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PERSPECTIVES

OPINION

Protein adaptation: mitotic functions for membrane trafficking proteins

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Abstract | Membrane trafficking and mitosis are two essential processes in eukaryotic cells. Surprisingly, many proteins best known for their role in membrane trafficking have additional 'moonlighting' functions in mitosis. Despite having proteins in common, there is insufficient evidence for a specific connection between these two processes. Instead, these phenomena demonstrate the adaptability of the membrane trafficking machinery that allows its repurposing for different cellular functions.

Understanding cell biology at the molecular level necessitates the careful characterization of protein function. It would be very convenient if one gene resulted in one protein that had one function, as predicated by the concept of one gene–one enzyme[1]. However, cell biology has long been known to be far more complicated. For example, the production of many different proteins from one gene by alternative splicing is now known to be the rule rather than the exception, with 95% of genes undergoing alternative splicing[2]. Moreover, the function of many proteins can be modulated by post-translational modifications such as phosphorylation or ubiquitylation. Adding further complexity to our understanding of protein function, it has become clear during the past 15 years that some proteins — the functions of which were thought to be safely confined within one cellular process — can also work in completely different pathways.

The term 'moonlighting protein' was coined by Constance Jeffery to describe proteins that have multiple roles[3]. The first examples included enzymes such as phosphoglucose isomerase, a cytosolic glycolytic enzyme that can act as an extracellular cytokine when secreted[3]. The list continued to expand and now includes components of the ribosome and proteasome, chaperones, receptors, ion channels and membrane trafficking proteins[4,5].

What constitutes a moonlighting function is debatable. It is clear that moonlighting functions do not include: functions that arise by gene fusion, alternative splicing or post-translational modification; functions of homologous but non-identical proteins; or a single function of a protein that operates in a different cellular location[3]. The most stringent definition requires that the new function is independent of the role originally assigned to the protein. Moreover, it has been proposed that the function that is 'younger' in evolutionary terms should be designated the moonlighting function; that is, the function that is most highly conserved is considered as the core purpose of the protein, and any functions that were acquired later are described as moonlighting[5]. These criteria are used here as a framework for discussion, although the term has been used more loosely in the literature. Whatever the definition, there are so many examples of proteins that feature in more than one cellular process that this behaviour can no longer be considered exceptional[5].

In this Opinion article, I focus on the putative moonlighting functions in mitosis of proteins the canonical function of which is in membrane trafficking. I describe the different ways in which the vesicular coat protein clathrin and other components of the clathrin-mediated endocytosis (CME) machinery are proposed to function during mitosis. Cytokinesis, a process that depends on membrane trafficking, is not considered in this context. I discuss to what extent these alternative functions can be regarded as true moonlighting functions. I also argue that despite the number of trafficking proteins that also influence mitosis, our current knowledge of their canonical and proposed alternative functions does not allow us to draw clear connections between these two cellular processes. Instead, I propose that membrane trafficking proteins are an adaptable set of molecules that makes them well suited to moonlighting and that the suppression of trafficking in mitosis facilitates their repurposing in cell division control itself.

An expanding group

As the cell enters mitosis, there is a marked reorganization of numerous cellular processes. Membrane trafficking and other pathways are suppressed, the cytoskeleton is reorganized and a bipolar mitotic spindle begins to form. Alternative functions during this time for various membrane trafficking proteins have been proposed and are summarized in TABLE 1. Many of these proteins are components of the CME machinery and include factors that act at distinct stages in this process, from nucleation of the clathrin-coated pit to uncoating of the clathrin-coated vesicle (BOX 1). These mitotic functions fall into three broad areas: mitotic spindle, centrosome and mitotic checkpoint (FIG. 1).

Mitotic spindle functions. Clathrin is probably the best-known example of a membrane trafficking protein that has an alternative function during mitosis[6]. Early immunolocalization studies using the first antibodies raised against clathrin revealed a distinct localization of clathrin at the mitotic spindle of dividing cells[7–9]. But an active role for clathrin during mitosis at the spindle was heretical at the time, and it was not until the GFP-tagged version of clathrin revealed a similar localization and mitotic defects in clathrin-depleted cells were described that a potential role for clathrin in mitosis was revisited[10] (for a review, see REF. 6).

Clathrin is recruited to the spindle apparatus at mitotic entry[10]. This pool of clathrin is not associated with membranes[6,10]. Clathrin is important for stabilizing

