Making, exporting, and modulating Wnts

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ABSTRACT:

Wnt proteins activate a conserved signalling pathway that controls development and tissue homeostasis in all metazoans. The intensity of Wnt signalling must be tightly controlled to avoid diseases caused by excess or ectopic signalling. Over the years, many proteins dedicated to Wnt function have been identified, including Porcupine, which appends a palmitoleate moiety that is essential for signalling activity. This lipid inevitably affects subcellular trafficking and solubility, as well as providing a target for post-translational modulation. Here we review the life history of Wnts, starting with progression through the secretory pathway, continuing with release and spread in the extracellular space and finishing with the various proteins that dampen or inactivate Wnts in the extracellular space.

A FAMILY OF CONSERVED PALMITOLEOYLATED SIGNALLING PROTEINS

Wnts are secreted proteins that control a huge number of processes in embryos and adults through activation of a highly conserved signalling pathway (Clevers et al., 2014, van Amerongen and Nusse, 2009). The first evidence for their roles in development came with the demonstration that Wingless, the main Drosophila Wnt, specifies cell fates within each larval segment (Cabrera et al., 1987, Rijsewijk et al., 1987). Since then, Wnt signalling has been implicated in many patterning processes, for example, in the specification of embryonic axes in vertebrates (Niehrs, 2010). In addition to specifying cell fates through transcriptional activation of target genes, Wnts also control asymmetric cell division by ensuring the localisation of determinants into one of the two daughter cells, as illustrated in the early C. elegans embryo (Goldstein et al., 2006), and embryonic stem cells (Habib et al., 2013). Wnts have also emerged as key factors that maintain a population of stem cells in various tissues such intestinal crypts, hair follicles, the hematopoietic system and the hippocampus (Clevers et al., 2014, Seib et al., 2013). These positive roles of Wnts are counterbalanced by a dark side, as
excess Wnt signalling is frequently associated with overgrowth and tumorigenesis (Clevers and Nusse, 2012). For example mutations in the adenomatous polyposis coli (APC) gene, which encodes a negative component of the Wnt pathway, are frequently associated with colorectal cancer. Furthermore, overexpression of positive regulators - or underexpression of negative regulators - of the pathway correlate with a variety of cancers (Anastas and Moon, 2013). It is clear that while Wnt signalling is essential for development and adult homeostasis, excess signalling is deleterious. It is likely therefore that a variety of mechanisms ensure “just-right” Wnt signalling.

Wnt ligands can exert their effects through the activation of several downstream signal transduction pathways. The two best characterised ones are the so-called canonical pathway, which regulates target gene expression, and the non-canonical planar cell polarity pathway (Sokol, 2015). Here we focus on canonical signalling, which is initiated by the binding of Wnt to a member of the Frizzled family of serpentine receptors and the coreceptor LRP5/6 (MacDonald and He, 2012). This leads, through an incompletely characterised process, to accumulation of β-Catenin within the nucleus and activation of target genes, which in turn contribute to cell fate specification, growth, or stemness depending on the context. The human genome encodes 19 Wnt genes, and homologs are found in all multicellular organisms ranging from sponges, worms and flies to higher vertebrates. One characteristic of (almost) all Wnts is that they are appended by palmitoleate (C16:1) in the endoplasmic reticulum (ER). No other secreted protein is known to undergo such modification (Hedgehog family members carry distinct lipids, namely palmitate and cholesterol). Several studies have investigated the role of Wnts’ palmitoleate (Komekado et al., 2007, Tang et al., 2012). It is now widely accepted that lipidation occurs at a single serine residue and is essential for Wnt function (Janda et al., 2012, Takada et al., 2006). The lipid adduct is required for signalling itself because it contributes to the interaction with Frizzled receptors (Janda et al., 2012, Nile and Hannoush, 2016). It is also required for progression through the secretory pathway by promoting physical interaction in the endoplasmic reticulum (ER) with the multipass transmembrane protein Wntless (WLS), which in turn escorts Wnts to the plasma-membrane (PM) (Bartscherer and Boutros, 2008, Yu et al., 2014). In addition to Wnt trafficking in secreting cells (Figure 1), much recent attention has been given to the constraints that lipidation imposes on the release, solubility, and spread of Wnts within tissues. Here we review the current state of knowledge of Wnt production and the modulation of its activity in the extracellular space, processes that are needed to ensure ‘just-right’ signalling activity within tissues.

