SIGNAL DISPERSAL AND TRANSDUCTION THROUGH THE ENDOCYTIC PATHWAY

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During cell signalling, information that is encoded by ligands travels from one place, the source, to another, the target, where signals are transduced by receptors. Evidence has emerged recently that uncovers a role for the endocytic pathway in the secretion of ligands at the source, their dispersion through developing target tissues and the transduction of the signals from endocytic compartments. As a result, endosomes have become the focus of attention in cell–cell communication studies.

PRIMORDIUM
Undifferentiated developing tissue.

MORPHOGEN
A molecule, the concentration of which endows cells with positional information that determines the fate of a developing cell.

HEPARAN-SULPHATE PROTEOGLYCAN
(HSPG). A protein that is bound to a complex polysaccharide (heparan-sulphate glycosaminoglycan) present at the cell surface or the extracellular matrix. When bound to ligand, it can have a key signalling role.

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Until recently, the predominant view of the role of endocytosis in cell signalling was that it downregulates signal responses by internalizing receptors either constitutively or on ligand binding. The endocytosis of receptors has two main consequences for cell signalling: first, it reduces the pools of receptors that are available to ligands at the plasma membrane; and second, it moves the receptors away from the location where they can elicit downstream signalling, the plasma membrane. This process, which is common among many signalling pathways, allows signalling levels to be regulated, by constitutively internalizing a pool of receptors from the plasma membrane and by allowing the signal to be switched off after ligand binding — signal transduction by the receptor would otherwise occur indefinitely once it had been initiated.

This review discusses recent studies that have uncovered new roles for endocytosis in signalling events. In particular, new data from studies in *Drosophila* indicate that ligands traffic through the endocytic pathway before being released in the extracellular space in membrane carriers, which are used as transport vehicles (FIG. 1). Other studies in *Drosophila* indicate that ligands can disperse by travelling through cells rather than by free diffusion. In each of these intermediate cells, ligands are internalized by endocytosis, traffic through the endocytic pathway and are then released before they enter the next round of endocytosis and exocytosis. The signalling range — that is, the distance over which the ligand can travel and signal — is controlled by adjusting the rates of recycling and degradation of the ligands at the receiving 'target' cells (FIG. 2). Finally, this review discusses recent data that indicate that there is a 'signalling endosome' where downstream signalling events are initiated (FIGS 3,4).

Dispersal of ligands

During organogenesis, each cell within the PRIMORDIUM acquires a specific fate and differentiates in a position-dependent manner. This positional information is conveyed in the graded distribution of MORPHOGENS[1,2] (FIG. 2a–c). Paradoxically, in spite of their ability to travel a long distance, some of the best-studied morphogens are either lipid-modified or bind tightly to membrane-associated proteins. For example, members of the Wnt family of proteins bind tightly to HEPARAN-SULPHATE PROTEOGLYCANS (HSPGs)[3,4], and HEDGEHOG (Hh) is covalently attached to cholesterol and palmitate[5,6]. This indicates that morphogens will associate readily with cell membranes, including those of the cells that produce the morphogens.

Ligand release from the membrane. So how can membrane-tethered signals travel a long distance? One possibility is that the membrane-anchored ligand is disengaged transiently from the plasma membrane, possibly with the help of another transmembrane protein.

HEDGEHOG (Hh). A morphogenetic ligand that is involved in patterning during development.

Such a mechanism has been suggested for Hh, which is anchored to sphingolipid rafts by its association with the outer-membrane leaflet through cholesterol and palmitate[7]. Hh is released from the producing 'source' cells by Dispatched, a 12-transmembrane protein that has sequence similarity to the Hh receptor, Patched (Ptc)[8] (FIG. 1a). Although Dispatched is needed for Hh to be released from the secreting cell, it is unclear whether it carries out its function by extracting cholesterol-modified Hh from the bilayer to yield a soluble protein (FIG. 1a).