Table 1 | **Mitotic functions of key membrane trafficking regulators**

Protein*	Membrane trafficking function	Alternative function(s)
Clathrin	Membrane coating during CME and other intracellular transport events[94]	Stabilization of microtubules in kinetochore fibres[10]; Centrosomal integrity[33]
β-arrestin 2	Adaptor for CME of GPCRs[50]	Localizes to centrosome and primary cilium; centrosome function[44,45]
RALBP1	Binds CME components AP2, epsin and NUMB[21]; precise function unclear	Mitotic spindle assembly[23]
ARH	Endocytic adaptor for LDLR[47]	Centrosome assembly and cytokinesis[48]; Mitotic spindle function[49]
Epsin	Polyubiquitin-selective clathrin-associated sorting protein[25]	Organizes mitotic ER membranes and influences spindle assembly[27]
EPS15	CME of EGFR[50]	Organizes pericentriolar material together with epsin[51]
ITSN2	Actin recruitment during CME[19]	Orientates mitotic spindle via CDC42 GEF activity[20]
Dynamin	Membrane scission in CME[34]	Microtubule dynamics in interphase[36]; centrosome cohesion[35]; cytokinesis[37,42,95]; actin polymerization[43]
SNX9	Recruited after scission in CME[93], implicated in actin recruitment during CME	Chromosome alignment during prometaphase[24]
GAK	Uncoating of clathrin-coated vesicles[96]	Promotes microtubule outgrowth during spindle assembly[31]; centrosome cohesion[30]; both functions possibly involve the regulation of clathrin–TACC3–ch-TOG complex[32]
RAB6A′	Retrograde transport from early endosomes to the ER[57]	Inactivation of spindle checkpoint[58]
RAB6C	Function unclear, high sequence identity to RAB6A′ (REF. 52); centrosomal localization in interphase	Localizes to centrosomes; required for cell cycle progression[52]
ZW10	ER–Golgi traffic[55]	Kinetochore localization through the RZZ complex; termination of the spindle checkpoint[54]

ch-TOG, colonic hepatic tumour overexpressed gene; CME, clathrin-mediated endocytosis; EGFR, epidermal growth factor receptor; EPS15, EGFR substrate 15; ER, endoplasmic reticulum; GAK, cyclin G-associated kinase; GEF, guanine nucleotide exchange factor; GPCRs, G protein-coupled receptors; ITSN2, intersectin 2; LDLR, low-density lipoprotein receptor; RALBP1, RALBP1-associated EPS domain-containing protein 1; RZZ, ROD–Zwilch–ZW10; SNX9, sorting nexin 9; TACC3, transforming acidic coiled-coil protein 3; ZW10, Zeste white 10. *CME proteins are listed in approximate temporal order (see BOX 1).

microtubules within the kinetochore fibres (K-fibres) of the mitotic spindle that run from the spindle pole to the kinetochores[10]. It is thought to achieve this by crosslinking microtubules within a K-fibre (FIG. 1e). Each K-fibre comprises 20–40 microtubules, and these are linked via electron-dense bridges[11], some of which contain clathrin[12]. In the absence of clathrin, K-fibres have fewer bridges and fewer microtubules which causes delays and defects in chromosome congression[10,12,13].

By itself, clathrin has no microtubule-binding capacity. Its spindle association is mediated through the formation of a complex with transforming acidic coiled-coil protein 3 (TACC3) and colonic hepatic tumour overexpressed gene (ch-TOG)[12,14–16]. Clathrin heavy chain binds TACC3, an event that is regulated by phosphorylation of TACC3 at Ser558 by Aurora A kinase[14,15,17]. This binding event brings together the amino-terminal domain of the clathrin heavy chain and the TACC domain of TACC3 to form a surface that can bind microtubules[17]. TACC3 binds to ch-TOG via a site that does not overlap with the clathrin interaction site[17].

Mitotic spindle functions have also been reported for intersectin 2 (ITSN2), RALA-binding protein 1 (RALBP1), sorting nexin 9 (SNX9), epsin and cyclin G-associated kinase (GAK; also known as auxilin 2) (Fig. 1a). ITSN2 is a multimodular protein that is involved in CME, presumably binding dynamin and SNAREs[18,19]. This protein can also act as a guanine nucleotide exchange factor (GEF) for CDC42 (REF. 20) (Fig. 1a). During mitosis, ITSN2 localizes to the spindle pole via its EPS15 (EGFR substrate 15) homology (EH) domains. Depletion of ITSN2 results in defects in mitotic spindle alignment owing to decreased activation of CDC42 (REF. 20) (FIG. 1d).

RALBP1 binds to several components of the CME pathway during interphase, including the medium mu2 subunit of the adaptor protein (AP2), epsin and the RALBP1-interacting protein REPS2 (REF. 21). During mitosis, it is found on the mitotic spindle and at spindle poles[22,23], where it is thought to have a role in spindle assembly. In addition, RALBP1 has been implicated in actin cytoskeleton regulation, suggesting that it is a multifunctional protein[22]. The mitotic spindle functions of clathrin, ITSN2 and RALBP1 all seem to be independent of their membrane trafficking functions.

In addition to its role in membrane trafficking during cytokinesis, SNX9 is important for mitotic progression and chromosome alignment[24]. However, SNX9 has no obvious spindle or centrosomal localization, and how it functions in mitosis is not clear. Interestingly, this role is specific to SNX9 as other sorting nexins such as SNX18 and SNX33, which function in membrane trafficking during cytokinesis, do not affect mitosis[24].