POST-TRANSLATIONAL MODIFICATIONS IN THE ER AND GOLGI APPARATUS
All Wnt proteins have a signal sequence required for secretion, and a characteristic pattern of conserved cysteine residues, which maintain secondary structure by forming intramolecular disulphide bridges (Janda et al., 2012, Willert and Nusse, 2012). All Wnts bar one (Drosophila WntD, see below) undergo post-translational glycosylation and acylation. The role of glycosylation is unclear, with some reports suggesting that it is largely dispensable (Mason et al., 1992, Tang et al., 2012), whilst others propose that glycosylation is important for efficient secretion (Komekado et al., 2007, Kurayoshi et al., 2007) but not for signalling activity (Kurayoshi et al., 2007). In contrast, acylation is essential for the secretion of Wnts as well as their signalling activity (Willert et al., 2003). Wnts are acylated in the ER by Porcupine, a transmembrane protein of the MBOAT (Membrane Associated O-Acyl Transferases) family, which catalyses the addition of palmitoleate (C16:1) or myristoleate (C14:1) to a conserved serine residue (S209 in Wnt-3a) (Hofmann, 2000, Rios-Esteves and Resh, 2013, Takada et al., 2006, Zhai et al., 2004). Mutation of serine 209 prevents acylation of human Wnt-3a and causes a significant impairment of secretion (Takada et al., 2006), and the small amount of Wnt-3aS209A that is secreted is poorly active, most likely because the acyl group contributes to the interaction with Frizzled receptors (Janda et al., 2012, Zhang et al., 2015) (Nile and Hannoush, 2016). Additional evidence suggests that palmitoleate is also required for progression through the secretory pathway (see below). In light of the essential role of acylation for secretion and signalling activity, it is understandable that mutations in PORCUPINE cause phenotypes associated with absent Wnt signalling. As one example of relevance to human health, one such mutation causes the X-linked developmental disorder, focal dermal hypoplasia (FDH) (Grzeschik et al., 2007, Wang et al., 2007) probably by reducing Wnt signalling. The requirement of Porcupine for Wnt secretion could be use in a therapeutic setting to reduce Wnt signalling. Indeed, small-molecule inhibitors of Porcupine show promise as possible treatments for Wnt-driven cancers (Liu et al., 2013, Proffitt et al., 2013). Other post-translational modifications of Wnts have been described besides glycosylation and acylation, and include tyrosine sulfation of Wnt5a and Wnt11 (Cha et al., 2009), and GPI anchor addition to Wnt-1 and Wnt-3a (Zoltewicz et al., 2009). However these modifications are likely to occur only on certain Wnts.

### BEYOND THE ER AND GOLGI APPARATUS

In the absence of WLS, which is also called Mig-14 (in *C. elegans*), GPR177 (in mouse), or Wntless, Evi, Sprinter (in *Drosophila*), Wnt secretion cannot proceed. It is thought that WLS binds to Wnt in the ER and chaperones it to the plasma membrane (Banziger et al., 2006, Bartscherer et al., 2006, Goodman et al., 2006), a function that is conserved from *C. elegans* to humans (Banziger et al., 2006). WLS appears to be dedicated solely to Wnt secretion, because release of other signalling proteins, including Sonic hedgehog (Shh), is not affected by the removal of WLS (Banziger et al.,
2006, Bartscherer et al., 2006, Goodman et al., 2006). It is thought that the lipid on Wnts is essential for interaction with WLS, explaining why Wnts accumulate in the secretory pathway of porcupine mutants. Indeed, molecular modelling predicts that WLS has a lipid binding β-barrel (Coombs et al., 2010). Organelle fractionation and immunofluorescence studies have revealed that endogenous WLS localises predominantly in the ER, where it associates with Wnt, in an acylation-dependent manner (Coombs et al., 2010, Yu et al., 2014). As expected, mutation of the lipidated serine or deletion of WLS leads to Wnt accumulation in the ER and, as shown recently, activation of the ER stress response (Zhang et al., 2016), most likely because of defective ER exit (Gao and Hannoush, 2013). It is worth noting that all Drosophila Wnts, except for WntD (the only Wnt that is known not to be acylated), require Wls for their secretion (Herr and Basler, 2012). It has been suggested that another class of protein required for Wnt secretion are the P24 protein family members, which act as cargo receptors for Wnt in the early secretory pathway (Buechling et al., 2011, Port et al., 2011) although this protein is unlikely to be exclusively required for Wnt secretion.

