Single proteins that serve linked functions in intracellular and extracellular microenvironments

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Preface

Maintenance of organ homeostasis and control of appropriate response to environmental alterations requires intimate coordination of cellular function and tissue organization. An important component of this coordination may be provided by proteins that can serve distinct, but *linked*, functions on both sides of the plasma membrane. Here we present a novel hypothesis in which non-classical secretion can provide a mechanism through which single proteins can integrate complex tissue functions.

Single genes can exert a complex, dynamic influence through a number of different processes that act to multiply the function of the gene product(s). Alternative splicing can create many different transcripts that encode proteins of diverse, even antagonistic, function from a single gene. Posttranslational modifications can alter the stability, activity, localization, and even basic function of proteins. A protein can exist in different subcellular localizations. More recently, it has become clear that single proteins can function both inside and outside the cell. These proteins often lack defined secretory signal sequences, and transit the plasma membrane by mechanisms separate from the classical ER/Golgi secretory process.

When examples of such proteins are examined individually, the multifunctionality and lack of a signal sequence are puzzling — why should a protein with a well known function in one context function in such a distinct fashion in another? We propose that one reason for a single protein to perform intracellular and extracellular roles is to coordinate organization and maintenance of a global tissue function. Here, we describe in detail three specific examples of proteins that act in this fashion, outlining their specific functions in the extracellular space and in the intracellular space, and we discuss how these functions may be linked. We present epimorphin/syntaxin-2, which may coordinate morphogenesis of secretory organs (as epimorphin) with control of protein secretion (as syntaxin-2), amphoterin/high mobility group box-1 (HMGB1), which may link inflammation (as amphoterin) with regulation of gene expression (as HMGB1), and tissue transglutaminase, which affects delivery of and response to apoptotic signals by serving a related function on both sides of the plasma membrane. As it is notable that all three of these proteins have been reported to transit the plasma membrane through non-classical secretory mechanisms, we will also discuss why coordinated inside/outside functions may be found in some examples of proteins which transit the plasma membrane through non-classical mechanisms and how this relationship can be used to identify additional proteins that share these characteristics.

Coordinating proteins: three examples

Morphogenesis and protein secretion. Epimorphin was initially identified as the target of a monoclonal antibody that blocked hair follicle morphogenesis in the dermal epithelium[1], but is known now to be involved in the morphogenesis and development of many other epithelial organs (Figure 1a; for review, see ref. [2]). In the mammary gland, epimorphin directs branching and luminal morphogenesis, where the orientation of its presentation to the mammary epithelium dictates the resulting tissue structure. Presentation to cells in a polarized fashion stimulates branching morphogenesis processes associated with increasing the complexity of the mammary ductal epithelium; presentation in an apolar fashion stimulates epithelial structures to increase the size of the ductal lumen[3]. Inappropriate expression of epimorphin in transgenic animals can lead to development of enlarged, cystic ducts, cell proliferation, and progression to cancer in aged animals[4].

Epimorphin is encoded by the same gene, and is likely to be the same protein, as syntaxin-2[5]. Syntaxins are members of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) superfamily of membrane proteins that mediate intracellular vesicle docking and fusion[6]. SNARE proteins are critical during the final stage of membrane fusion, a process that depends upon the formation of a protein complex that includes the vesicle-associated membrane protein (VAMP) on the vesicle surface, the 25 kDa synaptosome-associated protein SNAP-25 and a syntaxin on the target surface. When assembled into a functional complex, these three molecules form an intermolecular four-helix bundle that initiates the membrane fusion process. There are many distinct mammalian syntaxins, each associated with a particular vesicle fusion process. Syntaxin-2, discovered soon after the initial identification of epimorphin[7], is a plasma-membrane-associated protein, but its functional domain is oriented to the inside of the cell (Figure 1b). Syntaxin-2 has been shown to participate in specific and critical membrane fusion processes, including granule release from platelets[8], secretion of lung surfactant from alveolar type II cells[9], and regulation of midbody abscission during cytokinesis[10].