Two additional factors implicated in CME have also been linked to mitotic control, although it is not yet clear whether these represent true moonlighting proteins. One is epsin, a clathrin-associated protein that recognizes polyubiquitylated cargo[25] and may also contribute to membrane curvature at the forming clathrin-coated pit[26]. RNAi-mediated depletion of epsin results in dysregulation of spindle poles and a disorganized mitotic endoplasmic reticulum (ER) network[27]. However, it has been proposed that the regulation of mitotic membranes by epsin may explain its mitotic function. GAK, which promotes uncoating of clathrin-coated vesicles[28,29], has also been reported to affect mitosis. In one study, GAK depletion resulted in the accumulation of cells at prometaphase with disrupted mitotic spindle organization[30]. In another report, GAK was identified as a significant hit in an RNAi screen of Ser/Thr kinases that regulate mitosis[31]. But it seems likely that these mitotic defects result from loss of clathrin–TACC3–ch-TOG from the spindle rather than from a moonlighting function of GAK.

Indeed, depletion of GAK results in reduced free clathrin, arising from defective uncoating of clathrin-coated vesicles[32].

More work is needed to discern the precise function of many of these proteins at the mitotic spindle. In addition, the mitotic function of many trafficking proteins has not yet been analysed, and so we may see more players added to this list in the future.

Centrosomal functions. Several membrane trafficking proteins affect the organization and function of the centrosome in mitosis. In addition to its spindle function, clathrin has recently been shown to have a role at the centrosome[33]. Before mitotic entry, the clathrin–TACC3–ch-TOG complex also localizes to centrosomes, where it is important for maintaining centrosomal integrity[33] (FIG. 1b). Rapid inactivation of clathrin, using chemically induced dimerization of SNAP (SNARE attachment protein)-tagged clathrin light chains, triggers degradation of ch-TOG. This has led to the proposal that clathrin stabilizes ch-TOG and that this in turn maintains the integrity of the centrosome. But more work is now required to determine how this role for the clathrin–TACC3–ch-TOG complex relates to its microtubule-crosslinking function in K-fibres. For example, a key question that has not been addressed is whether or not the defects in centrosomal integrity involve microtubules.

Dynamins, large GTPases that are involved in membrane scission or remodelling, have also been implicated in centrosome control[34]. Dynamin 2 localizes to centrosomes through γ-tubulin association and contributes to centriole cohesion[35]. In addition, dynamin 2 has been implicated in modulating microtubules during interphase[36], and it associates with the mitotic spindle midzone during later stages of mitosis[37]. Dynamin 2 also affects cytokinesis[37–41], and there is good evidence that this role requires calcineurin-mediated phosphoregulation[42]. The role of dynamin 2 in cytokinesis is likely to be related to membrane scission and remodelling and may not therefore be a true moonlighting function. Dynamin 1 has also been reported to regulate the actin cytoskeleton[43], again hinting that dynamins may have other functions in the cell. Thus, a priority is to untangle all of these reported functions for dynamin and to clarify which relate to membrane trafficking and which, if any, represent moonlighting functions.

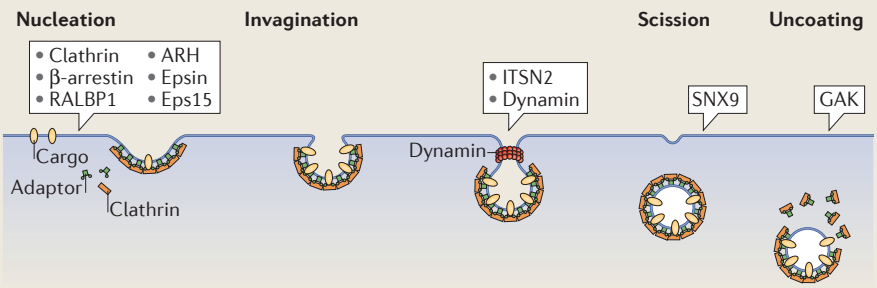
The arrestin family, which are established adaptor proteins for G protein-coupled receptors (GPCRs) during CME, have also been linked to the centrosome (FIG. 1c). Both β-arrestin 1 and β-arrestin 2 are constitutively associated with the centrosome[44,45], and depletion of either protein results in centrosome amplification and aberrant division, leading to multinucleate cells[45].

Some factors localize to multiple sites during mitosis. One example is ARH (also known as LDLRAP1), the accessory protein that interacts with the adaptor protein AP2 to mediate the uptake of low-density lipoproteins (LDL) via CME[46,47]. During mitosis, ARH is found at kinetochores, spindle poles and the midbody[48]. Loss of ARH results in mitotic defects presumably owing to dysregulation of the spindle poles[48,49]. Similarly, EPS15, which promotes CME in interphase[50], works in concert with epsin to organize the pericentriolar material during mitosis[51].

