ruffling, and lamellipodia extension. At the leading edge, PI3K activation also contributes to increased F-actin filopodia stability and increased cell motility, and obscurin blocks this activation.

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[75]. In the latter case, the lack of central stress fibers and actomyosin contraction likely prevents efficient force production and keeps the cells tethered to one location [74].

Obscurin's effect on cell motility shows how subtle alterations in RhoA activity influence cell migration. Since obscurin is one of many RhoA effectors, knockdown of either all of obscurin or just the RhoGEF domain does not completely ablate RhoA function, but instead merely dampens activity [31]. Either this amount of dampening, or the obscurin-dependent subcellular location of decreased RhoA activity, results in markedly increased cell migration [9,11,31]. This is in contrast with almost all other RhoA activators, whose activity tends to stimulate motility across a wide range of cell types, and whose down-regulation decreases RhoA activity and leads to a decrease in cell migration [76–81]. Other than obscurin, there are only a handful of specific instances where the down-regulation of a RhoA activator results in enhanced cellular migration [82,83]. While obscurin-mediated migration inhibition is linked to changes in actin dynamics [11,50], these other RhoA-linked migration inhibitors are associated with changes in microtubule regulation. The exact reason for obscurin's unique role in suppressing cell migration remains unknown, and the molecular mechanism of how obscurin uses RhoA to suppress cell migration is a current area of active research.

While RhoA has multiple downstream effectors that are involved in migration pathways, arguably the two most important are ROCK and mDia [43,84]. mDia is a formin protein that negatively autoregulates its own activity [85]. RhoA binds to mDia and releases this autoinhibition, allowing mDia to directly bind to and stabilize both microtubules and actin filaments [85–88]. In the context of cell migration, mDia accelerates actin fiber growth and actin nucleation, and plays a role in both filopodia formation and adherens junction stabilization [89]. Stress fiber formation requires both mDia and ROCK; neither protein, on its own, is sufficient to initiate this change in the cytoskeleton [43,90]. Given mDia's role in stress fiber formation, and given obscurin ability to both activate RhoA and stimulate stress fiber formation, it is likely that obscurin indirectly modulates mDia activity [11,31].

ROCK1/2 (Rho-associated kinase) are dimeric kinases that are activated upon RhoA binding to their C-terminal Rho-binding PH domain [91]. ROCK modulates the activity of dozens of downstream effectors via phosphorylation [92,93]. Here we will primarily examine how ROCK affects the actin cytoskeleton, but it also acts to destabilize microtubules via interactions with Map2, CRMP2, and Doublecortin, destabilize intermediate filaments via interactions with GFAP, vimentin, and NF-L, and control NO signaling, to name just some of its many roles [94–100]. There are over 1000 review articles on the RhoA/ROCK pathway; this minireview is only examining a small subset of these in relation to obscurin signaling. A more complete list of ROCK targets can be found in multiple other reviews including [93,101].

Since obscurin activates RhoA, it also indirectly activates ROCK. In the context of migration and changes in cytoskeletal dynamics, activated ROCK phosphorylates LIM kinase, which phosphorylates cofilin thereby inhibiting cofilin-mediated disassembly of F-actin [43]. ROCK also phosphorylates adducin, which stabilizes the actin-spectrin interaction near the plasma membrane and enhances cell contraction [102]. ROCK's activation of FHOD, another formin protein that is up-regulated in EMT, leads directly to stress fiber formation and increases cell motility [103,104]. ROCK activates ezrin, part of the ERM protein complex, that directly links the actin cytoskeleton to the plasma membrane [105,106]. On the other side of the actomyosin complex, ROCK both phosphorylates myosin light chain (MLC) and inhibits MLC phosphorylase through multiple pathways, which serves to amplify actin/myosin contraction [107,108]. As would be expected given these multiple pathways, chemical inhibition of ROCK inhibits cell migration. The two most common ROCK inhibitors are Y27432 and fasudil, and both are used extensively to study ROCK-associated pathways despite also having off-target effects on migrating cells [109]. Like RhoA, ROCK activation must be spatiotemporally regulated; for instance, the presence of stress fibers and actomyosin contraction at the leading edge stops migration [43]. Studies on how obscurin influences ROCK have suggested that while both unchecked ROCK activation or deactivation prevents cell motility, intermediate obscurin-dependent modulation of ROCK activity can actually lead to an increased rate of cell migration in some circumstances [50]. These studies suggest that attention to RhoA and ROCK subcellular localization, instead of simple western blot analysis on whole-cell activity, may resolve the discrepancies of how obscurin-dependent ROCK signaling fits into these otherwise well-studied pathways.

PI3K

PI3Ks are enzymes that phosphorylate the 3 carbon of the inositol headgroup in phosphatidylinositol 4,5 bisphosphate (PIP2), converting PI(4,5)P2 into phosphatidylinositol 3,4,5 trisphosphate (PIP3) [110]. Although

this phospholipid makes up <1% of the cellular plasma membrane, it is enriched in the inner membrane and in lipid rafts, and is linked to efficient membrane-associated signaling [111]. PI3K consists of a regulatory domain, most commonly p85, and a catalytic domain, most commonly p110 [112]. The p85 SH3 domain binds to the obscurin PH domain, and this inhibits PI3K catalysis [32]. The obscurin PH domain also preferentially binds to PIP2 [113]. This acts to sequester PIP2 away from PI3K and inhibits the formation of PIP3. Thus, the obscurin PH domain utilizes two independent mechanisms to inhibit PIP3 production.