The majority of proteins found outside the cell are trafficked through the ER/Golgi pathway; these proteins are initially targeted to the ER by the presence of a leader sequence, a hydrophobic signal peptide which is usually located at the N-terminus of the protein (Box 1). The discovery that the same gene encoded both epimorphin and syntaxin-2 provoked substantial controversy[11,12], as epimorphin/syntaxin-2 lacked a leader sequence that would target epimorphin to the extracellular space, and as there was no known mechanism for its secretion at that time. For many years thereafter, investigations of epimorphin/syntaxin-2 focused either on its extracellular or intracellular function[2], and the question of *how* the leaderless syntaxin-2 crosses the plasma membrane came to eclipse the question of *why* a single protein might have such different roles. We propose that the activity of extracellular epimorphin as a morphogen of epithelial tissues and the activity of intracellular syntaxin-2 as a mediator of the fusion of secretory vesicles with the plasma membrane may in fact both contribute directly to tissue organization. Since secretory epithelial organs depend on correct morphogenesis (to define sidedness of secretion) and on correct orientation of secretion, the functions fulfilled by epimorphin/syntaxin-2 are complementary. Given the potential importance of proper regulation of this protein in normal tissue function, perhaps it should

not be surprising that overexpression and misregulation of epimorphin/syntaxin-2 can cause organ pathology and induction of cancer[3,4] .

Cell communication and gene expression. Whereas epimorphin/syntaxin-2 coordinates maintenance and development of normal tissue structures, amphoterin/HMGB1 may act to coordinate the responses of tissues that are damaged or undergoing inflammatory processes (reviewed in [13-15]). Amphoterin is an extracellular protein (Figure 1c) that was first identified in the brain where it was shown to mediate outgrowth of neurites. Since then, it has been implicated as an inducer of chemotaxis and cell motility, and as a crucial cytokine that mediates inflammatory responses. Amphoterin stimulates motility and inflammation through binding to specific extracellular receptors, including RAGE (receptor for advanced glycation end products). The interaction of RAGE and amphoterin has generated particular interest because these two molecules also have been shown to regulate the invasiveness of several tumour cell types, providing insight into potential new therapeutic opportunities in the treatment of cancer. Amphoterin also lacks a classical secretion sequence, and while it can be released from necrotic cells, it also can be secreted from intact cells in response to specific stimuli, including the cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1)[16] or the extracellular matrix protein laminin[17]; investigations of non-classical secretion of amphoterin from monocytes have implicated a role for a vesicle-mediated secretory pathway[18] (Box 1) .

HMG (high mobility group)-box (HMGB) family proteins are non-histone components of chromatin; HMGB1 (formerly HMG-1) is one of the earliest-discovered members of this family. HMGB proteins function primarily as architectural facilitators in the assembly of nucleoprotein complexes[19,20]. HMGB1 is ubiquitously expressed and localized to the nucleus in most cell types (Figure 1d). While HMGB1 can bind with low affinity to single-stranded, linear duplex and supercoiled DNA, it binds with high affinity to cruciform DNA, a form of DNA that can be produced as an intermediate in chromosomal recombination[21]. Association of HMGB1 with DNA leads to the assembly of nucleoprotein complexes that affect and coordinate gene transcription[19,20,22]. Amphoterin/HMGB-1 thus acts when localized inside the cell nucleus as a modulator of chromatin function and gene expression, and when localized outside the cell as an inducer chemotaxis, cell motility, and

inflammation, cellular behaviors that depend upon coordinated modulation of gene expression.

Extracellular matrix and cell survival. Tissue transglutaminase (tTG) is found in the nucleus, cytoplasm, membrane and extracellular space, and has been principally characterized as a Ca^{2+}- and GTP-regulated protein cross-linking agent[23] (Figure 1e). Inside the cell, tTG has been implicated in an array of physiological processes, most notably the ability to regulate and to induce apoptosis. In the nucleus, tTG transamidates retinoblastoma protein (Rb), a process that results in covalent crosslinking of γ-carboxamide groups of glutamine residues; this modification of Rb provides protection against apoptotic stimuli[24]. However, when localized to the cytoplasm, tTG can induce apoptosis[24,25]. tTG is unique among transglutaminases in that it also possesses GTPase activity, and can serve as a signal transducing G-protein[26].