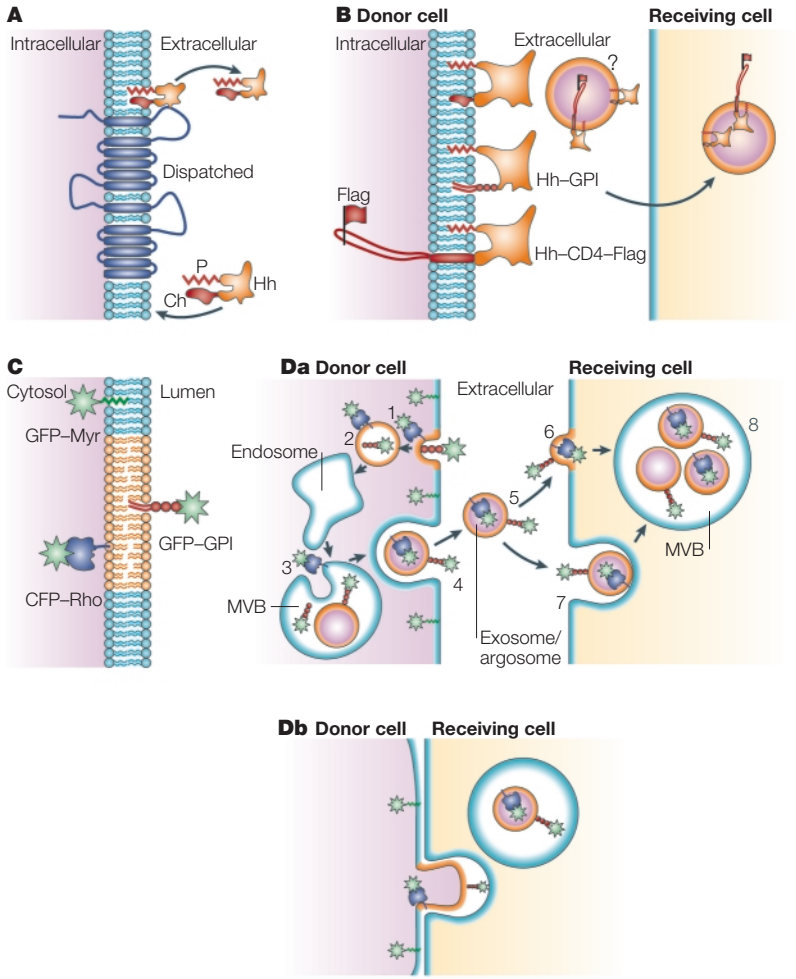


Figure 1 | **Dispersal of membrane-bound ligands. A** | Mature Hedgehog (Hh) is palmitoylated (P) and cholesterol-modified (Ch) at its amino- and carboxy-terminal ends, respectively, which allows it to become membrane associated. Dispatched has been proposed to interact with Hh, which causes its extraction from the plasma membrane, releasing it into the extracellular space to elicit signalling in target cells. **B** | Glycosylphosphatidylinositol (GPI)-anchored Hh (Hh–GPI) and membrane-tethered Hh (Hh–CD4–Flag) are released from donor cells and are found in vesicular structures in receiving cells, which implies that Hh could be released together with its surrounding membrane and possibly transported in membrane carriers (?). **C** | Argosomes. GPI-anchored green fluorescent protein (GFP)–GPI (green star, red tag) in the outer-membrane leaflet and geranylgeranyl-anchored cyan fluorescent protein (CFP)–Rho (geranylgeranyl, blue) in the inner leaflet are incorporated into the argosome membrane (orange), whereas myristoyl-anchored GFP (GFP–Myr) in the inner leaflet of the membrane is not. **D** | Two models of argosome formation. **Da** | The exosomal origin of argosomes. In the donor cells, argosomal membrane is endocytosed (1) and targeted to endosomes (2); invagination of argosomal membrane in the endosomes gives rise to multivesicular bodies (MVBs) (3). The outer membrane of MVBs fuses to the plasma membrane (4), which releases the internal vesicles, exosomes or argosomes (5). Exosomes fuse with the plasma membrane (6) or they are endocytosed by receiving cells (7). They are then internalized, form MVBs (8), and can be released again. **Db** | Argosome formation by transendocytosis. During transendocytosis, evagination of argosomal membrane at the donor cell is coupled to invagination of endocytic membrane in receiving cells. To move beyond the cells adjacent to the donors, the internal vesicle could fuse to the external membrane, which in turn would fuse to the plasma membrane and evaginate from there as in the donor cell.

Release in a membrane carrier. Experiments in cultured cells indicate that a different mechanism might be used; namely, that this morphogen might be released in a membrane carrier[9]. In the case of *Drosophila* Hh and vertebrate Sonic hedgehog (Shh), chimeric transmembrane versions that are generated by fusing the transmembrane domain of CD4 to the ligand — Hh–CD4 (REF. 10), Shh–CD4 (REF.11) — or glycosylphosphatidylinositol (GPI)-anchored versions[8,9] retain their signalling activity. These membrane-tethered ligands are, like the wild-type ligands[9,12,13], internalized in the receiving cells by Ptc-receptor-mediated endocytosis[9] (FIG. 1b). The addition of a Flag tag to the cytoplasmic tail of Shh–CD4 helped to confirm that the entire molecule, not just the extracellular domain, is released and endocytosed in receiving cells[9] (FIG. 1b). These observations introduced the idea that Hh is released while it is bound to the membrane moiety by which it is surrounded at the secreting cells. However, transmembrane- or GPI-anchored Hh ligands have shorter signalling ranges than wild-type cholesterol-modified Hh[8–11]. This indicates that there is a specific function for the cholesterol anchor in either the efficiency of ligand release from the secreting cell or its spreading kinetics throughout the target tissue.

Three other examples of membrane-bound ligands that are internalized into the receiving cells are Bride of sevenless (Boss), which is the ligand of the receptor tyrosine kinase (RTK) Sevenless[14], and the Notch ligands Delta and Serrate[15–17]. They are all transmembrane ligands the cytoplasmic domains of which colocalize with their receptors in intracellular vesicular structures in receiving cells, and are therefore probably released together with the membrane that surrounds them.

Argosomes, exosomes and transendocytosis. So, do membrane pieces travel from cell to cell? Greco *et al.* addressed this question by following the behaviour of a membrane-anchored green fluorescent protein (GFP) chimaera (GFP–GPI) in a developing epithelium, the *Drosophila* wing imaginal disc[18]. The GPI moiety of the chimaera tethers GFP to the outer leaflet of the plasma membrane and targets it to the basolateral outer surface of the expressing cells. GFP–GPI is found not only in the expressing cells but also in other cells within the epithelium, in which it is internalized into an endocytic compartment. This indicates that the protein is released from expressing cells, travels along the plane of the epithelium and ends up in endocytic structures.

Does GFP–GPI extracted from the lipid moiety of the plasma membrane become a soluble protein, or does it travel on membrane pieces that have become detached from the donor cells? The fact that a geranylgeranylated protein, a Rho–cyan fluorescent protein (CFP) chimaera, which is bound to the inner leaflet of

the plasma membrane, is also found in endocytic structures beyond the expressing wing cells indicates that complete lipid bilayers (that is, both the inner and outer membrane leaflet) are released (FIG. 1c). This implies that pieces of membrane can indeed travel from cell to cell. However, not every membrane can move in a similar way. For example, GFP anchored to the lipid moiety myristoil, which tethers proteins to inner plasma membrane leaflets, is not found beyond expressing cells (FIG. 1c). In the case of the developing wing, the travelling pieces of membrane were named argosomes (from Argo — the ship in which Jason and the Argonauts sailed to Colchis in search of the Golden Fleece).

How do argosomes form? Two models for the intercellular transfer of membrane have been proposed (FIG. 1d). Model one is transendocytosis, which was first proposed for the transfer of Boss; it implies that there is simultaneous pinching-off of the membrane from donor cells and that Boss is internalized by endocytosis in acceptor cells, as shown in electron micrographs[14,19] (FIG. 1Db).

An alternative method for the transfer of membrane — model two — is the formation of exosomes (FIG. 1Da; for a review, see REFS 19,20). Exosomes are membrane vesicles with a diameter of 40–100 nm that are secreted into the extracellular environment by many cell types. They correspond to the internal vesicles of an endosomal compartment — the MULTIVESICULAR BODY — and are released on exocytic fusion of this organelle with the plasma membrane[21,22]. Intracellularly, they are formed by the inward budding of the endosomal membrane[22]. Exosomes have a unique lipid and protein composition, which is consistent with the idea that they include membrane rafts (for reviews, see REFS 19,20). In particular, they are enriched in cholesterol[23], which is crucial for the formation of membrane rafts. Furthermore, raft proteins such as GPI-anchored proteins and TETRASPANINS are sorted efficiently to exosomes[24,25]. After binding target cells, exosomes — like the argosomes — can be endocytosed, because the diameter of CLATHRIN-coated vesicles (150 nm) is greater than that of the exosomes[20].

Argosomes have been proposed to function as carriers of morphogenetic ligands while they spread throughout target tissues and form concentration gradients[18]. In particular, WINGLESS (Wg) has been suggested to travel on argosomes during wing development (see above). Argosomes move at a speed that is consistent with the kinetics of Wg dispersal. In addition, Wg colocalizes with argosomes in intracellular vesicular structures in the receiving cells. Wg does not have a GPI anchor and, therefore, it might not interact directly with the lipid milieu of argosomes. However, Wg has been shown to interact with two GPI-anchored HSPGs, Dally and Dally-like, which are essential for Wg signalling[26–28]. This indicates that Wg might be incorporated into the argosomes through its interaction with Dally or Dally-like HSPGs. As the lipid and protein composition of the argosomes is non-random, the potential for specific protein sorting and concentration

