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#### Acknowledgements

The authors are supported by grants to O.V. from the European Research Council, 'Frontiers of RNAI' 210890 and from the Fondation Bettancourt pour les Sciences du Vivant, and to O.V. and P.B. from the European Union (MC-EIF-25064-2005). The authors thank S. Pfeiffer, L. Navarro, B. Wulff and D. Gibbins for comments on the manuscript.

#### DATABASES

Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>  
 AP2 | *cog-1* | *CSD1* | *CSD2* | *DNMT3B* | *p16<sup>INK4A</sup>* | *PHB*  
 The miRNA Registry: <http://microrna.sanger.ac.uk/sequences>  
 let-7 | lin-4 | lsy-6 | miR-10a | miR-24 | miR-148 | miR-164 | miR-165 | miR-172 | miR-398 | miR-430 | miR-834 | miR-H2-3p  
 UniProtKB: <http://www.uniprot.org>  
 Dicer | Drosha | ICP0 | nanog | OCT4 | SOX2

#### FURTHER INFORMATION

Olivier Voinnet's homepage: [http://ibmp.u-strasbg.fr/index.php?id=336L=16tx\\_labstructdef\\_pi1\[showUId\]=96tx\\_labstructdef\\_pi1\[viewType\]=member&chash=67efd20d58](http://ibmp.u-strasbg.fr/index.php?id=336L=16tx_labstructdef_pi1[showUId]=96tx_labstructdef_pi1[viewType]=member&chash=67efd20d58)

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#### OPINION

## Mechanisms of regulated unconventional protein secretion

Walter Nickel and Catherine Rabouille

**Abstract** | Most eukaryotic proteins are secreted through the conventional endoplasmic reticulum (ER)–Golgi secretory pathway. However, cytoplasmic, nuclear and signal-peptide-containing proteins have been shown to reach the cell surface by non-conventional transport pathways. The mechanisms and molecular components of unconventional protein secretion are beginning to emerge, including a role for caspase 1 and for the peripheral Golgi protein GRASP, which could function as a plasma membrane tether for membrane compartments during specific stages of development.

Most secretory proteins contain amino-terminal or internal signal peptides that direct their sorting to the endoplasmic reticulum (ER). From the ER, proteins are transported to the extracellular space or the plasma membrane through the ER–Golgi secretory pathway<sup>1,2</sup> (BOX 1).

Although the ER–Golgi system is an extremely efficient and accurate molecular machine of protein export<sup>3</sup>, two types of non-conventional protein transport to the cell surface of eukaryotic cells have been discovered: these processes are known as unconventional protein secretion<sup>4</sup>. On the one hand, signal-peptide-containing proteins, such as yeast heat-shock protein 150 (Hsp150)<sup>5</sup>, the cystic fibrosis transmembrane conductance regulator (CFTR)<sup>6</sup>, CD45 (REF. 7), the yeast protein Ist2 (REF. 8) and the *Drosophila melanogaster*  $\alpha$  integrin subunit<sup>9</sup>, are inserted into the ER but reach the cell surface in a coat protein complex II (COPII) machinery- and/or Golgi-independent manner. On the other hand, cytoplasmic and nuclear proteins that lack an ER-signal peptide have been shown to exit cells through ER- and Golgi-independent pathways. Such proteins include fibroblast growth factor 2 (FGF2)<sup>10–13</sup>,  $\beta$ -galactoside-specific lectins, galectin 1, galectin 3 (REFS 12, 14–17), certain members of the interleukin family<sup>12, 18, 19</sup>, the nuclear proteins HMGB1 (REFS 20–22) and engrailed homeoprotein<sup>23–26</sup>, as well as the recently discovered *Dictyostelium discoideum* acyl-coenzyme A-binding protein (Acba)<sup>27</sup>. We refer to these proteins as cytoplasmic/nuclear secretory proteins. In the extracellular environment, these macromolecules are crucial regulators of the immune response, cell growth, differentiation and angiogenesis.

Here, we begin by discussing the possible mechanisms that underlie these two types of unconventional protein transport. We then address the emerging role of caspase 1 in the unconventional secretion of cytoplasmic cytokines. Finally, we highlight possible roles for GRASP, a Golgi-associated peripheral membrane protein<sup>28, 29</sup> that is involved in both types of unconventional secretion.

#### Signal-peptide-containing proteins

Some signal-peptide-containing proteins have been shown to traffic unconventionally; either their exit from the ER does not seem to involve COPII vesicles or their transport from the ER to the plasma membrane bypasses the Golgi apparatus.

**Bypassing COPII vesicles.** Although most of the signal-peptide-containing proteins use COPII-coated vesicles to exit the ER (BOX 1), a number of proteins have been shown to behave differently. For example, the yeast protein Hsp150 does not seem to depend on the COPII coat proteins Sec24 and Sec13 (REFS 5, 30, 31) to exit the ER *en route* to the plasma membrane. Overexpression of dominant-negative Sar1 mutants (Sar1 is one of the core yeast COPII proteins) does not block ER exit of the voltage-sensitive potassium channel (Kv4 K<sup>+</sup>) when associated with its interacting proteins (KChIPs). Therefore, ER exit of this protein does not seem to be mediated by COPII-coated vesicles<sup>32</sup>. Lastly, ER degradation-enhancing  $\alpha$ -mannosidase-like protein 1 (EDEM1), which is a crucial regulator of ER-associated degradation (ERAD), does not accumulate in the ER under normal conditions because it is removed from the ER lumen by specific sequestration into ER-derived LC3-I-coated