RAB6C, a homologue of RAB6A′ which is specific to primates, localizes to centrosomes, and its overexpression causes cell cycle arrest[52]. Depletion of RAB6C results in supernumerary centrosomes and thus gives rise to tetraploid cells. However, as the membrane trafficking functions of this protein are uncertain, it remains possible that the centrosomal function of RAB6C is its sole function. Again, more loss-of-function studies and mechanistic analysis for each of these centrosomal roles will be helpful to determine whether these proteins have related functions at this organelle.

Mitotic spindle checkpoint. During mitosis, quality control mechanisms ensure that all chromosomes are correctly attached to the mitotic spindle before anaphase is initiated. This spindle assembly checkpoint is mediated by MAD2 (mitotic arrest deficient 2) recruitment to kinetochores that are incorrectly attached to the spindle. This in turn inhibits the activity of the APC/C (anaphase promoting complex; also known as the cyclosome)[53]. The kinetochore component Zeste white 10 (ZW10) is part of this checkpoint[54]. During interphase, ZW10 has been implicated in membrane trafficking between the ER and the Golgi[55], and it is possible that both this interphase function and the mitotic checkpoint role may require ZW10 regulation of dynein[56]; spindle checkpoint silencing involves dynein-mediated removal of checkpoint proteins from the kinetochore[53].

Box 1 | Clathrin-mediated endocytosis and moonlighting



During clathrin-mediated endocytosis (CME), transmembrane receptors and other cargo are internalized by membrane-bound vesicles through the action of the endocytic machinery (see the figure).

Endocytosis occurs in four stages: nucleation, invagination, scission and uncoating. During the first stage, clathrin cannot bind to membranes or cargo directly and so uses adaptor proteins such as AP2 that recognize clathrin, membranes and cargo to nucleate the clathrin-coated pit. When the pit has invaginated sufficiently, the large GTPase dynamin acts to release the coated vesicle by causing scission at the neck. The vesicle must then be uncoated by enzymes such as cyclin G-associated kinase (GAK) and heat shock cognate protein 70 (HSC70) so that the vesicle can fuse at the target membrane and the coat components can be recycled.

Clathrin is a triskelion, comprising three clathrin heavy chains each associated with a light chain[92]. Triskelia can polymerize to form a polyhedral coat around the membrane as a pit forms, invaginates and eventually buds from the donor membrane. In fact, the origin of the name clathrin reflects the clathrate or lattice-like appearance of the vesicle coats first visualized by electron microscopy. Through association with different adaptor protein complexes, clathrin is involved in membrane budding events at the plasma membrane, endosomes or the *trans*-Golgi network. In this way, clathrin-mediated membrane trafficking influences numerous processes, including nutrient uptake, intracellular signalling, cell adhesion and migration, organelle biogenesis and pathogen entry[92]. CME proteins with moonlighting functions are depicted to show their temporal recruitment[77,93].

EPS15, EGFR substrate 15; GAK, cyclin G-associated kinase; ITSN2, intersectin 2; RALBP1, RALBP1-associated EPS domain-containing protein 1; SNX9, sorting nexin 9.

