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Review

Mitotic Moonlighting Functions for Membrane Trafficking Proteins

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In recent years, cell biologists have uncovered a number of new functions for proteins that were previously thought to operate solely in membrane trafficking. These alternative roles, termed moonlighting functions, can occur at distinct intracellular sites or at different stages of the cell cycle. Here, I evaluate the evidence for mitotic moonlighting functions of proteins that have membrane trafficking roles during interphase. The aim is to identify key issues facing the field and to outline important questions for future work.

Key words: clathrin, cytokinesis, dynamin, endocytosis, mitosis, mitotic spindle, moonlighting

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The term 'moonlighting' was introduced to describe proteins that have two or more unrelated functions which are not due to gene fusions or alternative splicing (1). In membrane trafficking circles, the term was first used by Di Fiore to describe the nucleocytoplasmic shuttling of proteins involved in endocytosis (2). Over the last few years, there have been numerous examples of proteins with membrane trafficking functions that have been found to have discrete functions. These moonlighting functions occur either in a distinct cellular location or during a different phase of the cell cycle. The aim of this review is to appraise critically the moonlighting functions of proteins that were initially characterized for their involvement in membrane trafficking. There have been several excellent reviews that cover comprehensively the moonlighting roles of endocytic adaptors in the nucleus during interphase (3,4). The emphasis here is on moonlighting roles that occur during mitosis – a time of reduced membrane traffic. Table 1 highlights some of the examples of proteins that have alternative mitotic functions. These include core components of the endocytic machinery: clathrin and dynamin, as well as more peripheral trafficking proteins. We will look at several examples in detail from this table in an attempt to identify key issues and important questions facing the field.

Clathrin

A number of early studies pointed to a moonlighting function for clathrin during mitosis (31). For example, Maro et al. showed that clathrin is found on the spindle apparatus in mitotic and meiotic cells (32) (Figure 1). These early studies (32,33) were revisited and confirmed by Okamoto et al. (34). In addition, clathrin was found in a gene trap as a spindle protein (35) and in a mass spectrometry screen of spindle proteins in assembled asters (36). The advent of RNAi led to the uncovering of a function for clathrin at the mitotic spindle (6). Clathrin depletion destabilizes kinetochore fibers (K-fibers) of the mitotic spindle. K-fibers are bundles of 20–40 microtubules (MTs) that connect the kinetochore to the spindle pole. As a result, they are responsible for much of the chromosome movement during mitosis. The destabilized K-fibers in clathrin-depleted cells cannot congress chromosomes efficiently, resulting in a mitotic delay due to a functional spindle checkpoint (6).

Importantly, this mitotic function of clathrin is independent of its role in membrane trafficking; membranes and adaptors are not found together with clathrin at the spindle and electron microscopy showed immunogold labeling near MTs and away from membranes (6). In fact, the spindle itself is devoid of membranes; endoplasmic reticulum (ER) and nuclear envelope (37), Golgi (38) and endocytic vesicles (39) are excluded from the region that contains the mitotic spindle. Membranes can be visualized around the spindle (17,40) and this membraneous matrix has been proposed to be important for spindle function (see below). Good evidence for the independence of clathrin's roles in membrane trafficking and mitosis comes from the demonstration that the functions of clathrin in endocytosis and mitosis can be separated by various clathrin mutants that can do one function and not the other (41–43). Depletion of clathrin by RNAi interferes with both endocytosis and mitosis (6). Re-expression of full-length clathrin heavy chain (CHC) in clathrin-depleted cells rescues both functions, whereas the re-expression of (i) CHC lacking residues 457–507 rescues endocytosis but not mitosis (42) (ii) a dimeric CHC–TFE3 protein rescues mitosis but not endocytosis (41). These observations indicate that first, the clathrin at the mitotic spindle is not a store of clathrin-coated vesicles, and second that simply inhibiting clathrin-mediated membrane traffic does not give rise to mitotic defects non-specifically (as suggested in 30).