Lacking a secretory signal sequence, tTG can be exported from the cell via non-classical secretion mechanisms, where it localizes to the cell surface and extracellular matrix (ECM)[23]. A number of ECM proteins have been shown to be glutaminyl substrates for tTG and these proteins, when cross-linked, become resistant to proteolytic degradation and mechanical damage, conferring increased structural stability and flexibility on the tissue architecture. Significantly, degradation of tTG by matrix metalloproteinases (MMPs) facilitates tumour-cell invasion and metastasis[27]. At the cell surface, extracellular tTG facilitates cell-ECM interactions by cross-linking ECM proteins laminin and nidogen[28], and also enhances integrin-ECM interactions through transglutamination-independent processes[29]. The crosslinking function of tTG also plays important roles in temporally-controlled, tightly regulated processes such as fibrin clot formation[30].

Attachment to the ECM provides important cell survival signals; nontransformed cells that become detached from the ECM undergo a specific form of apoptosis known as anoikis. tTG unifies extracellular structures and intracellular responses by modulating ECM organization when outside the cells and controlling apoptotic response within. These functions have been studied primarily as distinct phenomena, but a comparison of the functional roles of tTG in both compartments provides insight into how the processes may be directly connected with each other.

Identifying coordinating proteins

While epimorphin/syntaxin-2, amphoterin/HMGB1, and tTG are among the best studied examples of proteins that function on both sides of the plasma membrane, that are secreted by non-classical mechanisms, and that may act to link inside and outside signaling processes, many others have been investigated (Table 1). RHAMM coordinates proliferation and mitosis through its roles as a cell surface hyaluronan receptor and its association with mitotic spindles and mitotic signaling pathways[31]. Annexin II modulates cell-cell and cell-ECM interactions outside the cell[32], and cytokinesis and vesicle trafficking inside the cell[33,34]. Extracellular autocrine motility factor (AMF) is a potent cytokine/morphogen[35,36], while the identical protein inside the cell is known as cytoplasmic phosphohexose isomerase (PHI), and controls glycolysis[37]. Ku controls cell adhesion outside the cell[38], and DNA repair in the nucleus[39]. While there are considerable differences between the sequences, structures, and functions of these proteins, several commonalities can be observed: separate structural motifs for distinct functions of the protein, and utilization of non-classical secretion for crossing the plasma membrane.

Different motifs for distinct functions. One striking feature of these multifunctional proteins is that the different mechanisms of action can be separated into distinct protein motifs. This is particularly evident with epimorphin/syntaxin-2 (Figure 2). The SNARE motif is a C-terminal helical domain highly conserved within the syntaxin family, and is essential for formation of competent membrane fusion complexes[6]. The active site of epimorphin for epithelial morphogenesis has been mapped to an independently folded N-terminal domain with a three-helix bundle structure[40]. Similarly, the acidic C-terminal domain of HMGB1 appears to be critical for regulation of binding to DNA and for formation of ternary structures[41], and a specific DDDDE motif in the C-terminus of HMGB1 is required for transcription stimulation[42]. By contrast, the extracellular function of amphoterin is dependent on a distinct receptor binding motif encompassing amino acids 150-183[43].

The utilization of different motifs for distinct functions has been found for other proteins that function of both sides of the plasma membrane as well: phosphoglucose

isomerase (PGI), a member of the sugar metabolism pathway, functions outside the cells as the morphogenic cytokine autocrine motility factor (AMF), and also possesses distinct structural domains for the distinct activities of PGI and AMF[36]. Similarly, C-terminal truncation of thioredoxin enhances its mitogenic, cytokine-like activities even though oxido-reductase function is compromised[44]. However, such utilization of different motifs is not an absolute requirement for the successful integration of multiple distinct functions, as in the case of tTG, where the consequences of a single enzymatic activity differ dramatically according to intra- or extracellular localization.

Non-classical mechanisms of secretion. Despite diversity of function and apparent lack of significant primary sequence homology between the proteins listed above, these proteins lack exocytosis-targeting signal sequences and yet clearly exit the cell. While epimorphin/syntaxin-2 exists primarily on the cytoplasmic side of the plasma membrane[45], recent studies have defined some of the key features of the non-classical secretion of epimorphin[46]. When localized to the cytoplasmic surface, epimorphin/syntaxin-2 associates with synaptotagmin and annexin II; when the cell is exposed to stress stimuli, the complex is translocated and released from the cell. Similar mechanisms have been identified for other non-classically-secreted proteins[31,47,48].