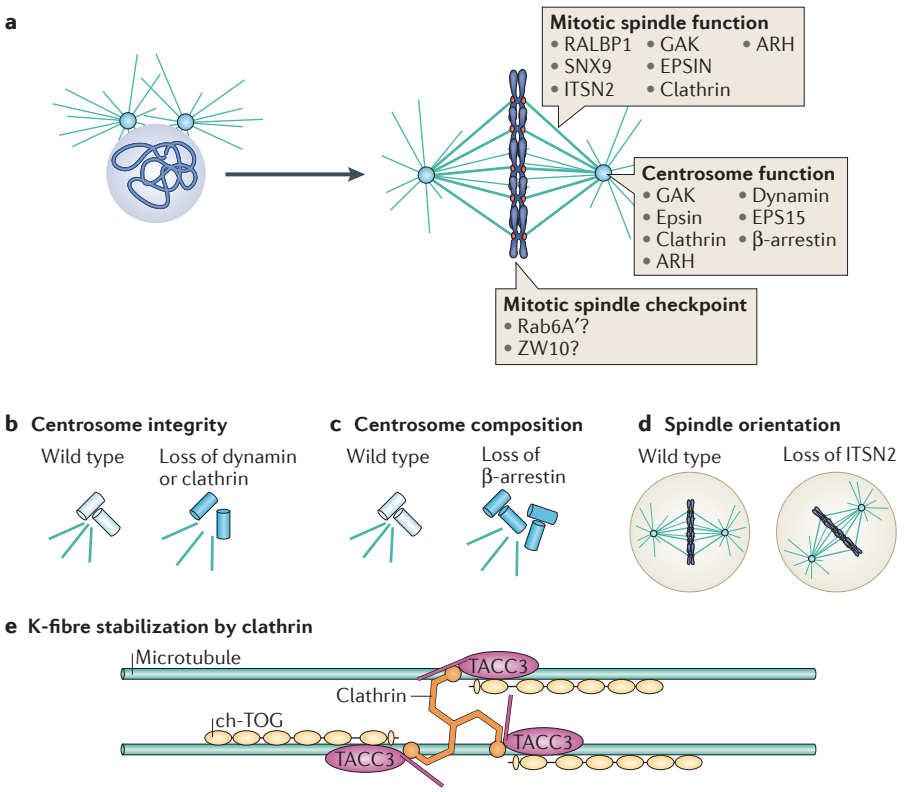


Figure 1 | **Mitotic functions for membrane trafficking proteins.** **a** | Several proteins that regulate clathrin-mediated endocytosis (CME) have alternative functions in interphase compared with mitosis. The different sites at which these factors act are indicated. The question marks denote those factors for which the proposed function may be related to the interphase function. The centrosome functions may also be active in interphase. **b** | Dynamin 2 and clathrin function in maintaining centrosome integrity. In wild-type cells, the centrosome is a compact assembly of two centrioles at the spindle pole. In the absence of dynamin or clathrin, the centrosome becomes fragmented. **c** | β-arrestins maintain correct centrosome composition; in the absence of β-arrestin, multiple centrosomes are seen. **d** | Intersectin 2 (ITSN2) is required for correct spindle orientation. In the absence of ITSN2, the spindle no longer aligns parallel to the direction of cell division which causes disorganization of luminal epithelial cells. **e** | Moonlighting functions may involve the formation of distinct regulatory complexes. For example, clathrin acts in a complex together with transforming acidic coiled-coil protein 3 (TACC3) and colonic hepatic tumour overexpressed gene (ch-TOG) to crosslink microtubules of kinetochore fibres (K-fibres) in the mitotic spindle. EPS15, EGFR substrate 15; GAK, cyclin G-associated kinase; RALBP1, RALBP1-associated EPS domain-containing protein 1; SNX9, sorting nexin 9; ZW10, Zeste white 10.

Similarly, the small GTPase RAB6A′ has membrane trafficking and checkpoint functions, but these may also be related. During interphase, RAB6A′ is involved in retrograde transport from early endosomes to the ER[57]. Depletion of RAB6A′ (but not RAB6A) causes the accumulation of cells at metaphase[58]. This MAD2-dependent block in the cell cycle was probably caused by a failure to remove spindle checkpoint proteins from the kinetochore by dynein–dynactin.

Establishing independence

For each trafficking protein listed in TABLE 1, a key question is whether the alternative function is independent of its canonical role in membrane trafficking. There are two reasons why it is important to determine this. First, this addresses whether the alternative function represents a true moonlighting function (that is, through a distinct mechanism). Second, it is important to rule out the possibility that the 'alternative function' is not genuine and results indirectly from perturbation of the canonical role. Once independence is established, how the switch between the two functions occurs can be assessed, and it then becomes possible to consider which of the two functions should be designated the moonlighting function.

True moonlighting functions. In the case of membrane trafficking proteins with roles in mitosis, it can be argued that because many of the alternative functions occur at stages of mitosis when membrane trafficking is shut down[59,60], they are likely to be independent of this process. Taking clathrin as the best-studied example, there is good evidence that its mitotic function is independent of its role in membrane trafficking. First, the functions can be separated by clathrin heavy chain mutants that can perform one function but not the other[6]. Second, the mitotic function requires binding to the spindle and association with proteins that have no role (that we know of) in membrane trafficking[10,12–17]. Third, the amount of membrane surrounding the spindle microtubules is variable in mammalian cells and is not sufficient to account for the localization of clathrin[10,61–64]. Last, the model proposed for microtubule crossbridge formation by the clathrin–TACC3–ch-TOG complex is very different from clathrin-mediated formation of lattices on membranes during membrane trafficking[6]. The spindle function of clathrin can therefore be regarded as a true moonlighting function, and it is likely that the centrosomal function of clathrin is also independent of membrane trafficking. It is important to note that clathrin also acts in its membrane trafficking capacity during late mitosis and cytokinesis[65,66], but this important function cannot be considered a moonlighting role.

Conversely, for several proteins that have been discussed (TABLE 1), the independence of functions has not yet been demonstrated. Indeed, in some cases, there is evidence that they may not be moonlighting functions at all. For example, it is likely that the roles of ZW10 and RAB6A′ in membrane trafficking and in spindle checkpoint silencing both mediate control of dynein function. For others, such as epsin, it is possible that the contribution to spindle function is via a role in membrane organization or, in the case of GAK, by effects upstream of clathrin. For RAB6C, a role in membrane trafficking is not clear, so its mitotic effects may reflect its primary function. For most other examples, including arrestins, RALBP1, EPS15, SNX9, ARH and dynamin 2, their alternative functions may well represent true moonlighting functions. However, more mechanistic understanding of how these factors act is needed to definitively conclude that these additional functions are independent of their membrane trafficking role.