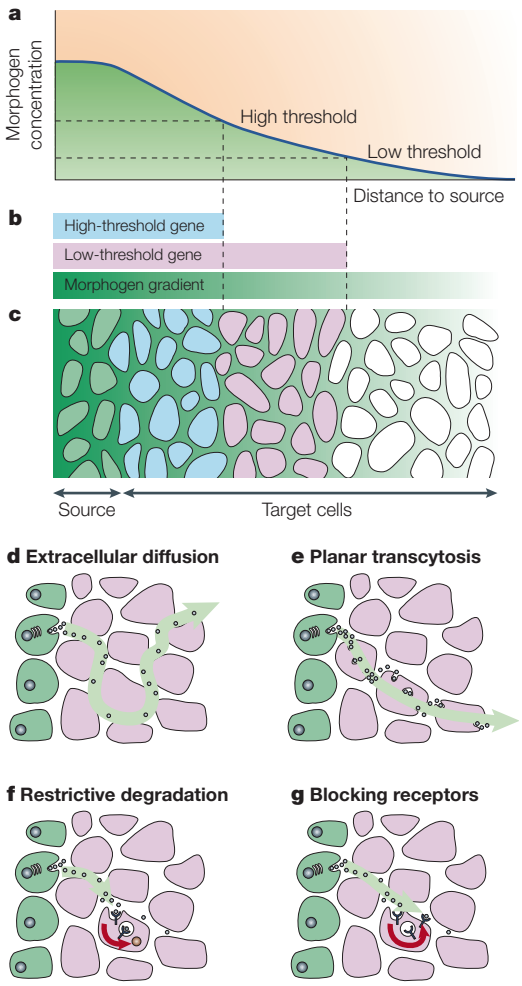


Figure 2 | **Morphogenetic signalling and dispersal of morphogens. a–c** | Positional information is conveyed by the graded distributions of morphogens. Morphogens are expressed in a restricted territory, the source, and are spread through the target tissue (**c**) to form a concentration gradient (**a**,**c**). Above different concentration thresholds (**a**), different genes are activated (high- or low-threshold genes) (**b**) and are expressed in cells at different distances from the source. Therefore, the information about the distance to the source is encoded as morphogen concentration. **d–g** | Models of morphogen trafficking. **d** | Extracellular diffusion. The morphogen is diluted as it moves away from the source and it forms a gradient. It can be degraded in the extracellular space to form a stable gradient. **e** | Planar transcytosis. When extracellular diffusion is restricted, the uptake and release of the morphogen at the receiving cells allow the morphogen to move far from the source. Intracellular degradation in lysosomes accounts for the stability of the gradient. **f** | Restrictive degradation. The morphogen can spread by free diffusion, but it is also taken up by cells and released from cells. This latter process ensures the access of the morphogen to the intracellular lysosomal degradative compartment, which restricts the range of the gradient. **g** | Blocking receptors. The morphogen traffics extracellularly. Its dispersal is restricted by binding to its receptor at the plasma membrane. The receptor is internalized and recycled, and if endocytosis is blocked, the receptor accumulates at the plasma membrane, titrates out the moving morphogen and reduces its range of dispersal.

GPI
(Glycosylphosphatidylinositol). A lipid species that is characteristic of lipid rafts.

MULTIVESICULAR BODY
A vesicular compartment that contains membrane vesicles and that is an intermediate in the lysosomal degradative pathway. It can also be an intermediate in exosome formation.

TETRASPANINS
A family of proteins that have four transmembrane domains and are associated with lipid rafts.

CLATHRIN
The main component of the endocytic vesicle coat.

WINGLESS
(Wg). A morphogenetic ligand that is involved in patterning during development.

into these membrane carriers makes them an attractive vehicle for the spread of membrane-bound morphogens that act at a long distance.

Argosomes can be found beyond the cells that produce them, many cell diameters away from the source. Argosome uptake by transendocytosis would explain only a short range of dispersal, into the cells adjacent to the source. Dispersal beyond these cells might indicate that there are other rounds of transendocytosis into the next cells. Similarly, although exosomes could in principle navigate extracellularly until they reach distant target cells, they could also be dispersed by an iteration of internalization and release events through the receiving cells until they reach distant target cells. In both cases, the dispersal of membrane and morphogens would occur through a mechanism of 'planar transcytosis' that involves endocytic trafficking rather than simple extracellular diffusion. Recent data on the dispersal of Wg and the transforming growth factor (TGF)-β homologue DECAPENTAPLEGIC (Dpp) give experimental support to this hypothesis.

Spreading Dpp by endocytic trafficking

Dpp forms a long-range concentration gradient across 40 cell diameters in the *Drosophila* wing[29,30]. The Dpp concentration gradient specifies cell fates along the anterior–posterior axis of the *Drosophila* wing by activating different target genes, including the zinc-finger transcription factors Spalt and Optomotor blind, at distinct concentration thresholds[31,32] (FIG. 2a–c). In the process of forming the concentration gradient, Dpp disperses rapidly (at a rate of more than five cells per h) and in all directions[29,30]. This is consistent with the extracellular dispersal of Dpp by free diffusion (FIG. 2d). However, a GFP fusion that uses the same signals for cleavage and release from the producing cells as Dpp itself is unable to generate a stable gradient[29]. This free-diffusing molecule merely fills the extracellular space with a flat distribution. In a different cellular context (the *Xenopus* animal cap assay), a TGF-β homologue — activin — is able to form a long-range concentration gradient by free diffusion across 200 μm, which instructs cells with positional information[33]. So, different morphogens and different cellular environments may generate gradients using distinct cellular mechanisms.

The mechanism of long-range Dpp dispersal. Dpp seems to generate its long-range concentration gradient by its internalization into early endosomes through a mechanism that involves receptor-mediated DYNAMIN-dependent endocytosis[29] (BOX 1). Patches of cells that lack the Dpp type I receptor Thickveins (Tkv), or that express the thermosensitive dynamin-mutant *shibire*, fail to endocytose Dpp. In mosaic animals in which producing cells are normal, but receiving cells do not internalize Dpp because they lack dynamin, Dpp distribution is restricted to the cells that are adjacent to the source. Furthermore, if the spreading of Dpp is challenged with a patch of dynamin-mutant cells, the ligand is unable to pass across this endocytosis-defective territory and forms a shadow with no Dpp found behind the mutant clone. These two observations indicate that endocytosis is required for the long-range dispersal of the Dpp ligand. This is consistent with a planar transcytosis model (FIG. 2e) in which extracellular Dpp does not move far[29]. Instead, Dpp is internalized in the receiving cell, traffics through the endocytic pathway, is secreted again and is thereby dispersed forwards (as well as backwards) to signal in adjacent cells.

An alternative model is that Dpp diffuses extracellularly and endocytosis-defective patches of mutant cells cast shadows because receptors accumulate at the plasma membrane in the absence of internalization[34] (FIG. 2g). A mathematical approach to this model predicts that high levels of receptors at the surface that bind Dpp would hamper its diffusion at the extracellular space, thereby causing the formation of shadows behind the mutant clones[34]. The same model predicts a large accumulation of the receptors at the plasma membrane and a 40-fold accumulation of extracellular Dpp[34]. However, neither surface receptors (M.G.G., unpublished observations) nor extracellular Dpp (see figures 4 and 5 in REF. 29) accumulated. Another prediction of such a model is that, in the absence of receptors in *Tkv* mutant clones, Dpp dispersal should be potentiated, because the surface receptors block its movement. By contrast, *Tkv* mutant clones impair Dpp movement and cause an accumulation of extracellular Dpp[29]. So, these data do not support the model in which Dpp is dispersed by extracellular diffusion and shadows in dynamin-mutant clones are caused by high levels of surface receptors when Dpp internalization is blocked.