HMGB1/amphoterin escapes the cell by at least two distinct mechanisms: passive release following necrotic cell death or active secretion in response to certain stimuli. When released from necrotic cells, HMGB1 functions as an immediate trigger for inflammation[49]. Active secretion of HMGB1 can occur in response to inflammatory stimuli, and is controlled by acetylation of the protein, which results in a shift in the intracellular equilibrium of the protein that favors the nucleus in unstimulated cells and the cytoplasm following activation[18,50]. In one study, HMGB1 was shown to accumulate into secretory lysosomes, Ca^{2+}-regulated organelles that are released from the cell by exocytosis[18].

A mechanistically distinct route for extracellular transport has been suggested for the multifunctional protein thioredoxin, an enzyme that catalyses thiol-disulfide exchange reactions intracellularly and exerts cytokine- and chemokine-like activities in the extracellular space[51]. Extracellular release of thioredoxin from T lymphocytes occurs rapidly

following exposure to redox agents, and is dependent upon the redox-sensitive site of thioredoxin[51].

The characteristic of non-classical secretion may be a key aspect of molecules that serve distinct, but linked, functions on both sides of the plasma membrane. One possibility is that transport through the ER/Golgi may result in protein modifications that interfere with the extracellular function of a protein, as has been shown for FGF-2[52]. However, this is not be a general rule, as forcing transit of epimorphin/syntaxin-2 through the ER/Golgi results in a glycosylated protein that is nevertheless capable of directing morphogenesis[40]. Another possibility is that the lack of an exocytosis signal sequence in proteins with paired functions allows for greater flexibility in compartmental distribution, as well as membrane translocation through non-classical secretory pathways, and thus more intimate linkage of their intracellular and extracellular functions. Moreover, the presence of a strong exocytosis signal in the protein would require extraordinary mechanisms by the cell to retain the protein intracellularly. The non-classical secretion process may have been developed early in evolution, prior to the formation of the ER/Golgi pathway; for these early cells, it would have been critical to coordinate cellular function with intercellular communication. Such a mechanism may have been retained because it allows for rapid connection between diverse processes.

An important implication of this possibility is that there should be a common mechanistic link between the intracellular and extracellular functions and the control of secretion; for example, there may be key posttranslational modifications that regulate retention within the cytoplasm versus exportation. Identification of specific protein modifications or other signaling mechanisms that trigger protein secretion by non-classical mechanisms is a critical area for future investigation. A number of mechanisms have been shown to be involved in the process of transiting the plasma membrane for proteins secreted by non-classical mechanisms (reviewed in refs. [31,47,48]), but defining the specific modifications that signal for these proteins exit the cell has been delayed by the lack of clear consensus regions that govern the process, although recent computational efforts have begun to address this problem (Box 2).

Consideration of how these protein relationships may have developed can provide direction towards their identification. Studies of large structurally homologous but

mechanistically divergent protein families have suggested that new protein functions are often evolved through an opportunistic process known as "recruitment", wherein the preexisting structural features of an active site or ligand binding site are exploited for a new purpose[53,54]. Evidence suggests that protein speciation often proceeds through intermediates with promiscuous functionality, capable of binding multiple ligands and facilitating multiple biological processes[53]. Seen in this light, acquisition of dual topology would provide an additional mechanism to acquire multifunctionality. For a molecule originally evolved to carry out an intracellular function through the selective binding of a particular ligand from among the array of potential intracellular binding partners, extracellular localization would result in exposure to a novel pool of potential ligands, allowing conscription of a preexisting protein binding site for new functional interactions. In some cases, the intracellular and extracellular functions of the nonclassically secreted proteins would become uncoupled, and the protein functions would diverge. For proteins such as those described here, the topologically distinct functions of the proteins would remain linked, providing an additional mechanism for controlling important cellular functions. A prediction of this model is that, for proteins which maintain linked functions, the different domains conferring the distinct functions would be preserved in evolution, as is seen for the epimorphin/syntaxin-2 secretion motif (Figure 2C). Another prediction is that mutagenic inactivation of one functional domain would equally affect the linked process as inactivation of the other functional domain; such investigations are critical for testing the model presented here.