Indirect effects. The second related concern is whether an alternative function actually results from apparent chronic inhibition of the canonical function. For example, cells go through several cell cycles during an RNAi experiment, and it is possible that defects can

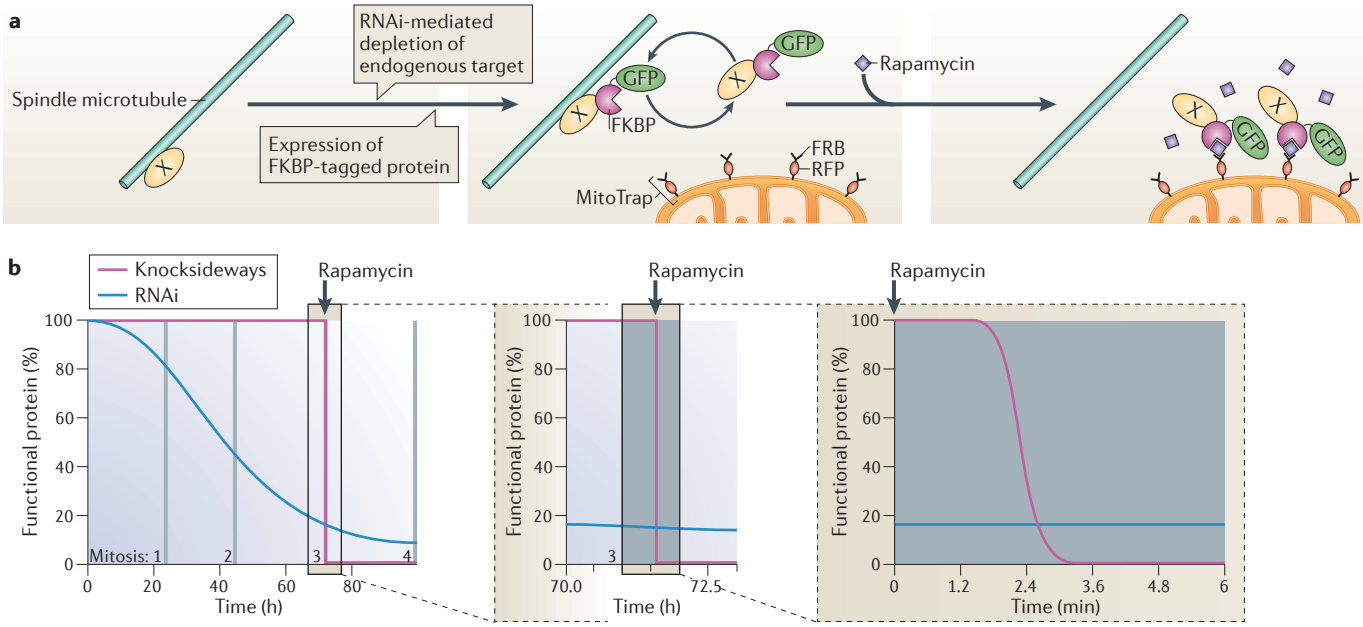


Figure 2 | **Rapid inactivation techniques for studying moonlighting roles.** Characterization of moonlighting functions requires rapid inactivation of proteins. **a** | The knocksideways technique[68], which relocalizes the target protein to the mitochondria, has been applied to microtubule-associated proteins in mitosis. After depletion of the endogenous protein, an FK506-binding protein (FKBP)-tagged RNAi-resistant protein is expressed in cells together with MitoTrap. This trap contains an FRB (FKBP–rapamycin-binding) domain that is targeted to the mitochondria. Both the target protein and MitoTrap are tagged with fluorescent proteins for visualization (GFP and RFP respectively). Thus, if the microtubule-associated protein cycles between the cytoplasm and the spindle, the protein can be rerouted to mitochondria[13]. If this new localization is not compatible with function, as is the case for microtubule-associated proteins, then the protein is inactivated. **b** | The knocksideways technique works on a faster timescale than RNAi. The graph on the left shows a cell line with a 24 hour cell cycle; mitosis (grey boxes) lasts for 1 hour. A protein of interest can be depleted by RNAi to ~10% of endogenous levels within 72 hours. During this time, the cell goes through several mitotic divisions with a gradual decline in protein function. Any canonical functions in interphase are therefore also impaired during this time. By contrast, in the knocksideways technique, the addition of rapamycin to cells (arrow) typically removes all the target protein within a few minutes[13] (right plot). Other inactivation methods such as the SNAP method for dimerization (using SNARE attachment proteins)[33] or the degron method[70] operate on similar timescales.

accumulate and give a misleading picture of protein function. Indeed, protein depletion via RNAi occurs slowly (1–3 days) compared with the length of mitosis (1–2 hours) (FIG. 2). Furthermore, depletion of a protein involved in CME is likely to cause problems in cytokinesis[51]. At the next division, a multipolar spindle may form, and this could lead to an erroneous conclusion regarding centrosome amplification[67]. This problem can be solved by selectively rescuing one function using mutant proteins expressed in cells depleted of the protein of interest. However, this approach is only possible if detailed molecular understanding of both functions is available. Another strategy is to use rapid inactivation methods that allow specific interference with protein function at defined stages of the cell cycle. In addition to the SNAP-tag approach[33], which can be used to induce rapid dimerization of proteins, the recently developed 'knocksideways' technique that rapidly inactivates proteins by rerouting them to mitochondria[68] and chemically inducible degradation methods[69,70] are viable approaches to inactivate proteins by their aggregation, relocalization or degradation, respectively.