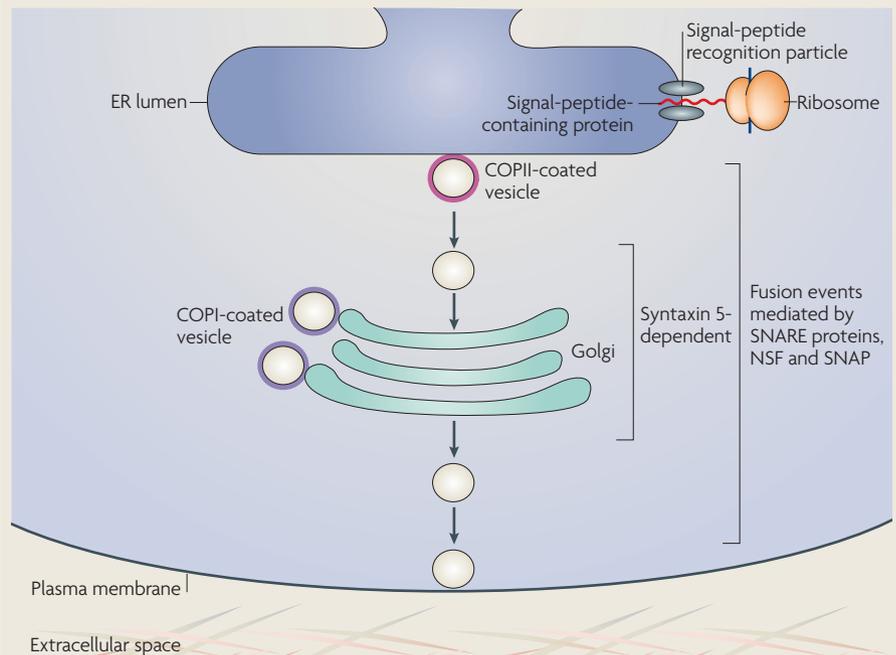
vesicles. LC3-I is a small protein that can form *trans*-oligomers and is required for autophagy<sup>33</sup>. However, EDEM1 sequestration is clearly distinct from autophagy and provides the first evidence for novel, possibly coated structures at the ER<sup>34</sup>.

**Bypassing the Golgi.** Cell surface transport of most plasma membrane proteins is sensitive to the drug brefeldin A (BFA). BFA is a potent inhibitor of membrane recruitment of the small GTPase ADP-ribosylation factor 1 (ARF1), which is the first step in the formation of COPI-coated vesicles from Golgi membrane<sup>35</sup> (BOX 1). COPI-coated vesicles mediate retrograde transport of resident enzymes in the Golgi apparatus and back to the ER<sup>36</sup>, but they are possibly also involved in anterograde transport of cargo proteins in the Golgi apparatus<sup>37</sup>.

However, the transport of a number of proteins does not seem to be sensitive to BFA. First, a pool of CD45, which is a receptor protein Tyr phosphatase that is essential for T-cell development, has been shown to be transported rapidly to the plasma membrane in a BFA-insensitive manner, which suggests that CD45 bypasses the Golgi<sup>7,38</sup>. This is supported by the high mannose content of its N-linked oligosaccharide chains — a characteristic feature of ER glycosylation — which suggests that these chains have not been modified in the Golgi apparatus. The simian rotavirus Rhesus monkey rhadinovirus (RRV), which is transported to the apical side of human tissue-cultured colon cancer Caco-2 cells<sup>39</sup>, was observed to exit the ER in vesicular structures but was undetectable in the Golgi or endosomal compartments. Finally, the antiviral secreted protein ovine *MX1* is also secreted by uterine epithelial cells in a BFA-insensitive manner<sup>40</sup>.

The best-documented example of a protein that bypasses the Golgi is the transmembrane protein CFTR, which reaches the plasma membrane of baby hamster kidney (BHK) and Chinese hamster ovary (CHO) cells (but not HeLa cells) by direct transport from the ER by COPII carriers<sup>41</sup>. This pathway does not depend on *syntaxin 5* (a t-SNARE (target soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) accessory protein (SNAP) receptor), which is required for the movement of newly synthesized proteins to and through the Golgi in all eukaryotes<sup>42</sup>) (BOX 1). CFTR deposition in the plasma membrane does not depend on ARF1, or on the small GTPases RAB1A and RAB2 that are otherwise essential for anterograde transport in the early secretory pathway. However, it is blocked by overexpression of

### Box 1 | The conventional secretory pathway



Most of the signal-peptide-containing proteins that are directed to the cell surface or the extracellular space are transported through the conventional secretory pathway (see the figure). Proteins enter the endoplasmic reticulum (ER) as nascent proteins through the signal-peptide recognition particle. The newly synthesized proteins then exit the ER at specialized membrane domains called ER exit sites or tER sites, from which cargo-containing coat protein complex II (COPII)-coated vesicles form. The yeast COPII coat, which consists of the small GTPase Sar1, Sec23–Sec24 and Sec13–Sec31, also ensures cargo selection either via Sec24 (REF. 83) or via interactions with cargo receptors<sup>84,85</sup>. The newly synthesized proteins reach the Golgi apparatus, where they are modified, processed, sorted and dispatched towards their final destination.