Conclusions

Here we have described examples of proteins that may act during epithelial morphogenesis to create the sidedness that provides meaning to polarized secretion, that link gene expression and inflammatory responses in damaged or inflamed tissues, or that modulate cell survival and tissue function. That single proteins could serve distinct but linked functions on opposite sides of the plasma membrane is not at present a widely-recognized concept. However, it is important to remember that the idea of a single protein possessing distinct but linked actions in different *intracellular* locations, as exemplified by β-catenin (a structural molecule at adhesion plaques and a transcription factor in the

nucleus), was once regarded as controversial, but through careful and detailed investigations of the interdependence of function and localization, this concept has opened up entire new fields of study. Higher organisms have developed many mechanisms for increasing the complexity of a relatively small genome, and the notion that a gene codes for a single protein that denotes a single function has been discarded following the recognition of the importance of gene splicing and post-translational modifications on protein function.

Many proteins coordinate distinct signaling pathways within the cells by performing multiple functions; the concept of "moonlighting" was advanced to describe this phenomenon[55]. This was an apt description initially, since the newly discovered function of an already defined protein appears to be secondary; however, this term gives the inevitable impression that one set of functions is more central than the others. We propose this is not so for the molecules described here. Although the locations of their distinct molecular activities are separated by the physical barrier of the membrane, their dual roles may act to unify a single tissue function, and thus be equally important in an integrated overall purpose. We suggest that these proteins may instead be considered as liason proteins, a term that may better reflect their shared function across the plasma membrane.

It is possible that many proteins might exist both inside and outside the cell, showing distinct but related functions in these different cellular contexts, and it is intriguing to consider how many potentially serendipitous observations have been disregarded as a consequence of prior identification of a given protein in a different location and with a different function. The phenomenon of proteins with distinct functions inside and outside the cell might not prove to be as common as alternative splicing or post-translational modifications, but given the importance of robust linkage of cellular functions and tissue processes, we expect that linkage of function across the plasma membrane might be much more common and important than previously suspected.

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Boxes

Box 1. Classical and non-classical protein secretion. Several different mechanisms have been identified by which cytosolic proteins can cross the plasma membrane. A. Classical protein secretion. (a) During polypeptide assembly by the ribosome, the presence of a membrane-targeting signal sequence leads to translocation of the ribosome-polypeptide complex to the endoplasmic reticulum (ER) by the signal recognition complex. (b) Following validation of correct protein folding in the ER, proteins are captured within transitional vesicles that bud from the ER and translocate to and fuse with the Golgi. (c) Protein maturation within the Golgi involves posttranslational modifications, including attachment of sugar molecules that can significantly alter the surface structure and function of the protein. (d) Once fully processed, proteins are transported to the cell surface through targeted vesicles. B. Non-classical secretion. Proteins lacking signal sequences can directly transit the plasma membrane by coordinating with specialized cotransport complexes (a), as is the case for fibroblast growth factor-1 (FGF1) and (FGF2). Some proteins can be collected into secretory lysosomes/vesicles that fuse with the plasma membrane, releasing their contents into the extracellular space (b) a mechanism that has been shown for HMGB1/amphoterin and interleukin-1β (IL-1β). An alternative method involves membrane blebbing to generate exovesicles (c); these become lysed to release their contents to the extracellular space. It should be noted that these are only a subset of mechanisms implicated for non-classical secretion. Additional mechanisms include secretory lysosomes and vesicle shedding; more complete discussion of current understanding of non-classical secretion mechanisms can be found in refs. [31,47,48].

Box 2. Identifying non-classically secreted proteins. Although a simple motif that defines proteins that are secreted by nonclassic mechanisms has not yet been identified, several methods have been developed that use computational approaches to identify leaderless proteins, which might be secreted by nonclassic methods in eukaryotic[56] and prokaryotic[57] organisms. Bendtsen et al. created a neural network that used sequence-derived features such as presence of potential sites of post-translational modifications, predicted secondary structure, abundance of charged residues, presence of predicted propeptides and other transmembrane helices, and regions of low complexity; they identified many proteins known

to be secreted by non-classical mechanisms, including FGF family members, thioredoxin, and galectin[56,57]. (Automated evaluation of known proteins or unknown protein sequences can be made online at http://www.cbs.dtu.dk/services/SecretomeP/.) A recent study has implicated caspase-1 as a mediator of non-classical protein secretion, and a screen to identify proteins that transit the plasma membrane in response to caspase-1 activation identified several proteins that are known to exit the cell by non-classical means, including annexin A2, macrophage migratory inhibitory factor, and HMGA2[58]. Identifying key protein motifs responsible for recognition by the non-classical secretory pathway(s) will be essential for defining how secretion and/or release are regulated.

