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Review

Specialised endocytic proteins regulate diverse internalisation mechanisms and signalling outputs in physiology and cancer

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Although endocytosis was first described as the process mediating macromolecule or nutrient uptake through the plasma membrane, it is now recognised as a critical component of the cellular infrastructure involved in numerous processes, ranging from receptor signalling, proliferation and migration to polarity and stem cell regulation. To realise these varying roles, endocytosis needs to be finely regulated. Accordingly, multiple endocytic mechanisms exist that require specialised molecular machineries and an array of endocytic adaptor proteins with cell-specific functions. This review provides some examples of specialised functions of endocytic adaptors and other components of the endocytic machinery in different cell physiological processes, and how the alteration of these functions is linked to cancer. In particular, we focus on: (i) cargo selection and endocytic mechanisms linked to different adaptors; (ii) specialised functions in clathrin-mediated versus non-clathrin endocytosis; (iii) differential regulation of endocytic mechanisms by post-translational modification of endocytic proteins; (iv) cell context-dependent expression and function of endocytic proteins. As cases in point, we describe two endocytic protein families, dynamins and epsins. Finally, we discuss how dysregulation of the physiological role of these specialised endocytic proteins is exploited by cancer cells to increase cell proliferation, migration and invasion, leading to anti-apoptotic or pro-metastatic behaviours.

Introduction

Endocytosis has a pleiotropic role in eukaryotic cells influencing most physiological cellular functions, including receptor signalling and turnover, cell proliferation and migration, tissue morphogenesis and mechanics and polarity and stem cell regulation [Sigismund et al., 2012; Sigismund and Scita, 2018]. The term 'endocytosis' encompasses multiple highly specialised internalisation mechanisms that depend on the nature of the cargo and the cell context, and are controlled by a wealth of endocytic adaptors, membrane lipids and different regulatory

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Abbreviations: AP2, adaptor protein 2; AP180/CALM, adaptor protein 180 / clathrin-assembly lymphoid myeloid leukaemia; ARH, autosomal recessive hypercholesterolemia; BAR, Bin-Amphiphysin-Rvs; β1AR, β1-adrenergic receptor; β2AR, β2-adrenergic receptor; CCPs, clathrin-coated pits; CG, CLIC/GEEC; CLCs, clathrin light chains; CLI, clathrin-independent carriers; CME, clathrin-mediated endocytosis; Dab2, disabled homolog 2; EGFR, Epidermal growth factor receptor; ENTH, Epsin N-terminal homology; Epn1, Epsin1; Epn2, Epsin2; Epn3, Epsin3; ER, endoplasmic reticulum; DPW, aspartate-proline-tryptophan; DR4/5, death receptor 4/5; Dyn1, Dynamin1; Dyn2, Dynamin2; Dyn3, Dynamin3; EMT, epithelial-to-mesenchymal transition; E-Cad, E-Cadherin; FCHO1/2, Fer/Cip4 homology domain only protein 1 and 2; FEME, Endophilin-mediated endocytosis; GEEC, GPI-enriched early endosomal compartments; GED, GTPase effector domain; GPCR, G protein-coupled receptors; GTPase, GTP hydrolysis domain; HRB, HIV-1 rev binding; IL-2R, interleukin-2 receptor; KO, knockout; LDLR, low density lipoprotein receptor; N-Cad, N-Cadherin; NCE, non-clathrin endocytosis; NPF, asparagine-proline-phenylalanine; PDZ, Postsynaptic density 95/Discs large/Zona occludens-1; PH, pleckstrin homology; PM, plasma membrane; PRD, proline-rich domain; Rtn3, Reticulon-3; SNX9, sorting nexin 9; TfR, transferrin receptor; TGFb, transforming growth factor b; TRAIL, TNF-related apoptosis-inducing ligand; UIM, ubiquitin interacting motif; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; Vim, vimentin

signals [Sigismund et al., 2012). In this chapter, we describe endocytic adaptors and other components of the endocytic machinery that have evolved to exert specialised roles in the different internalisation mechanisms, significantly shaping signalling output and cellular behaviour. We also provide examples of how this functional specialisation is exploited by cancer cells to aberrantly regulate endocytic pathways, leading to proliferative, anti-apoptotic or invasive/metastatic phenotypes.