Rate of Dpp dispersal. At what speed does Dpp move across a cell? And could intracellular trafficking account for this speed? As a full Dpp concentration gradient across 40 cells is formed in 8 h and Dpp movement is non-directional[29], a theoretical estimate that is based on Fick's law for a 'random walk' would predict that Dpp must take between 50 and 150 s to be dispersed across a single cell[34]. However, in the case of activin, which seems to be dispersed by free diffusion[35], it reportedly has lengthy time constants for its dissociation from the receptors and its trafficking[36]. Another consideration is that within the cell (or at the plasma membrane) a random walk would take longer than the 50–150 s predicted for Dpp[34]. So, the 'intracellular walk' of Dpp in carrier vesicles must be very different from the random walk throughout the full volume of the cell, as predicted in simplified models of morphogen gradient formation.

Dpp gradient formation. So how can a stable morphogen gradient be established? In pulse-chase experiments, in which the formation of the gradient is monitored *in vivo*, the Dpp gradient extends from the source until it reaches its maximal range of 40 cells in less than 8 h[29], after which it does not extend further. This means that the gradient has reached a steady state: only a minor percentage of the Dpp molecules secreted

DECAPENTAPLEGIC
A TGF-β-like morphogenetic ligand that is involved in patterning during development.

DYNAMIN
A GTPase that is involved in the fission of the coated pit.

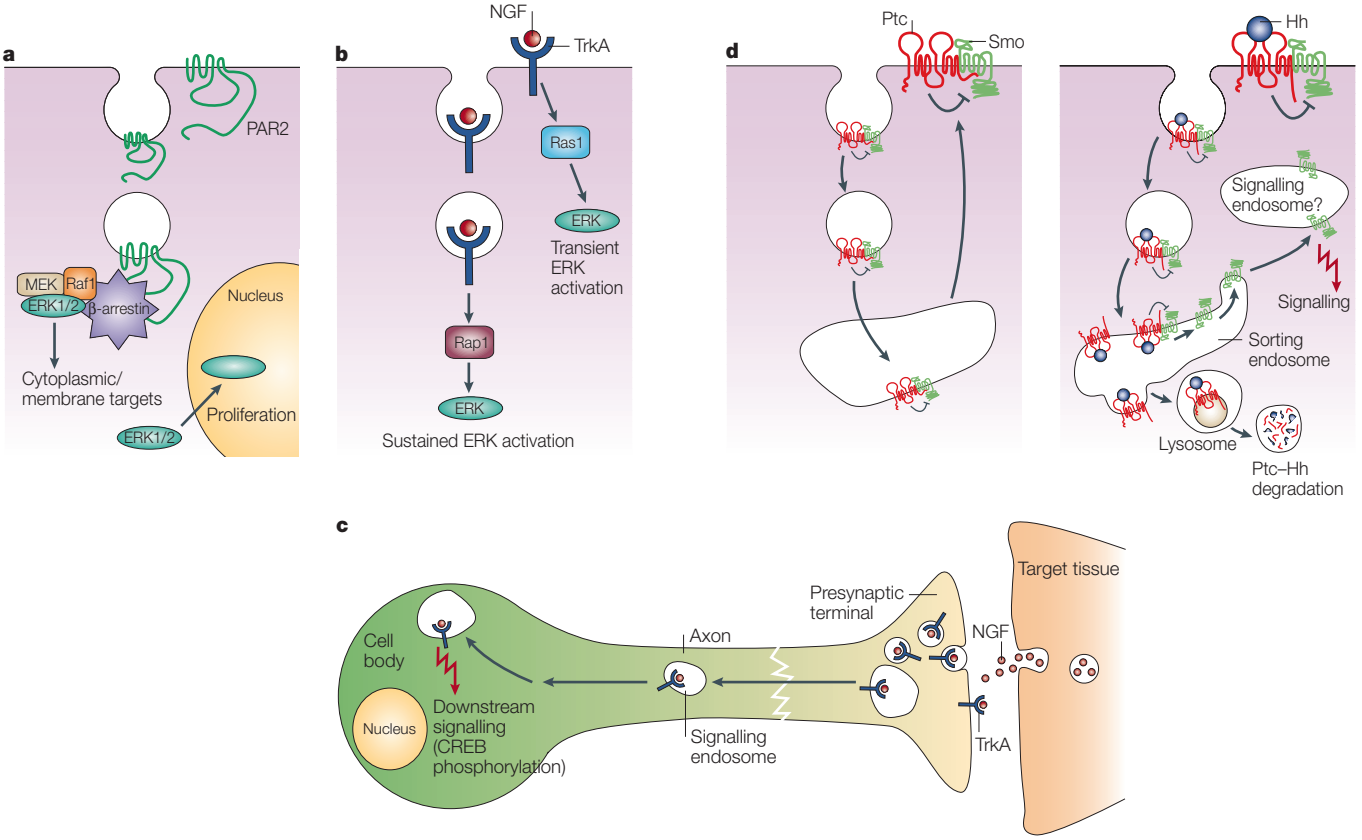


Figure 3 | **A possible rationale for signalling from endosomes. a** | Selecting different targets downstream of the receptor. Phosphorylated extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPKs; shown as ERK in the figure) can translocate to the nucleus, where they stimulate proliferation, or stay in the cytosol, where they can phosphorylate cytosolic or membrane targets. Activation of the PAR2 receptor by proteolysis causes phosphorylation of ERK/MAPKs. But activated PAR2 forms a complex with the adaptor protein β-arrestin, Raf1 and activated ERK/MAPKs, which retains activated ERK/MAPKs in the cytoplasm and prevents them from translocating into the nucleus. **b** | Determining the signalling kinetics. In PC12 cells, nerve growth factor (NGF) binds its receptor TrkA to promote either survival or differentiation. At the plasma membrane, activated TrkA causes a transient activation of ERK/MAPK through a pathway that involves Ras1, and transient activation of ERK/MAPK elicits survival. If it is internalized by endocytosis, activated TrkA causes a sustained activation of ERK/MAPK through a pathway that involves Rap1 (a Ras-like small GTPase). Sustained activation of ERK/MAPK elicits differentiation. **c** | Transport of signals from the synapse to the soma. From right to left, NGF emanating from target tissues binds TrkA at the presynaptic terminal. Activated TrkA is internalized into endocytic vesicles that fuse with a signalling endosome. The signalling endosome carries out retrograde trafficking through the axon and reaches the cell body where it elicits downstream signalling events including CREB (cAMP-response-element binding protein) phosphorylation. **d** | Signalling by freeing the signalling effector and targeting the repressor for degradation. During Hedgehog (Hh) signalling, Smoothened (Smo) signals by default. In the absence of Hh, its receptor, Patched (Ptc), represses Smo signalling (left). Incardona *et al.* proposed that in the presence of Hh (right), Hh binds Ptc and the Hh–Ptc–Smo complex is internalized into a sorting endosome. It is here that Ptc–Hh is sorted for degradation, whereas Smo is targeted to another compartment (a signalling endosome?) where it is freed from Ptc and can elicit downstream signalling.

RAB PROTEIN FAMILY
A family of small GTPases that function as key, specific regulators of membrane trafficking.

RAB7
A small GTPase that controls the targeting of endocytic cargo to the lysosome.

RAB5
A small GTPase that controls clathrin-coated pit formation from the plasma membrane and fusion to the early endosome.