Table 1: Moonlighting functions for proteins involved in membrane trafficking

Protein	Membrane trafficking function	Moonlighting function(s)
Clathrin	Coating membranes during trafficking (5)	1. Stabilizing MTs in K-fibers (6) 2. Nuclear function? (7)
Dynamin	Membrane fission (8)	1. Dynamic instability of MTs in interphase (9) 2. Centrosome cohesion (10) 3. Role in cytokinesis (11) 4. Actin polymerization (12)
Rab6A′	Retrograde transport from early endosomes to ER (13)	1. Inactivation of spindle checkpoint (14) 2. Rab6C required for cell cycle progression (15)
Epsin	Generating membrane curvature (16)	Organizing mitotic membranes/influencing spindle assembly (17)
ARH (LDLRAP1)	Adaptor for LDL receptor endocytosis (18)	Centrosomal assembly and cytokinesis (19)
GAK/Auxilin 2	Uncoating clathrin-coated vesicles (20)	1. Promotes MT outgrowth during spindle assembly (21) 2. Centrosome cohesion (22)
Intersectin (ITSN2)	Involved in clathrin-mediated endocytosis (CME) (23)	Orienting mitotic spindle via cdc42 GEF activity (24)
ZW10	Trafficking between the ER and Golgi (25)	Kinetochore component, termination of the spindle checkpoint (26)
RLIP76 (RALBP1)	Binding partner of AP-2, epsin and Numb in interphase (27)	Mitotic spindle assembly (28)
AP-2	Adaptor for CME (5)	1. Mitosis? Interaction with BubR1 (29) 2. RNAi gives mitotic phenotype (30)

How does clathrin stabilize K-fibers? On the basis of the triskelion structure of clathrin and the finding that the N-terminal domains of CHC are required for spindle-binding, it was proposed that clathrin could act as an inter-MT bridge to physically cross-link MTs within the K-fiber (6). The bridge hypothesis has been tested indirectly by replacing endogenous CHC with CHCs that cannot form bridges (43). These experiments agreed with a bridging function for clathrin but a more direct test was required. Using electron microscopy, Booth et al. found recently that clathrin depletion results in the loss of inter-MT bridges from K-fibers. Moreover, inter-MT bridges are labeled by anticlathrin immunogold (44). This suggests that clathrin is indeed an inter-MT bridge and therefore stabilizes K-fibers by physically tethering adjacent MTs. But how does clathrin associate with the spindle? As far as we know, clathrin cannot bind MTs directly and therefore clathrin would require some kind of adaptor to bind MTs. Two candidates have been proposed to fill this function, B-Myb (45) and cyclin-G associated kinase (GAK) (21). However, neither of these proteins is associated with MTs (46) and so, while it is possible that they can modulate clathrin binding to the spindle, they are unlikely to mediate this binding event. A more likely candidate is transforming acidic coiled-coil protein 3 (TACC3). A number of groups have reported that CHC can bind to the phosphorylated form of TACC3 (47–49). Although, these studies concluded that it is clathrin that localizes TACC3 to the spindle (47–49), it seems that the converse may be true. First, TACC3 is a known MT-associated protein and clathrin is not (47). Second, only a subset of clathrin is targeted to the mitotic spindle with the remainder in clathrin-coated structures, whereas TACC3 is almost entirely bound to the spindle. To address this paradox, Booth et al. purified the spindle clathrin complex and found that clathrin is associated with TACC3 and colonic, hepatic tumor overexpressed gene (ch-TOG) (44). Overexpression and RNAi analysis indicated that TACC3 and ch-TOG act as the adaptors for clathrin to bind MTs, and not the other way around. Clathrin binding to TACC3 and ch-TOG appears to act as a lock to stabilize the complex on adjacent MTs (44).

The mitotic function of clathrin has been confirmed in a number of different systems: human (6), mouse (45,50), rat (6), pig (51), frog (47) and plant (52). The CHC paralog found on chromosome 22 in humans can also function in mitosis, but only when overexpressed in cells depleted of endogenous CHC (42). In normal cell lines, however, the more highly expressed CHC dominates at the spindle, over the paralog (53). An apoptosis-resistant variant of DT40 chicken B-cells showed only very mild mitotic defects following CHC removal (54). It is unclear whether the moonlighting function of clathrin is not conserved in birds or if a compensatory mechanism is at work in this unusual cell line. The conservation of clathrin's mitotic role in lower eukaryotes has yet to be determined. For example, in *Caenorhabditis elegans*, clathrin (T20G5.1) removal in oocytes results in a 'jumpy' phenotype (55), but it is unclear whether or not this is a result of aberrant mitotic spindle division. It remains an open question as to when the moonlighting function of clathrin arose. The