The nature of the trafficking steps that take Wnt and WLS to the PM for release are poorly understood (for a comprehensive discussion of possible routes of WNT exocytosis see (Hausmann et al., 2007)). For example, it is not known whether WLS takes Wnts from the Trans Golgi network (TGN) to the cell surface directly, or via an endosomal compartment. It is also not clear whether Wnts undergo further trafficking steps after reaching the PM and before being released. It has been suggested, for example, that upon reaching the PM, Wnts might be endocytosed, possibly in association with WLS, before being released by secreting cells (Pfeiffer et al., 2002). More complex pre-release trafficking steps could take place in polarised epithelial cells. In wing imaginal discs of Drosophila, wingless (wg) mRNA accumulates apically (Simmonds et al., 2001, Wilkie and Davis, 2001), while most extracellular Wg protein is basolateral (Strigini and Cohen, 2000). This polarised distribution likely arises by Wg transcytosis, whereby Wg transits at the apical PM before being trafficked to the basal surface for release (Yamazaki et al., 2016). In Madin-Darby Canine Kidney (MDCK) cells, it has been suggested that different Wnts could be secreted at different surfaces, e.g. Wnt11 is secreted apically and Wnt-3a is secreted basally, with glycosylation possibly determining the trafficking route (Yamamoto et al., 2013). Thus, the route taken by Wnt prior to release likely depends on a specific Wnt and cell type. The significance of polarised Wnt secretion remains to be elucidated.

Wnt secretion requires AP-2 and Clathrin-mediated endocytosis of WLS (Pan et al., 2008). Following endocytosis, WLS is recycled to the TGN in a retromer-dependent process, to ensure that sufficient levels of WLS are maintained in the secretory pathway (Belenkaya et al., 2008, Coudreuse et al., 2006, Franch-Marro et al., 2008, Pan et al., 2008, Port et al., 2008, Prasad and Clark, 2006,
Yang et al., 2008). In the absence of retromer components, WLS is directed to lysosomes for degradation, and Wnt secretion terminates (Belenkaya et al., 2008, Franch-Marro et al., 2008, Port et al., 2008, Yang et al., 2008). Remarkably, WLS recycling does not rely on the sorting nexins SNX1-SNX2 and SNX5-SNX6, which are known to associate with the retromer complex to form the classical SNX-BAR retromer. Instead it relies on an alternative retrieval pathway that involves the retromer complex in association with SNX3 (Harterink et al., 2011, Zhang et al., 2011). The SNX3 retromer complex retrieves WLS from early endosomes via vesicular budding, while SNX-BAR retromer-mediated retrieval involves tubular budding from more mature endosomes (Harterink et al., 2011). Immunoprecipitation shows that SNX3 interacts physically with both WLS and the retromer component Vps35 (Zhang et al., 2011), suggesting that SNX3 acts as a retromer cargo adaptor. WLS undergoes further retrograde transport from the Golgi to the ER. This recently characterised process is mediated by a conserved ER targeting sequence at the C-terminus of WLS (Yu et al., 2014). It occurs via ARF-regulated COPI coated vesicles, and requires ERGIC2, an ER-Golgi intermediate compartment protein (Yu et al., 2014). Additional proteins that affect Wnt secretion through regulation of WLS trafficking include the myotubularin lipid phosphatase family members MTM-6 and MTM-9, which dephosphorylate phosphoinositides known to be involved in membrane trafficking. In C. elegans embryos lacking MTM-6 or MTM-9, WLS levels are reduced, and characteristic Wnt phenotypes ensue, including defects in the migration of the Q neuroblast descendants (Silhankova et al., 2010). In summary, a large body of evidence has highlighted the importance of WLS trafficking, in particular its ER-PM-ER cycle, in Wnt secretion.