RAB11
A small GTPase that controls the targeting of endocytic cargo to the recycling endosome.

at the source will get to the edge of the gradient, the rest must be degraded. Dpp is degraded in the lysosome of the receiving cells in a mechanism that involves one of the RAB PROTEIN FAMILY, the small GTPase RAB7 (REF. 29; BOX 1). The rate of lysosomal targeting controlled by Rab7 determines the maximal range of Dpp signalling, probably by setting the number of Dpp molecules degraded at each cell and thereby the range of the Dpp gradient. Similarly, the rates of trafficking through the early endosome, which are controlled by another small GTPase, RAB5 (BOX 1), also affect the range of Dpp signalling[29]. Therefore, in a system in which the ligand is dispersed by planar transcytosis, a stable gradient is maintained by controlling the rates of recycling and degradation at the lysosome in the receiving cells. In turn, those two rates determine the range of the gradient, its slope and thereby the coordinates of positional information. In the future, it will therefore be interesting to address the specific role of the recycling Rabs, Rab4 and RAB11, during morphogen spreading.

Spreading Wg by endocytic trafficking? Planar transcytosis was first proposed as a mechanism of morphogen dispersal in the case of Wg signalling during pattern formation of the embryonic epidermis of *Drosophila*, in

which Wg is able to elicit signalling at a distance of up to four cell diameters away from the source[37,38]. Wg is found in intracellular vesicular structures[37] in which it is internalized by dynamin-dependent endocytosis[38]. When endocytosis is impaired using the *shibire* mutation, Wg is only able to elicit signalling in cells that are adjacent to the source. These results support a situation in which Wg is dispersed by planar transcytosis[38]. However, this model of Wg dispersal is still controversial, and it has been argued that Wg is dispersed by extracellular diffusion during wing development[39]. In either case, recent data from the laboratory of Jean-Paul Vincent show that, like Dpp, the Wg signalling range is restricted by degradation at the endocytic pathway[40].

Restricting the signalling range by degradation

During embryogenesis, Wg is expressed as a one-cell-wide ectodermal stripe in each segment. From this source, Wg is secreted and dispersed symmetrically towards the anterior and the posterior sides of the source[41,42]. Later, Wg is spread asymmetrically: five cells towards the anterior side and one cell towards the posterior side[41,42]. Asymmetric Wg distribution confers polarity to Wg signalling during patterning of the epidermis. So, for example, Wg signalling represses the expression of a transcription factor, Shavenbaby, the activation of which is then restricted to a stripe, which is not centred between the Wg stripes[43]. The Shavenbaby domain is positioned asymmetrically, because long-range repression by Wg in the anterior direction means that Shavenbaby is 'displaced' towards the anterior direction. Ultimately, the Shavenbaby expression domain is necessary and sufficient to trigger, in these cells, the differentiation of DENTICLES in each segment.

Lysosomal degradation of Wg in cells that are posterior to the Wg-expressing stripe contributes to this asymmetric signalling event[40]. This was shown using a chimeric fusion of Wg with the enzyme horseradish peroxidase (HRP), HRP–Wg. HRP can be used to follow the trafficking of Wg. Furthermore, HRP remains undegraded after Wg is digested in the lysosome of the receiving cells. So, this fusion uncovers the asymmetric rates of Wg degradation. In anterior cells, the ranges of distribution of Wg and the HRP moiety is the same. By contrast, in posterior cells, Wg is actively degraded beyond the cells that are adjacent to the source, and further away from this source only the HRP moiety remains as a relic of the intracellular degradative event.

Wg degradation at the endocytic pathway. Wg degradation requires two proteins that are essential for endocytic trafficking — clathrin and DEEP-ORANGE[40] (Dor; BOX 1), which is the *Drosophila* homologue of the yeast vacuolar protein sorting factor Vps18 (REFS 44,45). Mutations in either clathrin or Dor cause an expansion in the signalling range of Wg, which indicates that intracellular trafficking might indeed control Wg distribution[40]. Clathrin-coated vesicles are involved in trafficking through the secretory pathway, in which the AP1 adaptor complex initiates formation of the clathrin coat on the *trans*-Golgi network (for a review, see REF. 46). Clathrin-coated vesicles are also involved in the early steps of trafficking along the endocytic pathway mediated by the AP2 and AP3 adaptor complexes[46] (BOX 1). Therefore, clathrin-dependent Wg degradation might be initiated by its endocytosis. Dor has also been implicated in trafficking through the degradative pathway during endocytic trafficking[44], which adds support to this hypothesis. Furthermore, chloroquine, an antimalarial drug that impairs degradation in the lysosomes, also causes an expansion of the Wg range[40]. Therefore, the distribution of Wg is restricted by intracellular protein degradation in the endocytic pathway, and the rate of degradation at the posterior side of Wg-producing cells confers the asymmetric distribution that is essential for pattern formation in the segmental unit. The active degradation of Wg in the posterior side is activated by the transcription of an unknown factor in response to epidermal growth factor receptor (EGFR) signalling[40].

Control of endocytic trafficking by EGFR. EGFR signalling has recently been shown to control endocytosis by regulating the activity of the small GTPase Rab5 during endocytic vesicle budding from the plasma membrane (BOX 1). The EGFR control of Rab5 involves the post-transcriptional regulation of the activity of RIN1, a Rab5 guanine nucleotide exchange factor (GEF), which acts as an effector of activated Ras[47] and RN-Tre, a Rab5 GTPase-activating protein (GAP), which is itself inactivated by active EGFR[48]. These, in turn, determine the kinetics of interconversion of the GDP-bound (inactive) and GTP-bound (active) Rab5 at the plasma membrane. Interestingly, the effect of epidermal growth factor (EGF) on Rab5-mediated internalization affects specifically the endocytosis of the EGFR when activated by the ligand, but not the constitutive endocytosis of the transferrin receptor[47,48].

In the case of Wg signalling in the *Drosophila* embryonic epidermis, it is not known whether EGF enhances lysosomal degradation in general or specifically the degradation of Wg, as mediated by internalization signals that are present on the cytoplasmic tail of the Wg receptors. Sorting information in the cytosolic tail of receptors is present in short peptide sequences, such as YppΦ (where p is a polar and Φ a hydrophobic residue) and the dileucine motifs, which interact with different components of the clathrin tetrameric adaptor complex AP2 (for a review, see REF. 49). The atomic structures of the interaction between receptors and the adaptors have been established recently[50,51]. An alternative sorting mechanism is provided by the mono-ubiquitylation of receptors (for reviews, see REFS 52,53). In mammalian cells, on ligand binding, many signalling receptors are mono-ubiquitylated by the ubiquitin–proteasome machinery that includes either Nedd4 or Cbl ubiquitin ligases[52]. Mono-ubiquitin in turn targets the receptors for rapid entry into the endocytic pathway. Alternatively, instead of tagging the cargo, the ubiquitylation of an endocytic factor, EPS15 (BOX 1), or other components of the endocytic apparatus, can also regulate the specific internalization of the receptors[54–56]. In addition, the next step

DENTICLE
A specialization of the cuticle of the ventral hypodermis of the *Drosophila* larva, which has been used as a diagnostic marker of the segmentation of the embryo.

DEEP-ORANGE
(Dor). A component of the homotypic fusion and vacuole protein sorting (HOPS) complex that controls the fusion of multivesicular bodies to lysosomes.