Adaptors with specialised functions in clathrin-mediated endocytosis

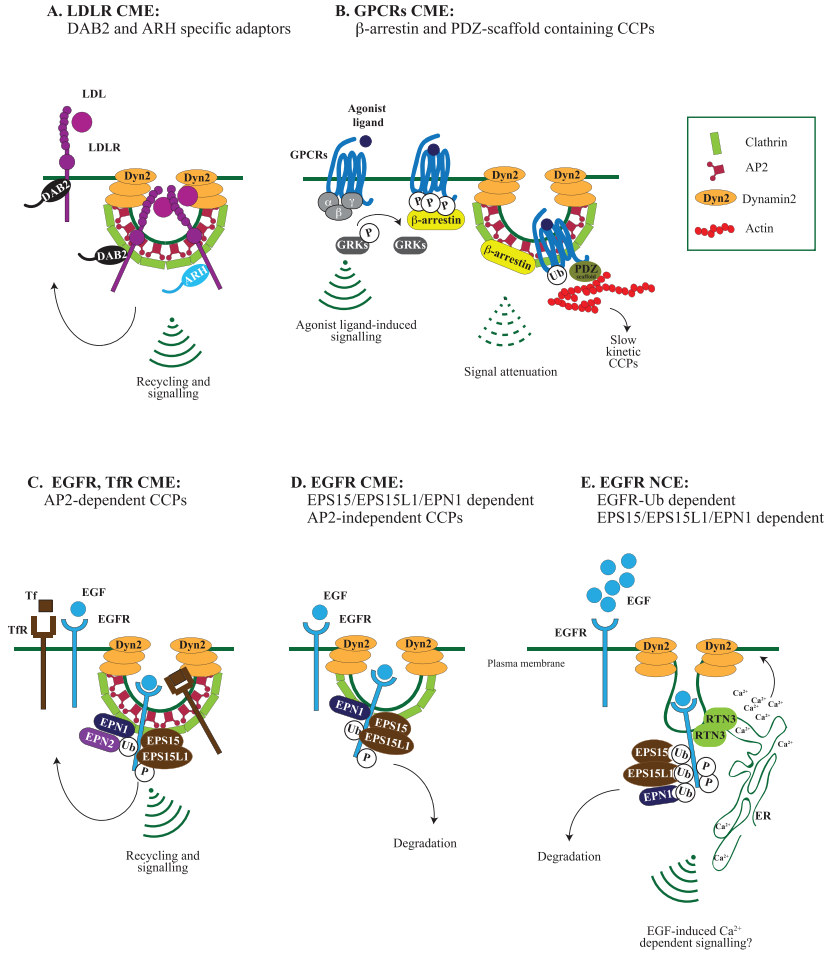
Endocytic adaptors are defined as those proteins that link the cargo to the endocytic machinery acting at different steps of the endocytic process, that is, cargo selection and clustering, membrane bending, maturation of the endocytic pit and vesicle fission [Traub, 2003]. They have been mostly studied in the context of clathrin-mediated endocytosis (CME), which is the best molecularly defined endocytic pathway. CME is responsible for constitutive, as well as many ligand-induced endocytic events in all cell types [Kaksonen and Roux, 2018; Kirchhausen et al., 2014; Mettlen et al., 2018]. The dynamics of adaptor recruitment have been extensively investigated, in particular, for constitutive CME, where a small subset of adaptors is recruited at initiator sites at the plasma membrane (PM), namely Fer/Cip4 homology domain only protein 1 and 2 (FCHo1/2), EPS15, EPS15L1 and adaptor protein 2 (AP2). Clustering of these 'pioneer' endocytic adaptors on the cytosolic portion of the PM defines the sites where clathrin-coated pits (CCPs) will assemble [Cocucci et al., 2012; Henne et al., 2010; Ma et al., 2016]. Interestingly, EPS15 and FCHo1/2 rely on weak, liquid-like interactions that allow for the rapid coalescence and the dynamic exchange of protein components required for efficient catalysis of CCP initiation [Day et al., 2019; Kozak & Kaksonen 2019].