RELEASE INTO THE EXTRACELLULAR SPACE

During development, the range of Wnt movement through the extracellular space determines the domains of expression of target genes, ensuring proper patterning. In wing imaginal discs of Drosophila, the range of Wingless is likely quite limited (Alexandre et al., 2014, Farin et al., 2016) although overexpressed Wingless can act over several cell diameters in this tissue (Zecca et al., 1996). Fully processed Wnts are unlikely to be soluble in the aqueous medium and it is probable that, in order to diffuse and transfer from secreting to receiving cells, Wnts need to shield their palmitoleate moiety. Several models have been proposed (Figure 1). One possibility is that Wnts travel on lipoprotein particles, large structures composed of a single layer of phospholipids surrounding a hydrophobic core containing triacylglycerol and other lipids (Palm et al., 2012). Such particles are structured by apolipoproteins (lipophorin in Drosophila), allowing functional genetic tests. Drosophila Wg has been shown to co-localise with lipophorin particles in wing imaginal discs; and RNAi-mediated knockdown of lipophorin narrowed the range of the Wg gradient, suggesting a role in long-range signalling (Panakova et al., 2005). However, pleiotropic effects can not be
excluded because loss of lipophorin interferes with larval growth. Lipophorin is expressed in the fat body, not the wing disc, raising the question of how Wg could be loaded onto lipophorin particles. This could occur either in the extracellular space or in endosomes, after internalisation of lipoprotein particles by wing disc cells (Hausmann et al., 2007). Wnt-3a also associates with lipoprotein particles in the medium of cultured mammalian cells (Neumann et al., 2009) but the functional significance of this association has not been tested. Overall, the relevance of lipoprotein particles to Wnt transport is provocative but remains unproven.

Recent work has suggested the involvement of another subcellular structure in Wnt transport. Exosomes are microvesicles that are released from cells upon fusion of multi-vesicular bodies (MVBs) with the PM (Lo Cicero et al., 2015). The topology of MVB formation ensures that Wnts would be on the extracellular surface of such microvesicles, possibly with palmitoleate inserted in their lipid bilayer. Therefore exosomes could act as Wnt carriers. Wnt-3a and Wg can be detected in exosome preparations from culture supernatant (Beckett et al., 2013, Gross et al., 2012), and such preparations are biologically active (Gross et al., 2012). Moreover, exosomes obtained from cultured Drosophila cells have been shown to contain WLS (Beckett et al., 2013, Gross et al., 2012, Koles et al., 2012), suggesting that the Wnt-WLS complex could be loaded on exosomes for release and possibly long-range transport. The best in vivo evidence for the role of exosome-mediated release of Wg and WLS is at the neuromuscular junction (NMJ) of Drosophila (Koles et al., 2012, Korkut et al., 2009). Knocking down Rab 11, which reduces exosome release from cultured Drosophila cells (Beckett et al., 2013, Koles et al., 2012), prevents the transfer of Wg and WLS across the NMJ. Exosome-mediated release has also been suggested to occur in wing imaginal discs of Drosophila although there is disagreement about the functional significance. Wg colocalises with exosomal markers (rab4 or exogenously produced CD63) in this tissue (Gross et al., 2012). However, RNAi against Rab11 in wing imaginal discs had no impact on extracellular levels of Wg, suggesting that exosomes do not play a role in Wg gradient formation (Beckett et al., 2013). Evidence for the roles of exosomes in Wg gradient formation comes from the effect of knocking down Ykt6. This treatment, which is purported specifically to interfere with exosome formation since it blocks the release of CD63-GFP in imaginal discs without seemingly affecting the extracellular level of the transmembrane proteins Patched and Flamingo, does prevent Wg gradient formation. However, Ykt6 is a well characterised SNARE recognition protein involved in ER to TGN vesicular transport (Daste et al., 2015), and is likely therefore to contribute to progression of many secreted proteins, including Wg, through the secretory pathway. Therefore the effect of Ykt6 knockdown cannot be taken as incontrovertible genetic evidence for the role of exosomes in Wg transport in vivo.
It is conceivable that Wnts could be rendered soluble in the extracellular space by forming a complex with a specific protein that shields their lipid. Indeed, the serum glycoprotein Afamin effectively maintains Wnt soluble and active (Mihara et al., 2016). This discovery has great practical implications since it provides a means of keeping Wnts active in solution but its physiological relevance remains untested as Afamin is not known to be expressed specifically in Wnt-producing cells. Another protein shown to interact directly with Wg and to increase its solubility is Swim (Secreted Wnt Interacting Molecule), a member of the Lipocalin family of proteins (Mulligan et al., 2012). Knockdown of Swim by RNAi narrows the Wg gradient, which reduces long-range, but not short-range Wg target gene expression in imaginal discs (Mulligan et al., 2012). Knockdown experiments suggest that Swim is not required for Wg secretion (Mulligan et al., 2012), raising the possibility that Swim and Wg could come together extracellularly. However, all functional studies of Swim have been based on RNAi and rigorous genetic tests will require the analysis of null mutant tissue.