AP2
An adaptor complex that interacts with the cytosolic tail of plasma-membrane receptors and recruits the clathrin coat.

EPS15
An adaptor protein that binds to AP2 when phosphorylated by the EGF receptor and recruits clathrin to the endocytic vesicle coat.

for cargo degradation, the targeting to the internal vesicles of multivesicular bodies and lysosomes (BOX 1), can be regulated by ubiquitylation [57,58].

The effects of degradation on Wg signalling. Regardless of the mechanism of Wg targeting to degradation, how does degradation affect the signalling range? One possibility is that high levels of lysosomal degradation might restrict the posterior dispersal of Wg (FIG. 2f). In this situation, Wg disperses by diffusion through the extracellular space and Wg 'endocytosis plus re-secretion' makes an intracellular degradative compartment accessible to Wg while it travels through the target tissue. However, the fact that HRP is seen far from the source on the posterior side argues against a role for degradation in dispersal[40]: HRP–Wg was indeed detected far from the source, but Wg was degraded. Nevertheless, it is possible that the HRP moiety is so resistant to degradation that its presence far away from the source reflects its accumulation during previous developmental stages. Wg dispersion by diffusion and Dpp spreading by planar transcytosis indicates that different morphogenetic signals are transported through target tissues using different strategies; they capitalize on their free extracellular diffusion versus their intracellular transport to different degrees.

A simple alternative model is that Wg reaches the plasma membrane of cells that are of equal distance from the source, both anterior and posterior, but that it is internalized and degraded in the posterior cells. As this posterior enhancement of the degradation rate has an impact on Wg signalling, an important proportion of Wg signal transduction must occur after its receptor-mediated internalization into the cell. In other words, Wg signal transduction probably occurs from an intracellular compartment as well as from the cell surface. Transduction from a 'signalling endosome' is becoming a leitmotiv in the signalling field, which seems to apply to cell communication events that are mediated by EGF, nerve growth factor (NGF) and TGF-β.

Signal transduction at the endocytic pathway

The textbook view of signal transduction is that ligands bind their receptors at the plasma membrane and the receptors elicit a downstream response *in situ*, from the plasma membrane. Subsequently, signalling is stopped by the internalization of the receptors by endocytosis. This concept is based on the idea that the signal-transduction machinery is restricted to the plasma membrane, so that on internalization the receptors are removed from the signalling site. It was supported by early work in the 1990s, which showed that receptors that can not be internalized can be biologically competent[59,60]. However, bound to its effectors, the receptor might traffic into the intracellular compartment and signal from there. In recent years, there have been observations made for several signalling pathways that point towards such a mechanism.

RTK signalling and endocytosis. The fact that the expression of a dominant-negative dynamin mutant, which blocks endocytosis, attenuates significantly the EGF-dependent activation of the extracellular signal-related kinase/mitogen-activated protein kinase (ERK/MAPK)1/2, indicates that endocytosis is required for signal transduction mediated by RTKs[61]. At what level is internalization by endocytosis required during

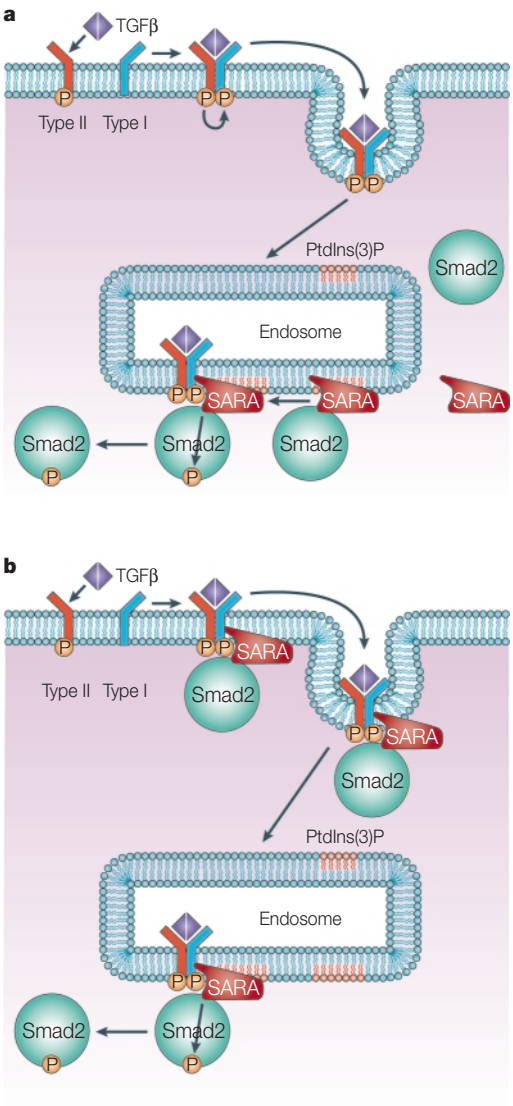


Figure 4 | **SARA mediates endosomal signalling. a** | TGF-β binds to the kinase-active type II receptor, thereby bringing it to and phosphorylating the type I receptor. Heterodimer formation activates the kinase in the type I receptor, after which the complex is internalized into endosomes. SARA (Smad anchor for receptor activation) interacts with Smad2, recruits it to the endosome and presents it to the activated transforming growth factor (TGF)-β receptor — SARA is recruited to the endosome by binding to phosphatidylinositol-3-phosphate (PtdIns3P) through its FYVE-finger domain. SARA binds to the type I receptor, thereby enabling the phosphorylation of Smad2. **b** | Activated type I receptor, Smad2 and SARA form a trimeric complex at the plasma membrane. When internalized into the endosome, SARA allows phosphorylation of Smad2 in a mechanism that involves the binding of its FYVE-finger domain to the endosomal PtdIns(3)P. In this situation, SARA confines the phosphorylation of Smad2 to the endosomal compartment.

RTK-mediated signal transduction? And what factor or factors need to be internalized? The observation that activated EGFR can be detected on endosomes[62] where it can activate Ras[63] indicates that it is the receptor itself that needs to be internalized to initiate signalling. *In vivo* localization studies of protein–protein interactions during signalling events using novel imaging techniques such as fluorescence resonance energy transfer (FRET) gave further support to this possibility[64]. FRET was monitored between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fused to EGFR and Grb2, respectively. Grb2 is an SH2-domain factor that binds activated EGFR and initiates signalling through Ras and ERK/MAPK. Stimulation by EGF resulted in the recruitment, as monitored by FRET, of Grb2–YFP to endosomes that contain EGFR–CFP.

Why does the EGFR relocate to the endosome? One reason could be that at the endosome the receptor can meet with downstream signal transducers. Furthermore, it is interesting to note that the internalized EGFR contains one extra threonine and three extra serine phosphorylated sites as compared with plasma-membrane-localized receptors[65], which opens up the possibility that further post-translational modifications add a level of control at the endosome.

However, in some systems, under conditions in which endocytosis is blocked and EGF activation is unable to regulate ERK/MAPKs, EGF still leads to the autophosphorylation of the EGFR and of Ras, Raf1 and MAPK kinase (MEK)[66,67]. This indicates that endocytosis is required in the signalling pathway after MEK1 activation and before phosphorylation of the ERK/MAPKs. In other words, endocytic trafficking somehow grants access of the signalling complex, which includes MEK1, to ERK/MAPK in an intracellular compartment.