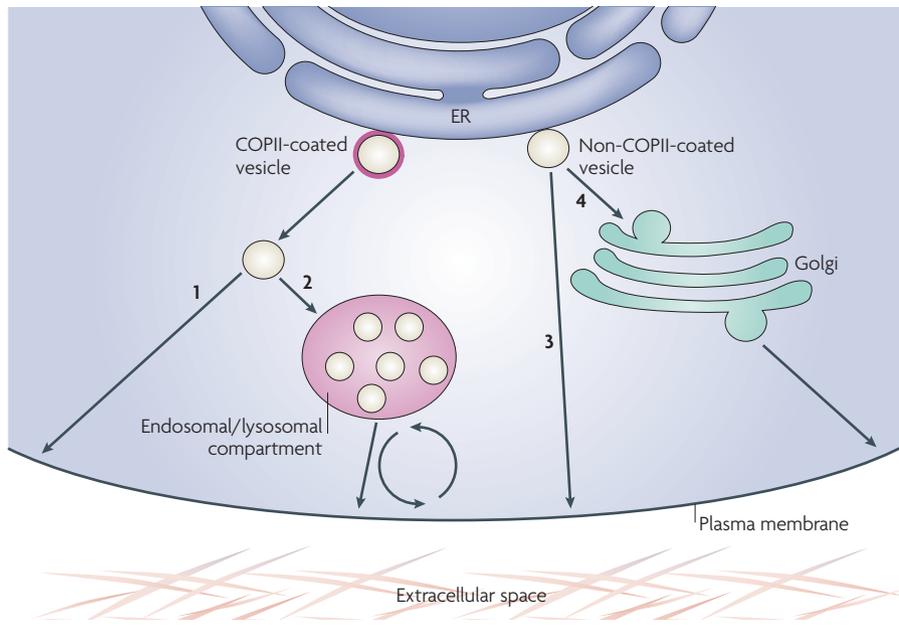
Transport in the secretory pathway depends on the formation of another coated vesicular intermediate, COPI-coated vesicles, which comprise the small GTPase ADP ribosylation factor 1 (ARF1) and coatomer, a heptameric protein complex. COPI vesicles are primarily formed in the Golgi and mediate retrograde movement of components in the Golgi and back to the ER<sup>36</sup>, as well as anterograde movement of certain components. The fungal metabolite brefeldin A blocks the recruitment of ARF1 to the Golgi and the formation of COPI-coated vesicles, and ultimately blocks ER–Golgi transport.

Transport within the secretory pathway involves a series of fusion events that occur between vesicular intermediates and organelles and that are catalysed by SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) accessory protein (SNAP) receptors). Membrane fusion is mediated by the formation of tight *trans*-SNARE complexes that are formed by  $\alpha$ -helical bundles of the cytoplasmic SNARE domains. These are derived from vesicle (*v*-SNAREs) and target (*t*-SNAREs). The opposing membrane is brought into close proximity, and eventually results in membrane fusion. The *trans*-SNARE complexes are then dissociated by the hexameric ATPase NSF, which binds to the complex via SNAP. This allows the recycling of *v*-SNAREs and their function in another round of vesicular transport. Syntaxin 5 is an example of a t-SNARE that has been shown to be crucial for transport to and through the Golgi in all model systems<sup>42</sup> (for a review on the machinery of tethering and docking factors, see REF. 86).

syntaxin 13, a t-SNARE that resides in late/recycling endosomes, which suggests that CFTR might be stored in, or cycle through, the late/recycling endosome compartment before its deposition at the plasma membrane<sup>6</sup>.

All of the proposed models that explain the unconventional secretion of signal-peptide-containing proteins to the plasma membrane (FIG. 1) involve the formation of

ER-derived carriers (whether COPII-coated or not). They directly fuse with the plasma membrane or with a late endosomal/lysosomal compartment, followed by transport to the plasma membrane. As is the case for most cellular fusion events, direct fusion of ER-derived vesicles to either the endosomal/lysosomal compartment or the plasma membrane is likely to depend on the typical fusion machinery, which comprises the formation



**Figure 1 | Unconventional transport of signal-peptide-containing proteins.** Following endoplasmic reticulum (ER) translocation, signal-peptide-containing proteins are packaged into coat protein complex II (COPII)-coated vesicles that fuse directly with the plasma membrane (mechanism 1). Alternatively, they can fuse with an endosomal or lysosomal compartment (such as late endosomes in the case of cystic fibrosis transmembrane conductance regulator) that, in turn, fuses with the plasma membrane (mechanism 2). Proteins can also be packaged into non-COPII-coated vesicles that can fuse directly with the plasma membrane (mechanism 3) or can be targeted to the Golgi apparatus (mechanism 4) before reaching the plasma membrane.

of cognate complexes of SNARE proteins, the ATPase NSF and its cofactor SNAP<sup>43,44</sup> (BOX 1). Interestingly, a comprehensive study on yeast SNARE pairing in artificial membranes (liposomes) has revealed an unusual but functional SNARE pair that involves the ER vesicle (v)-SNARE Sec22 and the plasma membrane t-SNARE Sec9. This suggests that fusion of the ER-derived membrane to the plasma membrane could occur<sup>45,46</sup>.

The yeast multispan membrane protein Ist2 has also been shown to reach the plasma membrane in a Golgi-independent manner. However, in this case, the transport route has been suggested to be independent of SNAREs, NSF and SNAP<sup>8,47</sup>, and seems to be mediated by direct transfer of Ist2 from cortical ER domains to the plasma membrane<sup>4</sup>.

It is currently unclear why signal-peptide-containing proteins bypass the Golgi apparatus. This prevents the processing of their oligosaccharide chains as well as potential proteolytic cleavages, two processes that can ultimately lead to a modulation of their biological activity. Therefore, it will be important to analyse whether Golgi-independent transport routes of signal-peptide-containing proteins are mechanisms that adapt properties of secretory proteins to specific physiological needs.

**Cytoplasmic/nuclear secretory proteins**

More than 20 cytoplasmic/nuclear secretory proteins have been described that are exported from cells by unconventional means<sup>4,12,48</sup>, and it is likely that a large number of further examples will be discovered in future studies. Among those that have been described are proteins with a main function in the extracellular space and those with both intracellular and extracellular roles<sup>4</sup>. Although the details of the secretory mechanisms of most of these proteins remain elusive, four different main pathways have been proposed to have a potential role in unconventional protein secretion (FIG. 2).