Figure legends

Figure 1. Models of linked intracellular and extracellular roles for molecules of dual topology/multiple function. a) Outside the cell, epimorphin mediates tissue morphogenesis, b) inside the cell, syntaxin-2 controls protein secretion from ER/Golgi-derived vesicles. c) Outside the cell, amphoterin stimulates cell responses that are mediated by altered gene expression, d) inside the cell, HMGB1 controls chromatin organization, influencing the expression of many gene products. e) Outside the cell, tTG controls ECM organization, f) inside the cell, tTG participates in pathways that determine cell survival or apoptosis that are responsive in part to cell attachment to the ECM.

Figure 2. Distinct motifs mediate the different functions of epimorphin/syntaxin-2. A. Ribbon drawing of the closed conformation of Syntaxin1a[59] (in complex with Sec1, in grey); molecular coordinates from PDB 1DN1. Syntaxins are composed of three α-helices (colored blue, green, and red), connected by a linker sequence (orange) to the SNARE helix (white). B. Distinct location of morphogenic and membrane fusion functional domains. Deletion analysis of epimorphin/syntaxin-2 have shown that mutant molecules containing helices A-C are sufficient to mediate the morphogenic activity, and that the SNARE domain is dispensible[40], while the SNARE domain is specifically required for membrane fusion activity[6]. C,D. Both syntaxin-1a (Syn1) and epimorphin/syntaxin-2 (E/S2) are synthesized as 34 kDa molecules, but only epimorphin is released from the cell surface as a 30 kDa molecule. Histidine-246 (H246) of epimorphin/syntaxin-2 is the key residue determining extracellular secretion[46]. C. Sequence alignment of epimorphin/syntaxin-2 from mouse (m), human (h), quail (q), and sheep (s) reveals that H246 is conserved, while the corresponding positioning nonsecreted syntaxins is occupied by an arginine in syntaxin-1, syntaxin-3 (Syn3), and syntaxin-4 (Syn4). D. Mutation of H246 in epimorphin/syntaxin-2 to arginine blocks secretion into the supernatant (Sup), whereas mutation of R246 to histidine in syntaxin-1a is sufficient to lead to extracellular secretion. (Data in C,D modified with permission from ref [46].)

Table 1. Molecules secreted by non-classical pathways that possess distinct intracellular and extracellular function

Protein name	Intracellular function	Extracellular function
Syntaxin-2/Epimorphin	Vesicle trafficking[9], cytokinesis [10]	Morphogen [2]
HMGB1/Amphoterin	DNA-binding protein [21]	Cytokine[14]
Tissue transglutaminase	Cell signaling[24,26]	ECM-modifying protein[29]
RHAMM/CD168	ERK1,2 signaling [60]	Cell surface hyaluronan receptor [61]
Annexin II	Cytokinesis and vesicle trafficking[33,34]	Cell surface receptor [32]
Phosphohexose isomerase/ autocrine motility factor	Glycolysis/homeostasis [37]	Cytokine/morphogen [35,36]
Thioredoxin/ADF	Redox reactions[62]	Immunomodulatory cytokine [63]
Ku	DNA repair [39]	Cell adhesion [38]