Functional significance of endocytic signalling. One advantage of the endocytic-signalling strategy might be to allow the choice between two alternative pathways for a signalling cascade that starts at the same receptor (FIG. 3a). So, activated ERK/MAPKs can be imported into the nucleus to elicit mitogenesis or, alternatively, they can remain associated with endocytic vesicles in a complex that includes the receptor and the endocytic factor β-ARRESTIN[68–70] (BOX 1). When at endocytic vesicles, the nuclear import of ERK/MAPKs is bypassed and ERK/MAPKs phosphorylate their cytosolic or membrane targets[68–70]. Another example is the effect of NGF-mediated signalling; the activated Trk receptor induces survival when at the plasma membrane, whereas it elicits differentiation of neurite outgrowth if internalized[71].

Another possible advantage is that the localization of the signal-transduction event can determine the kinetics of activation of the signalling pathway (FIG. 3b). This seems to be the case in PC12 cells in which the NGF-mediated activation of TrkA causes a sustained activation of ERK/MAPKs, which depends on endocytosis of the receptor, whereas transient activation of ERK/MAPKs occurs by transduction from the plasma membrane[72].

Transduction from the endosome also provides a mechanism by which signals that are generated by neurotrophic factors at the presynaptic terminal of neurons are communicated to cell bodies that are located far away from the source of the signal (FIG. 3c). NGF that is generated at target tissues activates presynaptic TrkA receptors, which induces the internalization of the ligand–receptor complex into clathrin-coated vesicles[71–74]. Subsequently, these clathrin-coated vesicles, which also contain activated signalling proteins of the Ras–ERK/MAPK pathway, reach a population of signalling endosomes[75,76] that undergo retrograde trafficking through the axon[73,77] until they reach the cell bodies where they carry out downstream signal-transduction events[78,79].

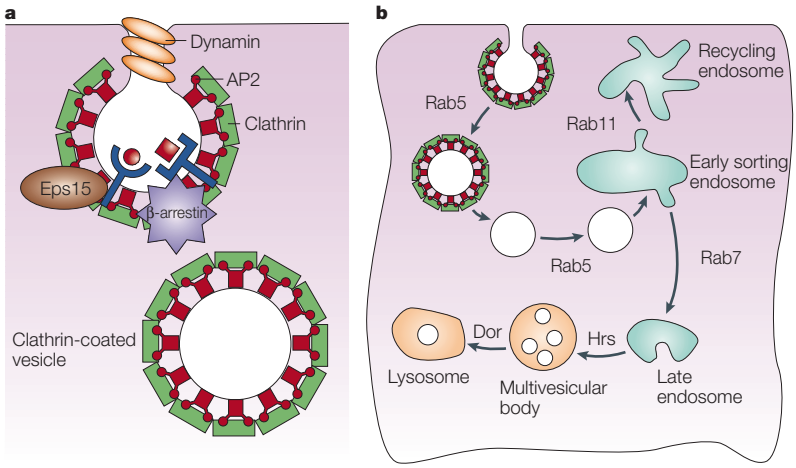
An interesting possibility has been proposed recently by Roelink and collaborators, who have postulated that for Shh signalling endocytic trafficking lies at the core of how signal transduction takes place[80]. It was shown previously that in the absence of ligand, the Shh receptor Ptc inhibits a latent, tonic signalling activity of another transmembrane protein, Smoothened (Smo; for a review, see REF. 81), and when Shh binds Ptc, the inhibition of Smo is released. Roelink and his collaborators showed that Ptc and Smo colocalize extensively in the absence of ligand and that they are internalized together after ligand binding. However, Smo becomes segregated from Ptc–Shh complexes that are targeted for degradation in the lysosome[80]. This uncovers a situation whereby Smo is inhibited by Ptc when they both are in the same compartment, whereas, on ligand binding and internalization of Smo and Ptc–Shh complexes, Smo becomes sorted from Ptc–Shh (FIG. 3d). The sorting of Smo requires an acid luminal endosomal environment in which the membrane is enriched in lysobiphosphatidic acid (LBPA), which indicates that a specific trafficking route along the endocytic pathway is involved in Hh signalling. Sorted away from Ptc–Shh, Smo can activate signal transduction in a Ptc-free compartment in which it can interact with downstream effectors. Therefore, on binding of Shh to Ptc, Ptc trafficking changes and it becomes targeted for degradation. The degradation of Ptc in turn prompts the sorting of Smo into a different (endosomal?) compartment in which the active signalling molecule Smo can elicit signalling.

TGF-β, SARA and the signalling endosome

It is still unclear whether intracellular signal transduction occurs from endocytic vesicles after the disassembly of the clathrin coat or from an intermediate sorting endosomal compartment. At least in the case of TGF-β or activin, signal transduction seems to occur from a sorting endosome. TGF-β induces the formation of a type I–type II heteromeric receptor complex with serine/threonine kinase activity (for a review, see REF. 82). The type I receptor kinase propagates the signal by phosphorylating transcription factors, the so-called receptor Smads (R-Smad; Smad1, 2, 3, 5 and 8). Phosphorylated R-Smads bind to another Smad, the Co-Smad (Smad4), and are translocated into the nucleus as a heteromeric complex;

β-ARRESTIN
An adaptor protein that binds to clathrin on interaction with phosphorylated β-adrenergic receptor.

Box 1 | **The endocytic pathway**



During the past 15 years, many of the events and players that are involved in the endocytic pathway have been uncovered. Endocytic trafficking is initiated by the formation of a clathrin-coated pit (see figure, part a). The cytosolic tails of receptors at the plasma membrane recruit clathrin in place to mediate the curvature of the membrane that will lead to its invagination. Binding of the receptor cytosolic tails to clathrin occurs by means of various adaptor proteins, including the AP2 heterotetrameric complex (which is composed of α-, β-, μ- and σ-adaptin), Eps15 and β-arrestin. Both Eps15 and β-arrestin bind to the receptors on binding of the ligand and subsequent phosphorylation. Once the membrane is invaginated into a pit, the fission of the coated pit to form a vesicle involves several proteins, including amphiphysin, dynamin and endophilin.

Downstream of endocytosis, membrane trafficking involves the endocytic pathway (see figure, part b). This pathway consists of several intermediate compartments, including the early sorting endosome, in which the decision is made whether the endocytic cargo is directed towards degradation in the late endosome or lysosome, or recycling the cargo back to the plasma membrane (directly or through the recycling endosome). Many of the key regulators of the distinct trafficking steps in the endocytic pathway have been identified. Rab5 controls the endocytic vesicle budding at the plasma membrane, as well as its motility along the microtubules and the fusion of the endocytic vesicles with the sorting endosome. Rab11 is involved in the step from the sorting to the recycling endosome, and Rab7 in the targeting from the sorting endosome to the late endosome. Hrs (hepatic growth factor-regulated tyrosine kinase substrate) has been implicated in the invagination of the endosomal membrane to generate the internal vesicle of the multivesicular bodies and Deep-orange (Dor), in later steps of the degradation pathway.

here the complex activates transcription. In the case of Smad2, phosphorylation by the receptor requires another factor, SARA (Smad anchor for receptor activation), that binds to Smad2 and brings it to the receptor[83].