**Translocation across the plasma membrane.**

Translocation across the plasma membrane is best shown by the secretion of FGF2 (FIG. 2, mechanism 1). FGF2 is first recruited by the phosphoinositide phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) at the inner leaflet of the plasma membrane<sup>49</sup>. This interaction is crucial for FGF2 export, because a reduction of cellular PtdIns(4,5)P<sub>2</sub> levels results in a substantial drop in FGF2-export efficiency. Accordingly, mutations in FGF2 that prevent binding to PtdIns(4,5)P<sub>2</sub> block secretion. In the extracellular space,

heparan sulphate proteoglycans (HSPGs) have recently been shown to participate in FGF2 secretion<sup>50</sup>, in addition to their known role in FGF2 storage and signalling<sup>51</sup>. We propose that membrane-proximal HSPGs are functioning as an extracellular trap that drives the net export of FGF2 (REF. 52). Furthermore, FGF2 membrane translocation does not depend on ATP hydrolysis or membrane potential<sup>53</sup>. Whether FGF2 translocation across the plasma membrane is mediated by a transporter or by an unrecognized ability of FGF2 to insert into membranes is unknown<sup>4</sup>.

**Lysosome-dependent pathway.**

The second pathway for unconventional protein secretion involves the sequestration of a soluble cytoplasmic factor by secretory lysosomes, a compartment that has features of both lysosomes and secretory granules (FIG. 2, mechanism 2). This has been best documented for interleukin 1β (IL-1β). During an inflammatory response (which can be triggered by several processes, including septic shock), a precursor form of IL-1β is synthesized in response to bacterial lipopolysaccharides. Both IL-1β and caspase 1, the protease that is known to convert the precursor into the mature form of IL-1β, are then translocated into secretory lysosomes, but the nature of the transporters remains elusive. A second trigger, extracellular ATP, is proposed to promote the fusion of secretory lysosomes to plasma membranes, which results in the release of mature IL-1β and caspase 1 into the extracellular space<sup>54,55</sup>.

**Microvesicle-dependent secretion.**

The third pathway for unconventional protein secretion (FIG. 2, mechanism 3), which might also explain the secretion of mature IL-1β, involves the shedding of microvesicles at the extracellular side of the plasma membrane<sup>56,57</sup>. In this case, caspase 1 activates IL-1β in the cytoplasm and is exported along with the mature cytokine. This is also true for the fourth mechanism (FIG. 2, mechanism 4). The formation of endosomal intraluminal vesicles was suggested to sequester the complex made of cytoplasmic mature IL-1β-caspase 1, thereby leading to the formation of IL-1β-enriched multivesicular bodies (MVBs). MVBs have well-characterized roles in receptor downregulation and protein degradation<sup>58,59</sup>, but they can also release internal vesicles into the extracellular space following fusion with the plasma membrane; these vesicles, which are known as exosomes, might contain activated IL-1β as well as caspase 1 (REF. 91).

Why do cytoplasmic secretory proteins use unconventional mechanisms of transport instead of the classical secretory pathway? In the case of galectins, it has been suggested that binding to oligosaccharide species in the lumen of the ER–Golgi complex might cause aggregation and misfolding. Similarly, signalling molecules could prematurely bind to their receptors in the lumen of the ER–Golgi complex, thus triggering potentially detrimental autocrine signalling. Last, the oxidative environment in the lumen of the ER and the Golgi apparatus might also cause misfolding of certain cytoplasmic secretory proteins, such as thioredoxin. As cytoplasmic secretory proteins do not seem to unfold during plasma membrane translocation, and, therefore, do not need to fold again in the extracellular space (as suggested for FGF2 (REF.4)), a possible misfolding in the lumen of the ER–Golgi-dependent secretory pathway might be detrimental for their function.

Experimental evidence further suggests that FGF2 cannot be secreted via the classical route in a functional form. FGF2 fused to an ER-signal peptide at its N terminus (SP–FGF2) can be efficiently secreted to the extracellular medium via the ER–Golgi-dependent secretory pathway, but it is not biologically active. This is due to non-physiological O-glycosylation of this chimeric protein<sup>60</sup>, which prevents both storage in the extracellular matrix and HSPG-dependent FGF2 signalling.

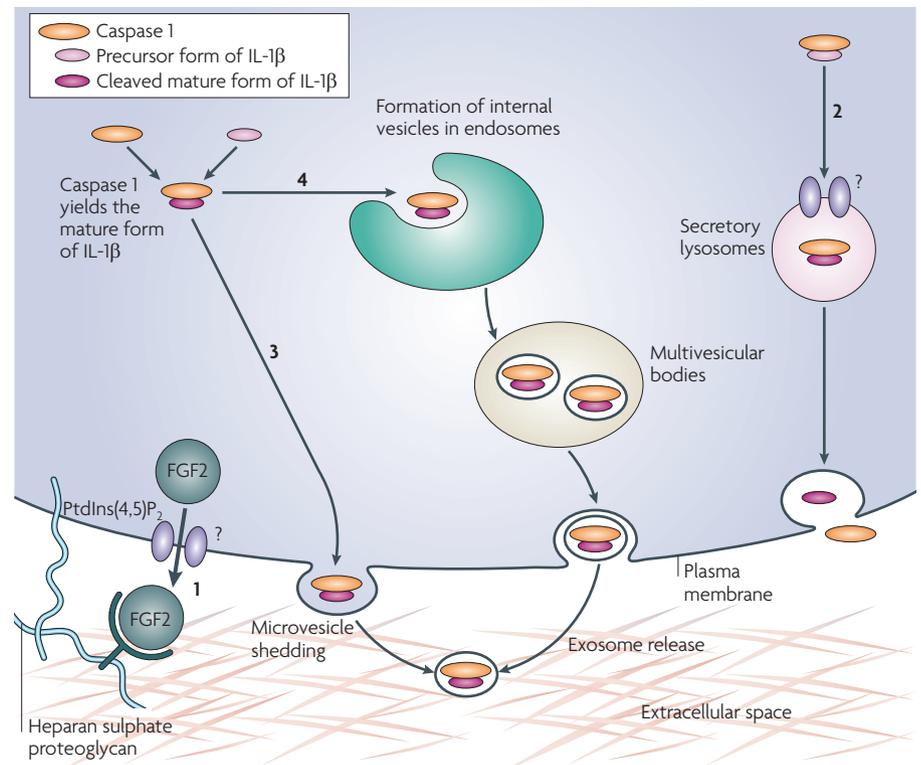
It is unclear at what point the process of unconventional secretion of cytoplasmic proteins emerged during evolution, but it might have preceded the so-called classical secretory pathway or it might have evolved concomitantly. If it preceded the classical pathway, this mechanism was kept during evolution because the emerging ER–Golgi pathway was incapable of secreting, for example, FGF2, in a functional form.