Via decreased PIP3 levels, the obscurin PH domain inhibits the AKT/mTOR pathway [114–116]. Under normal circumstances in epithelia, PIP3 recruits AKT to the plasma membrane, where it is phosphorylated by PDK, among others [117,118]. Activated AKT effectively activates mTORC1 [119]. This multifunctional complex consists of multiple proteins including mTOR, Rictor, SIN1, mLST8, and others, and is involved in many cell signaling cascades, but for the purposes of this review is associated with increased actin polymerization, filopodia extension, and increasing cell migration in epithelia [44–46]. Obscurin's ability to functionally repress mTORC1 may partially explain why obscurin down-regulation is associated with increased epithelial migration and other EMT phenotypes. AKT also activates RhoA by down-regulating the RhoA GAP DLC [120]. Thus, obscurin has the ability to both up-regulate RhoA activation through its RhoGEF domain and to indirectly down-regulate RhoA through its PH domain. Other than this and a handful of other examples, there is scant evidence of robust PI3K-RhoA pathway cross-talk [121]. Thus while many of the downstream actions are similar, these two pathways seem to largely act independently of each other. One outstanding question is how the cell synthesizes and incorporates information from these competing pathways to regulate migration.

Both PIP3 and the mTORC1 complex also activate the mTORC2 complex [122,123]. mTORC2 stimulates actin polymerization, leads to more cell protrusions and filopodia, and activates migration both in neutrophils and in gliomas [124–126]. This is brought about by mTORC2-dependent increases in actin polymerization and RhoGTPase activity [127]. Again, these downstream phenotypes align with changes in obscurin expression; obscurin can down-regulate mTORC2, leading to slower cell migration. Conversely, obscurin down-regulation is predicted to up-regulate mTORC2 activation, and this possibly accounts for some of the observed increase in cell motility.

Cadherin

Potentially one additional obscurin-mediated migration involves the kinase domain at the C-terminus of the obscurin B isoform. In skeletal muscles, this kinase (SK1) phosphorylates N-Cadherin at Ser-788, which disrupts the binding between N-cadherin and p120-catenin at the intercalated disk [36,37]. This disengages p120-catenin from the cadherin/catenin/actin complex and allows p120-catenin to interact with and down-regulate RhoA. In muscle, this series of events is associated with increased cell adhesion, altered actin remodeling, and altered cell-cell communication [37,128]. Normally epithelial cells do not express N-cadherin, but cells undergoing EMT switch from E- to N-cadherin, and this switch is associated with increased cell motility [129,130]. While obscurin-dependent phosphorylation of N-cadherin has only been shown in myocytes, the rest of this molecular mechanism, including N-cadherin phosphorylation, p120 dissociation, and the resulting increased migration, is known to occur in melanoma [30,130]. Since this hypothetical pathway feeds into the RhoA pathway, discussed above, it is possible that this could be another way for obscurin to modulate RhoA activity [31,50]. However, given the unique relationship between obscurin and the RhoA pathway, more studies are needed examining this still-hypothetical pathway's effect on cell migration.

Concluding remarks

Obscurin co-ordinates multiple independent motility pathways to signal epithelial cells to stop moving. This is driven through a RhoA interaction with the obscurin RhoGEF domain, a PI3K interaction with the obscurin PH domain, and perhaps through a cadherin phosphorylation via the SK1 domain in obscurin B. The cellular context of these downstream effectors matter; each of these independent pathways have the hypothetical capacity to either increase or decrease cell migration, depending on spatiotemporal factors. Likely due to these nuances, multiple studies now show that obscurin knockout and knockdown in epithelial cells leads to EMT and conversely that the addition of obscurin into epithelial-derived cancer cells could be a potential cancer treatment [13]; this is paradoxical to how many other RhoA effectors function but generally agrees with other PI3K signaling pathways. Many questions remain. Why is obscurin signaling so different from other RhoGEF signals? What upstream molecules regulate obscurin function? What role, if any, do the rest of the tandem structural domains play in regulating obscurin localization and function in epithelial-derived cells? Does

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obscurin co-ordinate these motility pathways or is each pathway independent? Is there additional cross-talk between these signaling pathways? The obscurin community is actively researching these topics; several recent papers describe proteins that phosphorylate obscurin and how this influences obscurin function [36,131,132]. Answering these questions will allow further exploration not only of the basic biology of how cells move, but may also lead to new anticancer applications that take advantage of these surprising obscurin-influenced pathways.

Perspectives

- Obscurin is a giant cytoskeletal protein that has recently been implicated in cell motility and cancer progression.
- Obscurin contains at least three signaling domains- A RhoGEF domain, a PH domain, and a kinase domain- that feed into various motility pathways and signal cells to stop moving.
- Future work will center on studying the molecular mechanisms of how obscurin fits into various motility pathways, disentangling the cross-talk between these pathways, and delineating what role the obscurin structural domains play in regulating motility.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

K.D.S. wrote, edited, and made the figures. Y.F.A. edited the manuscript. N.T.W. wrote, edited, secured funding, and proposed the idea.

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Abbreviations

EMT, epithelial to mesenchymal transition; GAP, GTPase-Activating Protein; GEF, guanine nucleotide exchange factor; MLC, myosin light chain; PI3K, phosphoinositide-3-kinase.

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