SARA contains a FYVE zinc-finger domain[83], a motif that has been shown to interact directly and specifically with phosphatidylinositol-3-phosphate (PtdIns3P) when it is inserted in the endosomal membrane[84]. PtdIns3P has an important function in endocytic trafficking (for a review, see REF. 85). It has been shown that a tandem FYVE domain functions as a probe for PtdIns3P, and that PtdIns3P is highly enriched in sorting endosomes and the internal vesicles of multivesicular bodies[86]. Furthermore, two FYVE-domain proteins, EEA1 and rabenosyn-5, are localized at the endosome where they act as bona fide mediators of endosomal dynamics[87,88]. A third FYVE-domain protein, HEPATIC GROWTH FACTOR-REGULATED TYROSINE KINASE SUBSTRATE (Hrs; BOX 1), has also been implicated in endosomal dynamics[89–91] and is thought to cooperate with SARA during TGF-β signalling[92].

Consistent with it having a FYVE domain, SARA is localized at the sorting endosome[93–95], where it recruits the transcription factor Smad2 (FIG. 4a). SARA can also interact with the TGF-β receptor complex[83], thereby presenting the bound Smad2 for its subsequent phosphorylation at a distinct intracellular compartment, the endosome[83,94,95]. Deletion of the FYVE domain from SARA[83]or depletion of endosomal PtdIns3P using wortmannin[94,95] leads to mislocalization of SARA, accompanied by mistargeting of Smad2 and the inhibition of TGF-β signalling. This implies that the SARA-dependent localization of the transcription factor to the endosome is essential for TGF-β signal transduction.

However, recent data have revealed a different situation, whereby SARA's main role is not to recruit Smad2 to the receptor at the endosome, but to sense the endosomal environment and activate signalling only there[96] (FIG. 4b). In experiments in which dynamin-dependent endocytosis was blocked, Leof and collaborators showed that binding of TGF-β to the heterodimeric receptor at the plasma membrane was by itself able to elicit phosphorylation of the type I receptor[96]. Furthermore, they showed that in the absence of endocytosis, a trimeric receptor–Smad2–SARA complex is formed at the cell surface, which implies that these factors bind to each other before they meet at the endosome. However, this complex is only active after dynamin-dependent endocytosis (that is, Smad2 phosphorylation requires internalization of the complex). These results open up the possibility that the FYVE domain of SARA senses the PtdIns3P-containing endosomal environment, and on PtdIns3P binding causes a conformational change in the trimeric receptor–Smad2–SARA complex, which elicits the phosphorylation of Smad2.

In any case, the early endosomal environment seems to be an essential prerequisite for TGF-β-regulated signal transduction. Indeed, the expression of a GDP-bound dominant-negative Rab5 mutant, which perturbs early endosomal dynamics, causes Smad2 phosphorylation, translocation into the nucleus and activation of Smad2-dependent transcription in the absence of the TGF-β ligand[95]. As inhibition of endocytosis does not cause the same phenotype, it is the altered endosomal dynamics, and not the impaired internalization of the receptors, which causes constitutive TGF-β signalling. It is unclear, however, how the receptor could be activated in the absence of the ligand and how Rab5 function could prevent this activation, although it has been suggested that it might be owing to the amplification of a ligand-independent, low-level activation of the kinase activity of the receptors[95].

Phosphorylation of Smad2 causes its dissociation from SARA and its association with the Co-Smad, Smad4. Consistent with this, SARA and Smad4 form mutually exclusive complexes with Smad2, which provides the system with processivity[83]. Furthermore, it has

HEPATIC GROWTH FACTOR-REGULATED TYROSINE KINASE SUBSTRATE (Hrs). A FYVE-domain protein that controls the invagination of the membrane to form multivesicular bodies.

been shown that in the absence of phosphorylation, Smad2 by itself can be imported into the nucleus, whereas SARA–Smad2 complexes can not[97]. In summary, this uncovers a situation whereby the nuclear import of non-phosphorylated Smad2 is inhibited by contact with SARA at the endosome. SARA in turn contacts the internalized activated type I receptor and thereby brings the transcription factor to its kinase. Receptor-mediated phosphorylation reduces the affinity of Smad2 for SARA at the endosome, which, first, releases Smad2, second, unmasks its intrinsic nuclear-import activity and, third, allows the formation of a phosphorylated Smad2–Smad4 active transcriptional complex that elicits expression of TGF-β-dependent factors.

Concluding remarks

The recently uncovered role of the endosome in signalling raises a number of important questions. Why does signalling occur from an endosome? Why do developing tissues spread their morphogens by passing through the endosomes of intermediate cells rather than moving around those cells? Are vesicular membrane carriers, such as the argosomes and exosomes, transporting ligands through the extracellular space? Are endosomes a device that integrates signalling events that are mediated by different pathways in a developing cell? To address these, and other, questions, protein trafficking needs to be studied at a time when cell signalling matters most — during morphogenesis — rather than in cultured cells. This will open up a new dimension during signalling events, as developing epithelial cells show an apico–basal polarization of their trafficking and intracellular compartments, and this adds an extra level of complexity. In this context, the relevance of the junction between cells, as the meeting place between the ligands and their receptors, and the relationship of polarized trafficking/junctions might be the next focus of this interface between intracellular trafficking and morphogenesis.

To address these questions, the dynamics of endocytic trafficking and cell signalling *in vivo* need to be studied during morphogenesis. Therefore, the monitoring of *in vivo* endosomal dynamics and following GFP-tagged ligands, receptors and effectors in *Drosophila*, fish or mouse in well-defined signalling systems during organogenesis will probably uncover how signalling and trafficking meet at the endosome.

Of particular relevance to unravelling the possibility of transduction from a signalling endosome will be to study the cellular compartment where protein–protein interactions, in particular receptor–effector interactions, take place *in vivo*. Some pioneering approaches to the *in vivo* visualization of signal transduction using FRET imaging techniques in cultured cells have already been undertaken[64,98–100]. An alternative and promising approach, which is based on the detection of conformational changes to effectors that are caused by signalling events, is the generation of chimeric proteins that are based on a circularly permutated GFP (cpGFP)[101]. Conformational changes in the protein to which cpGFP is fused cause it to acquire fluorescence. This feature would allow the cpGFP-based fusion to be used as a live sensor for the monitoring of the compartment of the cell in which ligand-dependent conformational changes of the transduction effectors take place.

Three fields are starting to merge: the cell biology of protein trafficking, the biochemistry of growth-factor signalling and developmental biology. In this new interface, trafficking through the endocytic pathway is one of the first focuses, probably because the processes of cell signalling, trafficking and development all largely consist of moving proteins through cells and tissues, in general, and in particular from the plasma membrane to intracellular compartments. Understanding the biochemistry of the different signalling pathways in the context of the different intracellular locations opens up a new dimension to understanding how cells can handle a multitude of regulatory signals and programmes during development. And yet another dimension is opening up because cells are integrated in developing epithelia, with their rich spatial and temporal specificity.

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Online links

DATABASES
The following terms in this article are linked online to:
Swiss-Prot: http://www.expasy.ch/
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