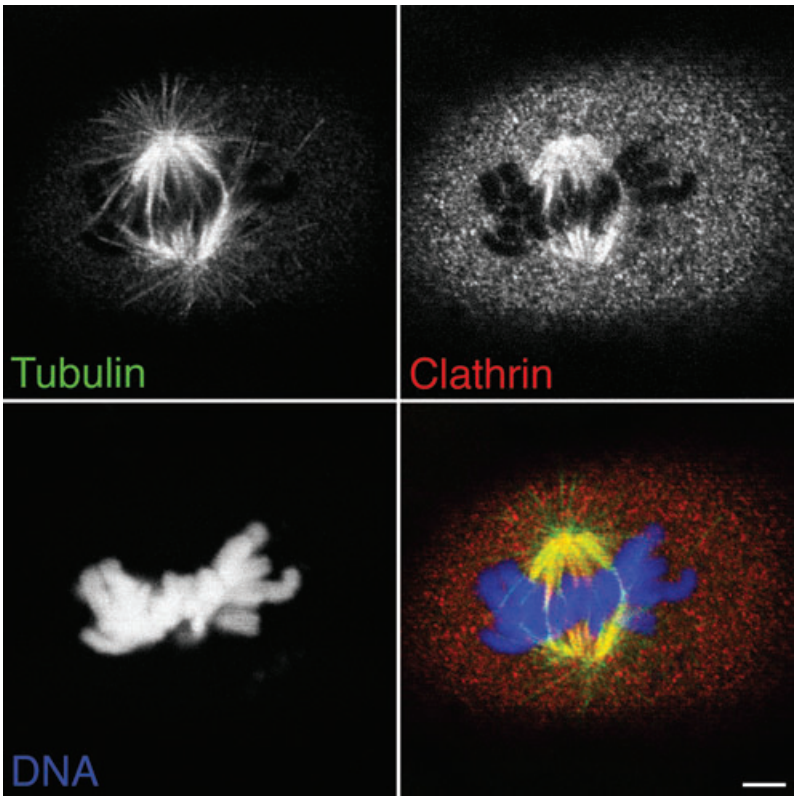

Figure 1: Moonlighting function of clathrin. Clathrin binds the mitotic spindle during mitosis. A rat kangaroo (PtK1) cell was fixed and immunostained with antibodies against CHC (red) and beta-tubulin (green). DNA is shown in blue. Scale bar is 10 μm.

need to organize membranes via membrane trafficking is certainly older in evolutionary terms than the requirement for inter-MT bridges in K-fibers. The budding yeast *Sacchromyces cerevisiae* uses single MTs to move its chromosomes and therefore requires no inter-MT bridges in its 'K-fibers' (56), yet clathrin is involved in membrane trafficking in this organism (57). Perhaps as centromeres and kinetochores increased in size and the number of MTs increased, a need for inter-MT bridges arose. Interestingly, the fission yeast *Schizosacchromyces pombe* has multiple MTs per K-fiber and also inter-MT bridges (58). Clathrin and ch-TOG are both well conserved in eukaryotes, yet a clear TACC3 homolog is absent in *S. cerevisiae*, but present in *S. pombe* (Mia1p/Alp7). This protein has cross-linking activity (59), and it will be interesting to determine how TACC3/ch–TOG/clathrin inter-MT bridges are conserved in lower organisms.

Unrelated to mitosis, but relevant here is an additional moonlighting function for clathrin that has been proposed recently. CHC is found in the nucleus where it is thought to enhance p53-mediated transcription (7). The amount of clathrin in the nucleus is very small – within the error of measurement. Others have noted that size isn't everything (3) and that even these low levels appear sufficient to alter p53 transcription. Later work by the same group proposed that monomeric but not trimeric CHC carries out this nuclear function and that the p53 binding site on CHC overlaps with that for clathrin light chain (60,61). This idea is rather controversial. Monomeric clathrin is not thought to exist in cells as CHC is an obligate trimer (62). In addition, clathrin light chains are tightly associated with CHC (63) and light chain-free clathrin is not thought to exist in cells (64). Finally, the same group have also suggested that p53 can modulate CME (65). On balance, these findings need to be confirmed and extended by others before we can consider another moonlighting function for clathrin that occurs in the nucleus.