The knocksideways method[68] has been used to study the function of the clathrin–TACC3–ch-TOG complex at the mitotic spindle[13] (FIG. 2). The removal of TACC3 from mitotic spindles using this approach causes TACC3 relocation to nearby mitochondria within ~5 min. This redirecting is specific, as clathrin and ch-TOG relocalize with TACC3, but other spindle proteins do not. If their relocation is induced just after nuclear envelope breakdown, this results in failed metaphase. By contrast, if spindle formation and metaphase are allowed to proceed normally before clathrin–TACC3–ch-TOG removal, the function of mature K-fibres is perturbed, with reduced tension across sister kinetochores. This therefore suggests that the clathrin–TACC3–ch-TOG complex is important for both the formation and function of K-fibres[13]. This example illustrates the functional insights that can be gained using such rapid inactivation methods and that ideally these should be the method of choice for studying moonlighting functions.

Understanding functional mechanisms. Better understanding of moonlighting roles will require mechanistic insights into how these switches in protein function are controlled. Again, using clathrin as the best-studied example, the formation of the clathrin–TACC3–ch-TOG complex explains the ability of clathrin to switch its function between interphase and mitosis. Whereas during interphase there is no phosphorylated TACC3 available, at the onset of mitosis, the level of TACC3 (phosphorylated at Ser558) increases, allowing the recruitment of clathrin into a complex that can bind and stabilize K-fibres[17]. Thus, the formation of a distinct protein complex is an effective method for switching to a moonlighting function. It remains to be seen whether other moonlighting proteins also use a similar type of control. This is true for the NUP107 nucleoporin complex, a subcomplex of the nuclear pore that moonlights at kinetochores during mitosis[71]. The mitotic localization of NUP107 depends on the nuclear division cycle 80 (NDC80) complex and centromere protein F (CENPF)[72], suggesting that its

mitotic localization and function is mediated by a distinct set of proteins.

Assigning the moonlighting function. In cases in which two independent functions have been demonstrated for a protein, the temptation is to assign the moonlighting function to the role that was discovered later. An excellent recommendation is that the moonlighting function should be the one that is younger in evolutionary terms[5]. However, for many proteins that have been proposed to have such dual function, we simply do not yet have enough information to determine which of the two is the moonlighting function. In the case of clathrin, orthologues are found in all eukaryotes[6,73]. The role of clathrin in endocytosis is well-conserved, actually becoming more important in higher organisms compared with yeast, for example[74]. However, it seems most likely that the function in K-fibre stabilization arose later. The function of clathrin in mitosis seems to be common to vertebrates, but it is not yet clear whether this role is shared in lower species. Homologues of TACC3 and ch-TOG, the factors that bind clathrin in mitosis, are found in *Schizosaccharomyces pombe* (altered polarity protein 7 (Alp7) and Alp14, respectively), but they function at the kinetochore rather than spindle fibres, and it remains to be determined whether clathrin is required at all[75]. Determining the order in which alternative functions arose during evolution is a key challenge for the future.

Repurposing in independent processes

In light of the fact that so many membrane trafficking proteins have alternative functions in mitosis (TABLE 1), it has been proposed that membrane trafficking and mitosis are deeply connected[76,77]. At first glance, a link between these two very different cellular processes seems unlikely, but it could make sense in evolutionary terms. The evolution of eukaryotes, which have their genetic material enshrined in a nuclear membrane, presented two related challenges: how to segregate the genetic material during mitosis and how to control membrane movement and remodelling in order to necessitate this (reviewed in REF. 78). Solving these problems, perhaps through the use of common components, may have been important for survival of early eukaryotic life.

A strong argument against mitosis and membrane trafficking being connected is that several of the alternative mitotic functions found for trafficking proteins have been shown to be true moonlighting functions. If a protein is doing something unrelated to its canonical role during mitosis, then it is difficult to argue that the cellular processes themselves are linked. Furthermore, if there was a special connection between membrane trafficking and mitosis, another prediction would be that the relationship between the two pathways would be exclusive. This also does not hold true. First, proteins from other cellular processes have been shown to moonlight in mitosis. For example, the nucleoporins NUP107, NUP160 and NUP358 moonlight in mitosis, operating in spindle assembly, checkpoint regulation and kinetochore assembly[79]. Moreover, the inner nuclear membrane protein SAMP1 (spindle-associated membrane protein 1) seems to function on K-fibres[80], and the integrin regulator kindlin 1 has a role in spindle assembly[81]. Second, membrane trafficking proteins have alternative functions in other cellular processes aside from mitosis. For example, clathrin has been proposed to affect signalling and transcription, roles that may also be independent of its role in membrane trafficking. Specifically, it has been suggested that clathrin restricts the basal activity of nuclear factor κB (NF-κB) in a process that is independent of CME[82]. In addition, clathrin may be involved in p53-mediated transcription[83], a function that might involve a non-trimeric form of clathrin[84].