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Figure 1

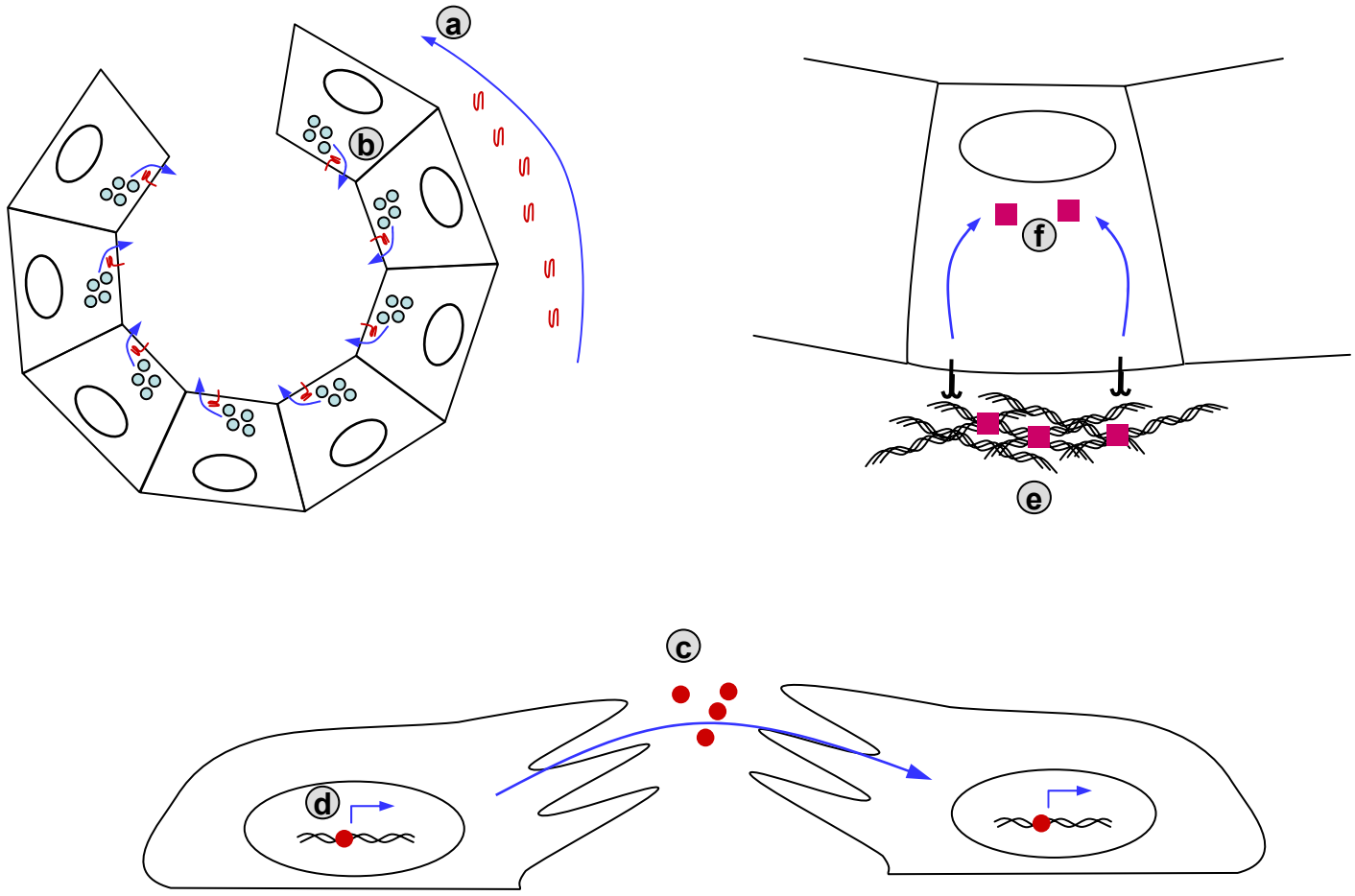
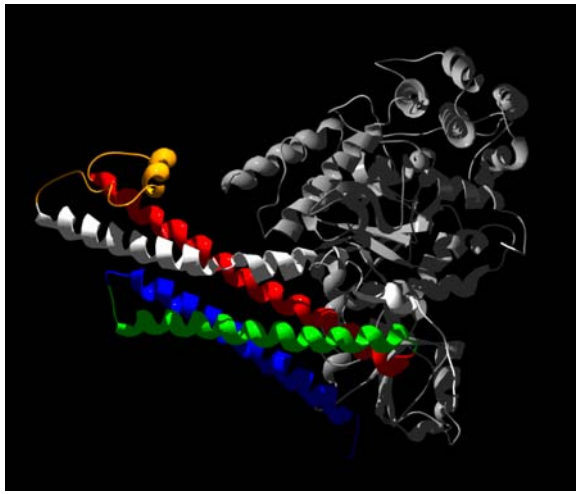


Figure 2

A



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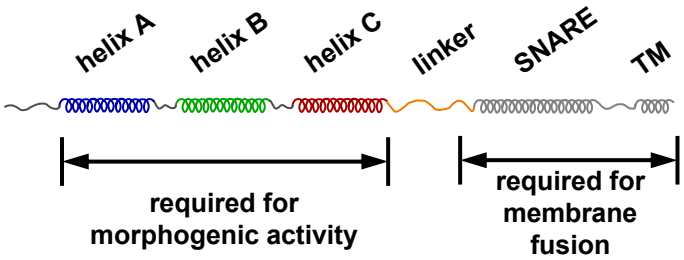