### Emerging roles of caspase 1

The number of factors (core machinery and regulators) that are known to have a role in unconventional secretion of cytoplasmic/nuclear proteins is small. However, caspase 1 has emerged as an important regulatory molecule. This goes much further than its role as the processing protease of IL-1 $\beta$  during inflammation to produce the mature and active form of this cytokine (see above)<sup>61,62</sup> (FIG. 3).

### The inflammasome and IL-1 $\beta$ secretion.

Caspase 1 is a key regulator of the inflammatory response because it cleaves, in addition to IL-1 $\beta$ , two crucial immunological

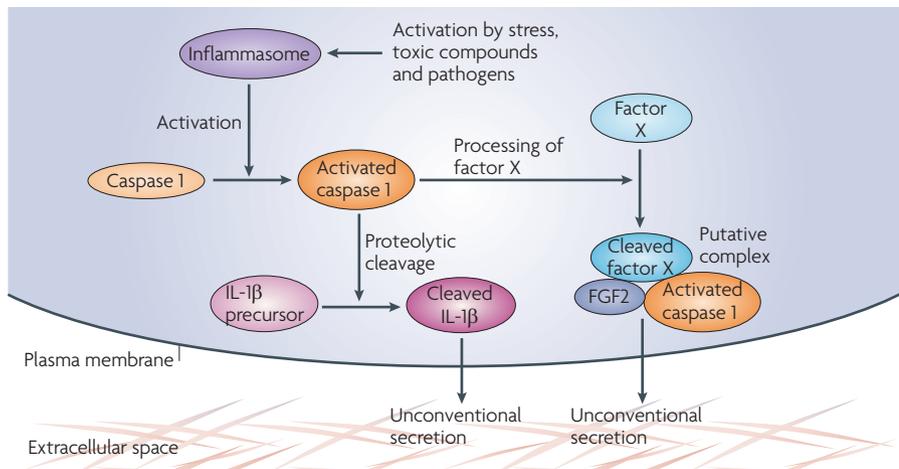


**Figure 2 | Unconventional secretion of soluble cytoplasmic proteins.** Four different mechanisms for unconventional secretion of soluble proteins have been proposed. The first (mechanism 1) is non-vesicular. Cytoplasmic proteins, such as fibroblast growth factor 2 (FGF2), can be directly translocated from the cytoplasm across the plasma membrane via recruitment by phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) and extracellular trapping by heparan sulphate proteoglycans (HSPGs), the sugar side chains of which are bound by FGF2. The remaining three mechanisms depend on vesicular intermediates and have been best shown with the cytoplasmic cytokine interleukin 1 $\beta$  (IL-1 $\beta$ ). IL-1 $\beta$  is translocated into secretory lysosomes together with caspase 1. This produces a mature form of the cytokine. The fusion of these lysosomes with the plasma membrane leads to the release of their content in the extracellular space. Caspase 1 cleaves IL-1 $\beta$  and is secreted along with the mature cytokine (mechanism 2). Microvesicle shedding from the cell surface can also lead to the release of mature IL-1 $\beta$  and caspase 1 into the extracellular space (mechanism 3). Lastly, the caspase 1–IL-1 $\beta$  complex can be captured from the cytoplasm during the formation of endosomal internal vesicles, which leads to the biogenesis of multivesicular bodies. These internal vesicles are then released as exosomes (mechanism 4). Note that in the last two scenarios (mechanisms 3,4), IL-1 $\beta$  and caspase 1 are released into the extracellular space surrounded by a membrane. The question marks drawn next to the transporters in steps 1 and 2 indicate that their identities have not been revealed.

cytokines of the interleukin family, IL-18 and IL-33. This proteolytic activation is a prerequisite for the secretion of these molecules.

Caspase 1 activity is itself regulated by the innate immune system. In macrophages, caspase 1 has recently been shown to be activated by inflammasomes<sup>63,64</sup>. Inflammasomes function as intracellular sensors and are multiple distinct protein complexes that are activated by specific internal and external triggers, such as endogenous danger signals (for example, extracellular ATP that is released from dying cells), exogenous toxic compounds and molecules that are associated to, or deposited by, pathogens (such as

lipopolysaccharide)<sup>63,65–69</sup>. In human primary keratinocytes, the inflammasome is triggered by exposure to ultraviolet B. In both macrophages and keratinocytes, inflammasome activation leads to the activation of caspase 1 and eventually the maturation and secretion of mature IL-1 $\beta$ <sup>70</sup>. The newly discovered NALP (nucleotide-binding domain-, Leu-rich repeat- and PYD-containing protein) family has been used to define different subtypes of inflammasomes that respond to different signals that lead to caspase 1 activation. Thus, caspase 1 is a key regulator of the inflammatory response as it links the activation of inflammasomes to the unconventional secretion of cytokines, such as IL-1 $\beta$  (FIG. 3).