Dynamin

Dynamins are a family of large GTPases involved in membrane scission and remodeling (8). Interestingly, dynamin was originally identified as a MT-binding protein (66), but clear evidence for its role in membrane fission rapidly eclipsed this initial characterization. Dynamin, like clathrin, is found in proteomic analyses of purified mitotic spindles (46). Dynamin-2 immunoreactivity is localized to the mitotic spindle, but its distribution differs from that of clathrin. Chiefly, it becomes concentrated at the midzone during telophase (11). Dynamin is important for cytokinesis in a variety of organisms (11,67–69). Small molecule inhibitors that target dynamin induce a failure in cytokinesis (70) and there is good evidence for a calcineurin-mediated phosphoregulation of dynamin activity in cytokinesis (71). The intercellular bridge between the two daughter cells at cytokinesis is a site of much membrane remodeling and therefore the extent to which this

moonlighting function of dynamin in cytokinesis is related to its role in membrane scission is a key outstanding question. In addition to the midzone localization, dynamin-2 is also localized to centrosomes and may contribute to centromere cohesion (10). More recently, dynamin-2 has been implicated in modulating MT dynamics during interphase (9). Previously, patients with Charcot–Marie–Tooth disease were found to have mutations in dynamin-2 (72). Tanabe and Takei found that one of these dynamin-2 mutants localized to MTs and caused an increase in the number of acetylated (stable) MTs (9). Knockdown of dynamin-2 reduced both the growth and shortening phases of interphase MTs. It remains to be determined whether this modulation of MT dynamic instability is specific to dynamin-2. Moreover, further work will hopefully uncover whether this function of dynamin at MTs in interphase is related to the previously proposed mitotic functions for dynamin.

An interplay between the actin cytoskeleton and dynamin has also been suggested recently (12). Dynamin-1 can bind directly to actin via a basic patch in the middle of the molecule that is well conserved in other dynamins. The dynamin–actin interaction enhances the polymerization of actin via the generation of free-barbed ends. Importantly, dynamin-1 mutants with altered actin-binding are competent for CME (12) which suggests an independence of this function of dynamin in actin polymerization and in endocytosis. It will be very interesting to determine first, whether these functions remain independent especially in lower species where actin is central to endocytic function (73). Second, does dynamin as an actin-binding protein play a role in actin remodeling that occurs during cytokinesis?

Epsin

Recent work from the Zheng lab indicates that epsin may have a moonlighting function in mitosis (17). Epsin is a protein that can induce membrane curvature *in vitro* and has been proposed to contribute to membrane curvature at the forming clathrin-coated pit (16). Depletion of epsin results in dysregulated spindle poles and also in disorganization of the mitotic ER network. Epsin was shown not to be associated with the mitotic spindle (17), which would have been predicted based on its apparent MT-binding activity *in vitro* (74) and the finding that epsin is a mitotic phosphoprotein (75). The membrane deformation property of the epsin N-terminal homology (ENTH) domain of epsin1 was found to be necessary for spindle function in *Xenopus laevis* egg extracts. These preparations are assembled plasma membrane-free which suggests that the endocytic function of epsin is dispensable for its mitotic function (17). How epsin organizes the mitotic ER network is not clear. The membrane deformation property of epsin1 is thought to rely on interaction with phosphatidylinositol 4,5-bisphosphate (16,76), a phospholipid which is enriched in the plasma membrane rather than the ER. Nonetheless, the authors proposed that epsin is important for the organization of intracellular membranes during mitosis (17) and that these membranes form a web or matrix around the spindle to assist its function (77,78). The existence of a mitotic spindle matrix has been rather controversial (79), if a membraneous matrix does indeed contribute to mitotic spindle function then this would provide a neat explanation for the continued abuttal of membrane trafficking and mitosis.