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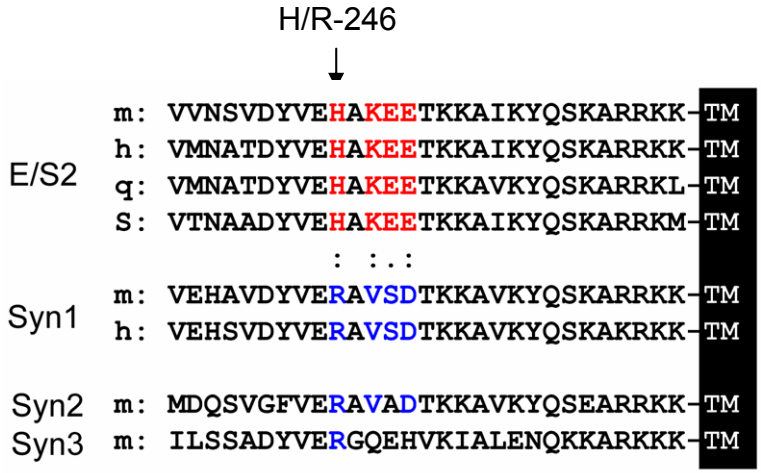

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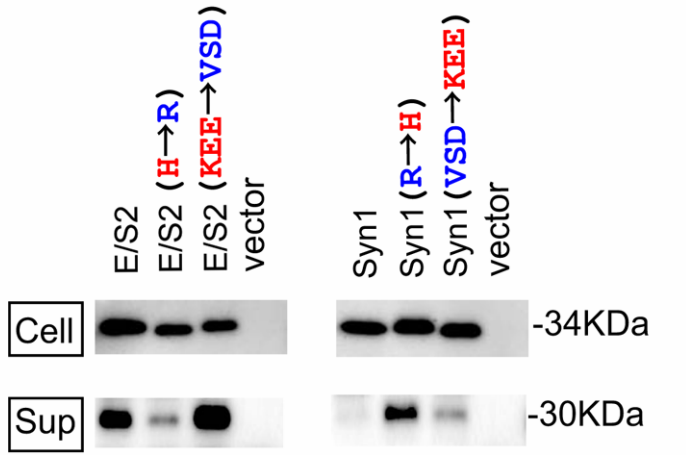
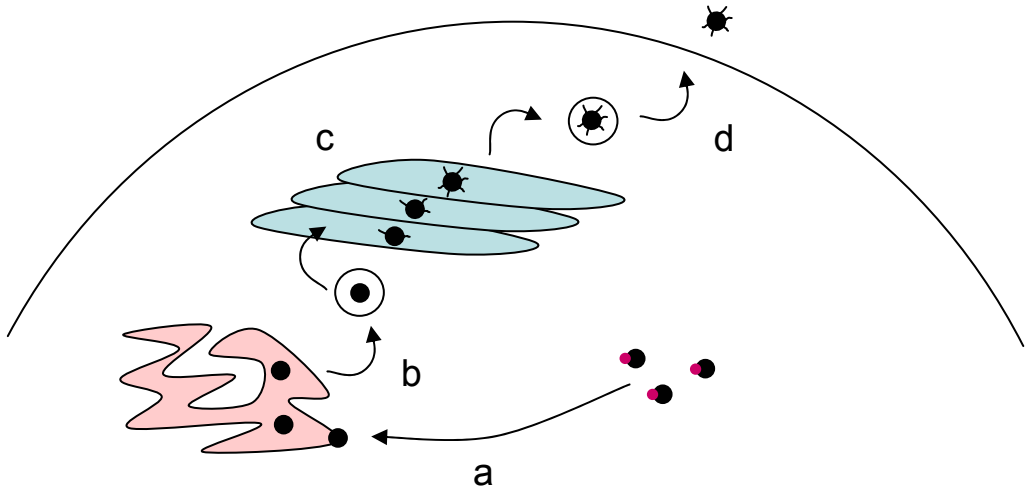


Box 1

A



B