**Figure 3 | Proposed roles for caspase 1 in the unconventional secretion of cytokines.** Innate immune protein complexes (inflammasomes) are activated by external or internal triggers (stress, toxic and pathogenic compounds). Activation of the inflammasome leads to the activation of caspase 1, which cleaves the interleukin 1 $\beta$  (IL-1 $\beta$ ) precursor form and produces its active mature form. This mature form is unconventionally secreted by three possible pathways (FIG. 2). Activated caspase 1 has also been shown to interact with other cytokines (such as fibroblast growth factor 2 (FGF2)). Although these cytokines are not cleaved by caspase 1, the proteolytic activity of caspase 1 is required for their secretion. It is therefore possible that caspase 1 cleaves an unknown substrate, factor X. Caspase 1, the cleaved factor X and these cytokines (for example, FGF2) might then form a complex, which is secreted through unconventional pathways.

**Secretion of cytoplasmic secretory proteins.**

A recent study has shown that caspase 1 activation does not only have a role in the secretion of IL-1 $\beta$ , but also for a number of other unconventionally secreted cytokines<sup>71</sup>, thereby adding an interesting twist to the caspase 1 story. Pharmacological inhibition or downregulation of caspase 1 by RNA interference blocked the secretion of IL-1 $\beta$  but also IL-1 $\alpha$ , macrophage migration inhibitory factor (MIF), galectin 1, galectin 3 and FGF2. Although the mechanistic details remain to be elucidated, these results suggest that there is a general regulatory role for caspase 1 and might provide a link between the distinct unconventional protein secretion pathways that are described above. The role of caspase 1 in the secretion of proteins such as FGF2 might be mediated by unknown factors that

are substrates of caspase 1, the cleavage products of which might have a direct role. These considerations suggest the potential existence of hetero-oligomeric protein complexes of caspase 1, novel caspase 1 substrates and unconventional secretory proteins (FIG. 3). Their biochemical and biological characterization will be a major goal for future research efforts in this field. Whether the inflammasome has a general role in the secretion of cytokines remains to be seen.

**Emerging roles of GRASP**

GRASP65 and GRASP55 are two mammalian myristoylated proteins that are associated with the cytoplasmic leaflet of Golgi membranes and they were originally identified as Golgi cisternae stacking factors *in vitro*<sup>28,29</sup>. GRASP65 can form *trans*-oligomers — the

formation of which is crucial for stacking *in vitro*<sup>72</sup> — but its role in Golgi stacking *in vivo* remains controversial<sup>73–76</sup>. However, based on their putative role in tethering<sup>72</sup>, both GRASPs have a clear role in Golgi organization, in particular in the formation of the Golgi ribbon in mammalian cells<sup>74,75</sup>. Both proteins are also heavily phosphorylated during mitosis in their carboxy-terminal domains and have a central role in cell-cycle control at the Golgi G2–M-phase checkpoint. Indeed, it was proposed that specific phosphorylation of GRASP65 and GRASP55 at G2 leads to Golgi ribbon unlinking, a process that is crucial for cells to enter mitosis<sup>77</sup>. In organisms such as yeast and *D. melanogaster* and in mammalian cells, GRASP proteins are largely dispensable for anterograde transport to the plasma membrane<sup>73,74,78,79</sup>. However, GRASP55 has specifically been implicated in the transport of transforming growth factor- $\alpha$  (TGF $\alpha$ ) to the plasma membrane. The two proteins interact and GRASP55 has been proposed to function as a chaperone<sup>80</sup>.

Surprisingly, the single homologue of GRASP65 and GRASP55 in *D. discoideum*, GrpA, has recently been shown to be required for the unconventional secretion of the soluble cytoplasmic AcbA<sup>27</sup>, whereas the *D. melanogaster* GRASP<sup>79</sup> is required for the unconventional secretion of integral plasma membrane  $\alpha$  integrins<sup>9</sup>.

**GrpA and unconventional secretion of AcbA.**

AcbA is one of the rare examples of an unconventionally secreted cytoplasmic protein in lower eukaryotes<sup>27</sup>, although the yeast *a*-factor, a small lipopeptide, is also known to be secreted without the involvement of the conventional secretory pathway (BOX 2).

AcbA is required for the terminal differentiation of *D. discoideum* prespore cells. It is proposed to be secreted from these cells by an unconventional secretory pathway, as it does not have a signal peptide<sup>81</sup>. Following release, it is proteolytically cleaved by TagC<sup>92</sup>. This leads to the formation of an extracellular peptide, Sdf2, which binds to a receptor at the surface of prespore cells and triggers their terminal differentiation. Intriguingly, this process is strongly reduced in a *D. discoideum* *grpA* mutant, which is probably due to a block in AcbA secretion. Indeed, the addition of exogenous AcbA to the *grpA* mutant prespore cells fully restores their differentiation.

The molecular mechanism by which AcbA is released from *D. discoideum* prespore cells is elusive. First, GrpA could mediate the direct non-vesicular translocation of