Autosomal Recessive Hypercholesterolemia

Autosomal recessive hypercholesterolemia protein (ARH) was first characterized as an accessory protein that interacts with adaptor protein complex-2 (AP-2) to mediate the uptake of low-density lipoproteins (LDL) via the LDL receptor (18,80). Recently, ARH was reported to have a moonlighting function during mitosis. ARH was found by immunostaining at the kinetochores, spindle poles and midbody (19). ARH was shown to interact with dynein and fibroblasts from ARH−/− mice had smaller or absent centrosomes. Mitotic defects were observed in these mouse embryonic fibroblasts (MEFs) and also in Rat-1 cells depleted of ARH by RNAi (19). In particular the cells took longer to undergo cytokinesis. From this study it was not clear whether these functions were related to the role of ARH in membrane trafficking, e.g. mediating membrane delivery to the site of cytokinesis, or represent discrete function(s) for ARH at the various sites on the mitotic spindle. It remains to be tested whether other adaptors have similar localizations and functions. Disabled-2, a functional homolog of ARH, does not co-localize with the mitotic machinery (81) suggesting that this moonlighting function is particular to ARH.

Cyclin-G Associated Kinase

GAK was first identified as a serine/threonine kinase that associates with cyclin-G (82). The C-terminal part of GAK has significant homology with auxilin, a protein that is required for the hsc70-dependent uncoating of clathrin-coated vesicles (83). GAK has similar uncoating properties and is now considered as an auxilin homolog, auxilin 2 (84,85). GAK can also bind and phosphorylate adaptor proteins (85), placing it as a key coated-vesicle kinase. Mitotic moonlighting functions for GAK away from membrane trafficking have recently been proposed. GAK-depleted cells have a number of mitotic defects. In the first report, depletion of GAK by RNAi results in an accumulation of cells at prometaphase indicating a problem in spindle organization and chromosome congression (22). In the second report, an RNAi screen of serine/threonine kinases was performed to identify novel regulators of mitosis and GAK was found as the only significant hit (21). Both papers showed that the amount of clathrin at the spindle was reduced and interpreted

the mitotic defects in GAK-depleted cells as being related to the function of clathrin at the spindle (21,22). It is possible that the reduction in clathrin at the spindle may simply reflect the loss of MTs observed in GAK-depleted cells (21) or the reduction in free clathrin caused by the inhibition of vesicle uncoating in GAK-depleted cells (21,86). In addition, GAK is not localized to mitotic spindles, so it is difficult to see how it could directly co-operate with clathrin at the mitotic spindle. Moreover, the mitotic defects in GAK-depleted cells (21,22) are more severe than in clathrin-depleted cells (6), for example the mitotic index is higher following GAK depletion (21,22) and GAK-depleted cells may have fragmented centrosomes (22), which is not obvious in clathrin-depleted cells. These observations suggest that GAK has mitotic moonlighting function(s) some or all of which are independent of clathrin.

Interestingly, loss of GAK function promotes outgrowth of cells in soft agar, a model for tumorigenesis (87). This could be related to deregulated cell division. In addition, GAK is essential in mice: deletion of GAK in developing or mature mice results in lethality (20). It is tempting to speculate that the death of GAK knockout mice, particularly of developing mice is due to perturbation of a putative moonlighting function for GAK. However, MEFs from these animals show a blockade of CME which indicates that there is no compensation for the deletion of GAK and suggests that blockade of CME is the primary cause of lethality (20).

Current Challenges

A major challenge facing the field is to convincingly show that in each case these moonlighting functions are real. It is understandable that the proposal of a new function for a well-studied protein is met with some skepticism. There are several reasons why it is not sufficient to simply deplete a membrane trafficking protein, observe a mitotic defect and conclude that this protein has a moonlighting function. First, mitotic defects are a common off-target effect in RNAi experiments. For example, in a recent RNAi screen for novel mitotic kinases, >10% of the 716 kinases erroneously gave a mitotic defect (21). In addition, the spindle checkpoint protein Mad2 has been reported to be particularly susceptible to an off-target depletion (88,89). Second, mitotic defects could arise not as an off-target effect of RNAi, but as a consequence of altered membrane trafficking. Cells that have been depleted of a membrane trafficking protein are no longer normal. For a typical 72-h knockdown period, the cell must transit through ~3 cell cycles with decreasing amounts of the protein. Other approaches are required to confirm that any mitotic defects observed are due only to the removal of protein from that process. Add-back or rescue experiments are very useful but rely on the design of constructs that can restore one function and not the other. Another way to circumvent this problem is to disrupt the protein on a more rapid timescale. In this way a protein could be targeted at the onset of mitosis and therefore any mitotic problems that might arise non-specifically through the interference of interphase function would be negated. Small molecule inhibitors are ideal, but relatively few are available for membrane trafficking proteins. The recently described 'knock sideways' approach is very promising (90). With this method, proteins can function normally and then be removed from their site of action by targeting them to the mitochondria upon application of rapamycin.