Although we have assumed in the above discussion that Wnts must be released from secreting cells in order to act at a distance, recent work has suggested that this does not need to be the case. It has been suggested that signalling molecules could be presented to distant cells on cytonemes, actin-based extensions extending for distances of up to 700µm (Stanganello and Scholpp, 2016). Several studies have suggested a role for cytonemes in long range signalling by Dpp and Hedgehog in Drosophila wing imaginal discs (Bischoff et al., 2013, Roy et al., 2014) although this remains a subject of debate. Cytonemes have also been implicated in long range Wnt signalling. For example, it was shown that Drosophila flight muscle progenitors extend Frizzled decorated projections towards wing imaginal disc cells that take up Wingless at a distance (Huang and Kornberg, 2015). Furthermore, Zebrafish Wnt8a was shown to be transported along cytoneme-like extensions to activate signalling in distant receiving cells during neural plate formation (Stanganello and Scholpp, 2016). Further information on the role of cytonemes in long-range Wnt signalling can be found in a recent review(Stanganello and Scholpp, 2016).

EXTRACELLULAR REGULATION OF WNT BY MEMBRANE-BOUND EXTRACELLULAR REGULATORS

Wnt signalling is subject to extensive regulation in the extracellular space. This is achieved by secreted as well as transmembrane agonists and antagonists (for a comprehensive review see (Cruciat and Niehrs, 2013)). Here, we focus on extracellular molecules that regulate Wnt signalling by acting upon Wnt itself (Figure 2). We discuss membrane-associated and secreted regulators in turn.
Glypicans are membrane-associated proteins that have long been thought to regulate the extracellular distribution and signalling output of several secreted molecules, including Wg, Hh and Dpp in the *Drosophila* wing (Yan and Lin, 2009). Glypicans comprise a GPI anchor, a stalk region, to which several glycosaminoglycan (GAG) chains are covalently linked, and a globular cysteine-rich domain at the N-terminus (Yan and Lin, 2009). Both glypicans encoded by the *Drosophila* genome, Dally and Dally-like protein (Dlp), bind Wg in cell culture (Franch-Marro et al., 2005, Yan et al., 2009), and extracellular Wg is reduced at the surface of wing imaginal disc cells lacking Dlp and Dally (Han et al., 2005) suggesting that glypicans contribute to Wg retention at the cell surface. Dally has been proposed to act as a co-receptor since *dally* mutants show a mild Wg loss of function phenotype (Lin and Perrimon, 1999). By contrast, Dlp has a more complex role in Wg signalling. It appears to negatively regulate Wg signalling in regions exposed to high levels of Wg, but to boost signalling in regions of low Wg (Baeg et al., 2004, Franch-Marro et al., 2005, Kirkpatrick et al., 2004, Kreuger et al., 2004). The primary role of Dlp could be to retain Wg on the cell surface, and the ultimate outcome on signalling activity may be determined by the relative levels of Wg, Fz and Dlp at the surface of any given cell (Yan et al., 2009).

Adenomatosis Polyposis coli down-regulated 1 (APCDD1) is another membrane-bound glycoprotein that regulates Wnt signalling. An APCDD1 mutation has been identified in human patients with hereditary hypotrichosis simplex (HSS), a rare genetic disorder causing hair loss (Shimomura et al., 2010). APCDD1 binds to Wnt-3a and to the Frizzled co-receptor LRP5. Overexpression of APCDD1 inhibits Wnt signalling in cell culture assays and in *Xenopus* embryos (Shimomura et al., 2010), perhaps by titrating Wnts away from the signalling complex.