Several endocytic adaptors, including arrestins, have roles in transcription and have also been shown to undergo nuclear–cytoplasmic shuttling[85,86]. This suggests that perhaps proteins involved in membrane trafficking do not have a specific link to mitotic control but are simply adaptable proteins that can be readily repurposed for diverse cellular processes. This adaptability may be due to their biochemistry. Some membrane trafficking proteins have a modular layout with various different domains and interaction motifs[50], and perhaps this organization increases the plasticity of these molecules. Alternatively, the adaptability may reflect the fact that the core membrane trafficking machinery is ancient[73], and this may have increased the possibility of advantageous repurposing during evolution from the last eukaryotic common ancestor.

The fact that only specific membrane trafficking factors affect mitosis also argues against the idea that these two processes have a special connection. First, the components of the trafficking machinery have varied mitotic functions. For example, aside from clathrin, no other components seem to have a role in K-fibre stabilization (TABLE 1). If the whole clathrin pathway had been repurposed, then similar roles would be expected for other proteins. Second, key components of the clathrin membrane trafficking machinery do not seem to have a mitotic function. For example, the adaptor complexes AP1–AP3 do not localize to spindles or centrosomes[10] and have no reported mitotic function. So, although more information is required about the moonlighting functions of membrane trafficking proteins, the hypothesis that the entire membrane trafficking machinery is repurposed wholesale to assist mitosis can be ruled out.

Cellular frugality

As moonlighting involves the reuse of certain proteins for very different cellular processes, it provides an economical strategy for the cell to recycle existing molecules for alternative purposes while avoiding the need to synthesize new proteins and the associated expense of having to carry an extra gene in the genome. Although this strategy makes sense, what is the cost to the canonical function when proteins are repurposed? In the case of membrane trafficking factors, the answer may be very little.

When cells enter mitosis, membrane trafficking and many other interphase functions are switched off[59,87]. The inhibition of CME begins within seconds of prophase entry, and the inhibition is relieved only in late anaphase[60]. Resumption of CME releases membranes for remodelling events during cytokinesis[88]. The shutdown of CME in early mitosis effectively liberates proteins involved in this process so that they are available for repurposing. An analogous example of this are the nuclear pore components NUP107 and NUP133, which relocate to kinetochores during mitosis[89]. As the nuclear membrane breaks down during mitotic entry, no nuclear transport occurs, and these nuclear pore components are therefore free to be used elsewhere. Thus, it might be a requirement that there is little or no cost to the canonical pathway in order for a protein to take on a moonlighting function. The shutdown of CME during mitosis may therefore explain the apparent adaptability of proteins involved in membrane trafficking.

An alternative view is that there is a cost to the canonical function and that the repurposing of membrane trafficking proteins for moonlighting roles actually causes the shutdown of membrane trafficking during mitosis[60]. However, this is unlikely to be the case. For example, clathrin is highly abundant (~500,000 triskelia per cell)[90,91], whereas there are far fewer phosphorylated TACC3

molecules; as such, only a fraction of clathrin is recruited to the spindle in the form of the clathrin–TACC3–ch-TOG complex, and a large soluble pool of clathrin is present during mitosis. Because the amount of clathrin that switches function is relatively small, the shutdown of CME during early mitosis is unlikely to be caused by a lack of available clathrin[60]. However, the precise mechanism by which the mitotic shutdown of CME occurs needs to be determined in order to fully exclude this possibility.

Conclusions

The list of membrane trafficking proteins that have alternative functions has continued to grow during the past decade. However, in many cases, we need better mechanistic understanding to determine whether they are independent of the canonical role of the protein. Methods that can rapidly inactivate proteins will be crucial to assess moonlighting functions. For each of the proteins discussed (TABLE 1), we still need to understand precisely how they contribute to mitosis. We also need to ascertain which function is the canonical, original role and which arose later in evolution.

As I have discussed, alternative functions for membrane trafficking proteins are not restricted to mitosis, and this argues against a special connection between these two cellular processes. Instead, I propose that membrane trafficking proteins may represent a set of adaptable molecules that are well suited to repurposing for different pathways. It will be fascinating to uncover what aspects of their biochemistry allow repurposing and what other processes they contribute to.

Although it is now clear that the idea of one gene that gives rise to one protein with one function in one cellular process is rare in reality, Occam's razor dictates that this logic must be used to interpret cell biology experiments. Membrane trafficking and mitosis are two highly intricate cellular processes, for which increasing complexity continues to be revealed. An appreciation of the propensity of proteins to moonlight is therefore important for the interpretation of protein depletion experiments in which the disruption of one process could affect another. This is also important for drug design to ensure that inhibition of off-target pathways does not cause unnecessary side effects. This has broad implications, as moonlighting is not restricted to these two cellular processes, and so the development of tools that cleanly define protein functions within a single process will be key for a better molecular understanding of cell biological processes.

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The authors declare no competing financial interests.