Once a bona fide moonlighting function has been described, it is important to determine whether or not this function is related to its interphase role. There is current evidence for proteins with moonlighting functions that are completely independent (e.g. clathrin) and other proteins with moonlighting functions that are somewhat related to their interphase role (e.g. epsin). The relatedness of membrane trafficking and mitosis needs to be established in order to define the moonlighting function in more detail, but also to test the recent proposal that a membraneous matrix is important for mitotic spindle function (77). One implication of the moonlighting hypothesis is that membrane trafficking proteins are free to have alternative functions because mitosis is a time when membrane trafficking is dramatically reduced (91,92); although the extent of endocytic shutdown has been questioned (30). A key question therefore is whether or not the shutdown is actually caused by the employment of membrane trafficking proteins in moonlighting functions. For example, it has been suggested that mitotic phosphorylation of epsin prevents its association with AP-2, thus blocking endocytosis (75). If this phosphorylation is key to its moonlighting function then the blockade of endocytosis could be due to redeployment of epsin. In the case of clathrin, such a small fraction of the total pool is targeted to the spindle that it is unlikely to be the cause of endocytic shutdown.

If the moonlighting function is not related to membrane trafficking then the next logical question to address is: how do proteins with moonlighting functions switch from one role to the other? One obvious mechanism is phosphorylation by mitotic kinases; especially given the examples we have encountered above. This mechanism is well understood in the regulation of GRASP65, a Golgi stacking factor whose mitotic phosphorylation is key for disassembly of the Golgi at mitotic entry (93,94). However, the answer to this question will likely be different for each moonlighting protein. In the case of clathrin, it seems that the 'switch' in function is due to the availability of the adaptor to put clathrin onto the spindle (44). First, the expression of TACC3 is low during interphase and is only increased in late G2/M-phase and destroyed by cdh1/anaphase promoting complex at the end of anaphase (95). Second, recruitment of clathrin to the spindle by TACC3 is regulated by the phosphorylation of TACC3 by the mitotic kinase aurora-A (44), a modification known to localize TACC3

to MTs (96). Third, the targeting of clathrin to the spindle seems to be specific for K-fibers and these, or bundles like them, do not normally exist in interphase. Any mitotic phosphorylation of clathrin itself (97) is likely to be secondary to these drivers for the alteration of clathrin function.

With so many membrane trafficking proteins having proposed mitotic moonlighting functions, it may appear that membrane trafficking and mitosis are intrinsically linked (98). This may indeed be the case; however, this pairing is neither unique nor exclusive. Looking from the other side, there are proteins that moonlight at the spindle who have roles in interphase that are unrelated to membrane trafficking. For example, some proteins involved in nuclear transport during interphase, such as Nup107, Nup133 (99), Nup358 (100) and RanGAP (101), localize to kinetochores or to the mitotic spindle to ensure orderly mitotic progression (100). Even non-protein components, such as poly(ADP-ribose) and mRNAs, are seemingly drafted into action at the spindle during mitosis (102,103). Undergoing mitosis is arguably the most important thing that a cell does during its lifetime and it could be that the cell mobilizes all of its disparate resources to complete the job.

Outlook

In this review, we have seen that many membrane trafficking proteins have moonlighting functions during mitosis (see also Table 1). How many more await discovery? A recent genome-wide RNAi screen for proteins involved in mitosis suggested that there may be more moonlighting functions for membrane trafficking proteins (104), e.g. AAK1 and AP1M1. This screen actually failed to identify a number of known mitotic proteins, suggesting that the screen was highly stringent and that other moonlighting proteins may have been missed. It does seem that the time is right for 'systems-level' approaches to identify new moonlighting functions (an anathema in a field where the power has rested with detailed study of individual protein function). We appear to be near saturation in terms of understanding all of the molecular components of membrane trafficking pathways and so these lists of proteins can now be tested systematically for potential mitotic moonlighting functions. The next few years promise to be an exciting time for those at the interface between membrane trafficking and cell division.

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