Unlike the two preceding examples, the transmembrane proteins Tiki1 and Tiki2 act enzymatically to inhibit Wnt signalling. Tiki1 was identified by cDNA expression screening for genes involved in anterior-posterior patterning in *Xenopus* embryos. Tiki1 overexpression results in an enlarged head phenotype, which mirrors that caused by overexpression of Dkk1 (Zhang et al., 2012), a known Wnt antagonist. Furthermore, knockdown of Tiki2 was found to enhance the activity of Wnt-3a in cultured human cells (Zhang et al., 2012). Protein sequencing and quantitative mass spectrometry showed that Tiki2 acts as a protease that removes eight amino acids from the N-terminus of processed mature Wnt-3a (Zhang et al., 2012). This causes the formation of inactive oxidised oligomers of Wnt-3a, thus accounting for Tiki’s inhibitory effect. Indeed, an engineered version of Wnt-3a lacking the residues cleaved by Tiki2 has little signalling activity (Zhang et al., 2012). Tiki proteins are the first extracellular Wnt enzyme shown to act directly on Wnt itself.
Besides membrane-associated proteins, several secreted (diffusible) proteins dampen Wnt signalling activity. Many such proteins act by binding to the receptors and/or the Wnt ligand. For example, the secreted Frizzled-related proteins (sFRPs) have a domain that resembles the Wnt-binding cysteine-rich domain (CRD) of Frizzled receptors. sFRP3 has been shown to bind Wnt1 and XWnt8 and to inhibit Wnt signalling (Leyns et al., 1997, Lin et al., 1997, Wang et al., 1997), probably by preventing Wnt from binding receptors. An alternative, though not exclusive, mechanism of action is that sFRPs could form heterodimers with Frizzled via their CRDs, causing the formation of inactive receptor complexes (Bafico et al., 2001, Rodriguez et al., 2005). sFRPs appear to be redundant during mammalian development; sFRP1-/- or sFRP2-/- mice showed no obvious developmental phenotype, but double-mutant mice die during embryogenesis with a severely shortened AP axis thought to be caused by a somitogenesis defect (Satoh et al., 2006), attributed to a defect in Wnt signalling (Satoh et al., 2006, Satoh et al., 2008).

Wnt-inhibitory factor 1 (WIF-1) is another secreted protein that dampens Wnt signalling during vertebrate development. WIF-1 had been shown to interfere with canonical as well as non-canonical (beta-catenin-independent) Wnts, including Wnt-3a, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt11 (Cruciat and Niehrs, 2013). The exact mechanism is unknown but, like sFRPs, WIF-1 is likely to prevent Wnts from associating with their receptors. The WIF-1 homolog in *Drosophila* (encoded by shifted) does not affect Wg signalling. Instead, it inhibits Hedgehog signalling by enhancing Hedgehog-glypican interactions, thus sequestering Hh away from its receptors (Glise et al., 2005, Gorfinkiel et al., 2005). Interestingly, zebrafish Wif-1 was found to inhibit Wg signalling in *Drosophila* imaginal discs by increasing the association of Wg to Dlp. Therefore, despite the differences in specificity, it is likely that Wif-1 inhibits Wnt or Hh by modulating the interaction of these ligands with glypicans (Avanesov et al., 2012).

Following its identification through genetic screens in *Drosophila*, it has been known that another secreted protein, Notum, acts as a feedback inhibitor of Wnt signalling (Gerlitz and Basler, 2002, Giraldez et al., 2002). Unlike sFRPs and WIF-1, Notum has enzymatic activity, although the nature of this activity was only recently elucidated. It was initially thought that Notum acted as a phospholipase, cleaving the GPI anchor of glypicans and causing their shedding, along with bound Wnt, from the cell surface (Kreuger et al., 2004, Traister et al., 2008). However, further characterization of Notum’s enzymatic properties revealed that it is a carboxylesterase (Kakugawa et al., 2015). Mass spectrometry and metabolic labelling experiments were independently used to demonstrate that Notum deacylates Wnt-3a by hydrolysing the carboxyester bond linking palmitoleic acid to S209 (Kakugawa et al., 2015, Zhang et al., 2015). Notum does not affect Wnt secretion, which depends on Wnt’s lipid moiety, suggesting that Notum primarily deacylates Wnt
extracellularly. Consistent with the essential role of the lipid moiety of Wnts to bind Frizzled (Janda et al., 2012), Notum expression was shown to perturb the Wnt-3a-Fz8 interaction (Zhang et al., 2015). In addition, like Tiki, Notum induces the formation of inactive oxidised oligomers of Wnt (Zhang et al., 2015), probably contributing to inactivation of signalling activity. Surface plasmon resonance (SPR) has shown that human Notum interacts with the GAG chain of glypican GPC3, suggesting that glypicans could retain Notum at the cell surface. Indeed, Notum is lost from the surface of Drosophila imaginal discs lacking Dally and Dlp. All evidence so far suggests that glypicans help bring Notum and Wnts in close proximity thus allowing deacylation and hence inactivation of Wnts (Kakugawa et al., 2015).