**Box 2 | Unconventional secretion of cytoplasmic factors in yeast**

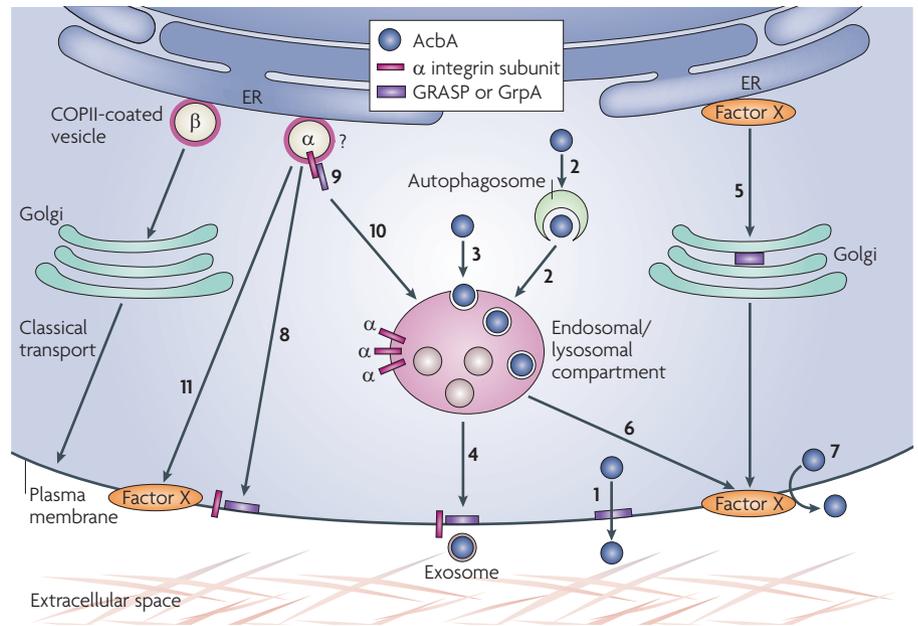
It is not clear whether unconventional secretion of soluble proteins exists in yeast. So far, the only known example of endoplasmic reticulum (ER)–Golgi-independent secretion in *Saccharomyces cerevisiae* involves *a*-factor, a small prenylated mating peptide of 12 amino acids, the secretion of which is mediated by Ste6 (REF. 87). Ste6 belongs to the family of ATP-binding cassette (ABC) transporters, which are involved throughout eukaryotes in, for example, the translocation of peptides across the ER membrane<sup>88</sup> or cholesterol across the plasma membrane<sup>89</sup> (by ABCA1).

There is no evidence that the export of the *a*-factor lipopeptide is related to direct membrane translocation of unconventional secretory proteins (see the main text). For example, in contrast to fibroblast growth factor 2 (FGF2), *a*-factor is secreted by an ABC transporter and, therefore, does depend on ATP hydrolysis. Furthermore, general inhibitors of various classes of ABC transporters do not block FGF2 secretion<sup>90</sup>. Finally, FGF2 is thought to be transported in a folded state, which is a mechanism that ABC transporters are unlikely to support.

AcbA across the plasma membrane (FIG. 4, pathway 1). Alternatively, AcbA could become captured from the cytoplasm by a specific autophagy-like process and be delivered to late endosomal or lysosomal compartments<sup>82</sup> (FIG. 4, pathway 2). AcbA could also be directly sequestered in internal vesicles of MVBs (FIG. 4, pathway 3). GrpA could have one of two roles. First, in preparation of membrane fusion, GrpA could mediate the tethering of the endosomal or lysosomal compartment to the plasma membrane (FIG. 4, pathway 4). Second, consistent with its subcellular localization as a Golgi-resident protein, GrpA could be required for the proper delivery of at least one specific factor to the plasma membrane (FIG. 3, pathway 5), thus mirroring the role of GRASP55 in mediating TGF $\alpha$  transport to the plasma membrane<sup>80</sup>. In turn, this factor could be crucial for the tethering of AcbA-enriched endosomal or lysosomal compartments to the plasma membrane, and would therefore be essential for the release of AcbA as part of exosomes (FIG. 4, pathway 6). Alternatively, during prespore differentiation, this factor could directly mediate AcbA translocation across the plasma membrane (FIG. 4, pathway 7), although the mechanism remains unknown.

#### GRASP-dependent secretion of $\alpha$ integrins.

In *D. melanogaster*, GRASP is also required for Golgi-independent cell surface transport of a signal-peptide-containing protein, the integral plasma membrane protein  $\alpha$  integrin. During remodelling, epithelial cells change their adhesion to the basal extracellular matrix, which might require the specific and spatially restricted deposition of  $\alpha$  or  $\beta$  integrins to the remodelled membrane. At the membrane, they induce the formation of new focal adhesion sites that are required for epithelium integrity<sup>9</sup>. In a *D. melanogaster* *grasp* mutant, however, the  $\alpha$  integrin (but not the  $\beta$  integrin) subunits are not properly deposited at the plasma membrane. Instead, they are retained intracellularly and, as a result, the epithelium is disrupted<sup>9</sup>. These results suggest the existence of a GRASP-dependent secretory route that is specific for  $\alpha$  integrin. Furthermore, this route bypasses the Golgi complex as it is not sensitive to BFA and is fully functional in the absence of syntaxin 5. Of note, the Golgi-independent transport of  $\alpha$  integrins occurs only during epithelium remodelling<sup>9</sup>. Under all other conditions,  $\alpha$  and  $\beta$  integrins are transported as heterodimers through the conventional secretory pathway. Why  $\alpha$  transport switches from



**Figure 4 | Proposed roles for GRASP in unconventional protein secretion.** In *Dictyostelium discoideum* and *Drosophila melanogaster*, the single GRASP orthologue (GrpA or GRASP, respectively) is required for unconventional secretion at specific developmental stages. During *D. discoideum* spore differentiation, GrpA is required for unconventional secretion of acyl-coenzyme A-binding protein (AcbA). GrpA might mediate direct transport of AcbA across the plasma membrane by a mechanism that remains to be defined (pathway 1). Alternatively, it might act as a tether for endosomal or lysosomal compartments that have engulfed cytoplasmic AcbA in exosomes, or to which AcbA has been delivered by an autophagy-like process (pathways 2–4). Another option is that GrpA is present at the Golgi apparatus, where it mediates the proper delivery of an unidentified factor X (pathway 5). This mechanism might reflect the role of GRASP55 in mediating transforming growth factor- $\alpha$  (TGF $\alpha$ ) transport to the plasma membrane in mammalian cells<sup>80</sup>. Such a factor might be required either for the tethering of AcbA-enriched endosomal or lysosomal compartments to the plasma membrane before their fusion (pathway 6), or for the direct transport of AcbA across the plasma membrane (pathway 7). At certain stages of *D. melanogaster* development, GRASP is required for the delivery of  $\alpha$  integrin (but not  $\beta$  integrin) subunits to the plasma membrane in a Golgi-independent manner. During these stages, GRASP is anchored to the plasma membrane and functions as a tether for either endoplasmic reticulum (ER)-derived carriers (pathway 8), or for endosomal or lysosomal compartments, both of which are enriched in  $\alpha$  integrin subunits (pathways 4,10). This mechanism is similar to the proposed mechanism for AcbA (pathways 3,4). Furthermore, GRASP could bind to the cytoplasmic tail of  $\alpha$  integrin in the ER-derived carriers (pathway 9), and given its *trans*-oligomerization properties, this might help the tethering of the incoming vesicles. Finally, similar to AcbA, factor X could help with the tethering of an endosomal or lysosomal compartment that is enriched in  $\alpha$  integrins (pathways 6,10), or in the tethering of incoming  $\alpha$  integrin-enriched ER vesicles (step 11).