**CONCLUDING REMARKS:**

Recent work has uncovered many steps required for the biosynthesis of mature Wnt ligands. Particularly interesting is the role of the palmitoleate moiety that is appended onto Wnts in the ER by Porcupine. This modification, which is essential for secretion and signalling, places constraints on the way Wnts progress through the secretory pathway and on their solubility in the extracellular space. Thus, acylated Wnts are dependent on accessory proteins such as WLS for secretion. The likely impact of lipidation on solubility raises the possibility that specific proteins or processes are needed to ensure release from secreting cells and action at a distance. Their nature is still the subject of intense study. Once outside secreting cells, Wnts are subject to a wide range of extracellular inhibitory proteins, including two enzymes, Tiki, a Wnt-specific protease and Notum, a Wnt deacylase.

The numerous proteins that contribute to Wnt biosynthesis, release and modulation in the extracellular space provide ample scope for physiological fine tuning of signalling, as well as targets for intervention. Trafficking and packaging of Wnt in the secretory pathway likely controls the amount of Wnt that is secreted, its solubility and possibly its specific site of delivery within developing tissues. Therefore trafficking and packaging likely contribute to ensuring appropriate expression of the target genes involved in patterning and stem cell maintenance. However, the extent to which these processes are regulated is poorly understood so far. Different estimates for the range of Wnt signalling have been suggested. For example, within intestinal crypts, Wnt acts over a short range (Farin et al., 2016) while in other situations, such as in vertebrate anterior-posterior patterning, Wnts are thought to act at a long range (Niehrs, 2010), perhaps through specific Wnt packaging within the secretory pathway. Further work will be needed to determine whether the range of Wnts is developmentally regulated through modulation of specific trafficking steps.
In light of the relevance of Wnt signalling to human health, there is great interest in developing small molecules that modulate Wnt signalling, both positively and negatively. So far, inhibitors of Porcupine have been shown to reduce Wnt signalling in vivo. In fact, one such inhibitor, LGK974, is currently undergoing clinical trials for the treatment of the subset of pancreatic adenocarcinoma and colorectal cancers that are caused by Wnt overproduction (https://clinicaltrials.gov/ct2/show/NCT01351103). Compounds that boost signalling, but not to a level to triggers cancer, could also be useful in the clinic. For example, mildly raising the level of Wnt signalling could help prevent or reverse age-related neurodegeneration since removal of Dkk, a Wnt antagonist, leads to enhanced self-renewal and increased generation of immature neurons in old animals (Seib et al., 2013). Dkk is not readily druggable but it is conceivable that Wnt signalling could be increased within a physiological range with chemical inhibitors of Notum or Tiki which, by virtue of being enzymes, are probably more amenable to chemical inhibition. This example shows that, as our understanding of physiological Wnt pathway modulation increases, we can hope to start developing means of controlling signalling in a therapeutic setting.

**FIGURE LEGENDS:**

**Figure 1. Post-translational modification and trafficking in Wnt secreting cells.** Most Wnts are appended by a palmitoleate moiety in the ER and require WLS for progression through the Golgi network. Upon reaching the plasma membrane, WLS is recycled to the Golgi apparatus by the retromer complex. Further retrograde transport of WLS from the Golgi to the ER relies on COP1 coated vesicles. In wing imaginal discs of Drosophila, Wg undergoes transcytosis from the apical to the basolateral surface. Whether this takes place in complex with WLS is still undetermined, as indicated by question marks. How Wnts are released from secreting cells is the subject of intense research with three mechanisms being considered, as shown. Note that release on exosomes could occur with or without WLS. Release mechanisms are shown to operate at the basolateral surface only for illustrative purposes.

**Figure 2. Dampening and inactivation in the extracellular space.** This process is achieved by a variety of transmembrane and secreted proteins. Among those, two so far are known to act enzymatically: the transmembrane protease Tiki1/2, and the secreted deacylase Notum.

**REFERENCES:**


Regulation in the extracellular space

(a) No inhibition

(b) APCDD1

(c) Tiki

(d) sFRP

(e) WIF

(f) Notum

Wnt  Glypican  LRP5/6  Fz

APCDD1  Tiki 1/2  sFRP  WIF  Notum