conventional to non-conventional mechanisms during epithelium remodelling is currently not understood.

Although this transport process is clearly different from AcbA secretion, GRASP might have a similar role to GrpA. For example, at certain stages in development, GRASP could act as a plasma membrane-resident tethering factor for ER-derived carriers that are enriched in  $\alpha$  integrins (FIG. 4, pathway 8). This scenario is supported by the fact that, during epithelium remodelling, GRASP localization is not restricted to the Golgi but is also present at specific plasma membrane sites<sup>9</sup>. In addition, it could also bind to  $\alpha$  integrin tails in the ER (FIG. 4, pathway 9). Given its

*trans*-oligomerization properties<sup>72</sup>, this could facilitate the tethering of ER-derived carriers.

However, it cannot be formally excluded that delivery of  $\alpha$  integrins to the plasma membrane occurs in a similar manner to CFTR (see above) and might involve endosomal or lysosomal intermediates that would fuse with the plasma membrane in a developmentally regulated manner or to which  $\alpha$  integrins could cycle (FIG. 4, pathways 4,10). In both cases, GRASP localized at the inner leaflet of the plasma membrane would have a general role in tethering membrane-bound compartments that are enriched in either ER-derived (FIG. 4, pathway 8) or endosomal (FIG. 4, pathways 4,10)  $\alpha$  integrin subunits.

Lastly, GRASP, as proposed for GrpA above, could be involved in the sorting or chaperoning of a fusion factor to the plasma membrane (FIG. 4, pathways 5,9,11), thereby mediating the fusion of late endosomes or the deposition of  $\alpha$  integrins at the plasma membrane (FIG. 4, pathway 11). Could GRASP have a common role in the unconventional delivery of  $\alpha$  integrins to the plasma membrane and in the release of AcbA into the extracellular space? This scenario would be similar to the proposed role for GrpA in tethering AcbA-enriched endosomes (FIG. 4, pathways 3,4). How GRASP anchoring at the plasma membrane is achieved, how GRASP tethers the incoming membrane compartment, and whether mammalian GRASPs have a similar function remains to be elucidated.

**Conclusions**

Two striking findings in the field of unconventional protein secretion have been the recent discoveries of general roles for caspase 1 and GRASP. Caspase 1 was shown to function as a general regulator of stress-induced unconventional secretion for a number of cytokines. In parallel, orthologues of mammalian GRASP proteins have been shown to have a crucial role in both types of unconventional secretion in *D. discoideum* and in *D. melanogaster*. Intriguingly, these two processes are restricted to specific developmental stages and, therefore, are thought to be triggered by unrecognized signalling pathways. Caspase 1 and GRASP are the first link in terms of the molecular requirements between unconventional pathways of protein secretion. Therefore, caspase 1 and GRASP could also be the key to understanding the molecular mechanism of other unconventional secretory routes, such as the ones for CFTR and CD45. Whether caspase 1 and GRASP function to mediate unconventional protein secretion independently, in parallel or in concert remains to be elucidated.

Given that the transport of these proteins is triggered by unconventional means and by signalling that is not always clearly defined, the main challenges for future studies will be to provide a comprehensive list of these proteins and to understand how signalling elicits their unconventional secretion. On these questions, a number of proteomics approaches have been used and the efforts in this direction will lead to new interesting discoveries. Understanding why these proteins are excluded from conventional transport mechanisms will also be of the utmost importance, as well as revealing the specific molecular requirements of these new trafficking pathways.

Walter Nickel is at the Heidelberg University Biochemistry Center, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany.

Catherine Rabouille is at the Cell Microscopy Center, Department of Cell Biology, UMC Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.

e-mails: [walter.nickel@bzh.uni-heidelberg.de](mailto:walter.nickel@bzh.uni-heidelberg.de); [C.rabouille@umcutrecht.nl](mailto:C.rabouille@umcutrecht.nl)

doi:1038/nrm2617

Published online 24 December 2008

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#### Acknowledgements

We are grateful to F. Reggiori, J. Klumperman and V. Malhotra for critically reading the manuscript.

#### DATABASES

UniProtKB: <http://www.uniprot.org>  
 AcbA | caspase 1 | CD45 | CFTR | EDEM1 | EGF2 | galectin 1 |  
 galectin 3 | GRASP | GrpA | Hsp150 | IL-1 $\beta$  | IL-18 | IL-33 | Ist2 |  
 MX1 | syntaxin 5 | TagC |

#### FURTHER INFORMATION

Walter Nickel's homepage: <http://bzh.db-engine.de/default.asp?fn=2329>  
 Catherine Rabouille's homepage: [http://www.cmc-utrecht.nl/People/Catherine\\_Rabouille/catherineindex.html](http://www.cmc-utrecht.nl/People/Catherine_Rabouille/catherineindex.html)

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