AP2 is the major clathrin adaptor involved in most constitutive endocytic events that promotes clathrin assembly, cargo capture into the nascent CCPs and pit maturation. However, alternative adaptors with the ability to bind the initiator complex, membrane, clathrin and/or cargo have also been described, which can either assist AP2 or function independently to drive CCP assembly, cargo recruitment and/or CCP maturation in constitutive or ligand-induced CME [Mettlen et al., 2018; Mettlen et al., 2009]. For example, FCHo1/2, a Bin-Amphiphysin-Rvs (BAR) domain-containing protein involved in membrane bending, can recruit the adaptor proteins, HIV-1 rev binding and disabled homolog 2 (DAB2), in addition to EPS15 and AP2 [Umasankar et al., 2012]. Likewise, EPS15 can interact with the adaptors, intersectins, epsins and adaptor protein 180/clathrin-assembly lymphoid myeloid leukaemia (AP180/CALM) [Chen et al., 1998; Morgan et al., 2003].

In addition to FCHo1/2, other BAR domain-containing proteins are recruited in CME and are involved in generating membrane curvature in co-operation with clathrin polymerisation. One of these proteins is sorting nexin 9 (SNX9) that is recruited at an intermediate stage of CCP formation [Taylor et al., 2011], while the BAR domain-containing proteins, endophilin and amphiphysin, are recruited in the final step of CCP formation and cooperate with the GTPase, dynamin, to facilitate CCP fission [Kaksonen and Roux, 2018; Peter et al., 2004]. Although dynamin has a critical role in the last step of fission in CME [Antonny et al., 2016], it has recently been shown to be recruited also in an early phase of CCP formation and to regulate cargo recruitment and CCP maturation [Loerke et al., 2009; Srinivasan et al., 2018] (see also Section 3.1).

The involvement of so many endocytic adaptors in CME raises the possibility that different types of CCPs with a specialised repertoire of adaptors could exist, with specific roles in cargo selection, regulation of receptor clustering, signalling and fate [Kaksonen and Roux, 2018; Lampe et al., 2016; Traub, 2003]. Indeed, cargo recruitment occurs through specific binding sites in the cytosolic tail of PM receptors, namely short linear sorting motifs or covalent modifications, such as phosphorylation and ubiquitination [Traub, 2009]. This allows the selective recruitment of specific cargoes into the nascent pit, as in the case of the low-density lipoprotein receptor (LDLR), for which cargo-specific adaptors exist, namely DAB2 and autosomal recessive hypercholesterolemia (ARH) proteins (Figure 1A) [He et al., 2002; Maurer and Cooper, 2006; Mishra et al., 2002a; Mishra et al., 2002b; Morris and Cooper, 2001; Tao et al., 2016]. ARH was also shown to act as a selective adaptor in β1-integrin endocytosis [Teckchandani et al., 2012].

Figure 1 | Cargo selective adaptors in CME and NCE. (**A**) DAB2 and ARH work as specific adaptors for LDLR endocytosis acting in concert with AP2 and leading to recycling and signalling. (**B**) GPCRs are internalised through a subset of CCPs containing the cargo specific adaptor, β-arrestin. Receptor ubiquitination and the PDZ-containing scaffold, linking the GPCR to the actin cytoskeleton, determine an increased surface retention of GPCR-containing CCPs and a slower kinetic, promoting cargo clustering. (**C**) EGFR and TfR are internalised through a subset of CCPs, absolutely dependent on AP2, which are destined to recycling. In the case of the EGFR, these CCPs are also involved in sustaining signalling leading to cell migration. (**D**) In addition to AP2-dependent CME, EGFR can be internalised through a subset of AP2-independent CCPs, which rely on EPS15/EPS15L1 and EPN1 adaptors and target the EGFR to degradation. (**E**) EPS15/EPS15L1 and EPN1 are also involved in EGFR-NCE through their ubiquitin-binding ability. Indeed, they possess ubiquitin binding motifs (UIMs) through which they recognise the ubiquitinated EGFR and target it for internalisation via NCE. This mechanism requires the formation of contact sites between the PM and the ER, which are mediated by the ER-tubulating factor, RTN3, and a local release of calcium (Ca^{2+}) from the ER to the PM needed for vesicle fission in concert with Dyn2. The interaction between UIM-containing adaptors and RTN3 is yet to be characterised. Importantly, this mechanism, on the one hand, targets the EGFR to degradation restricting MAPK/Akt signalling, on the other hand, it stimulates calcium signalling that might regulate additional unknown EGF-dependent functions in the cell.



A case in point is represented by some G protein-coupled receptors (GPCRs) that, upon agonist stimulation, have been shown to internalise through a distinct subset of CCPs specifically containing the adaptor protein, β-arrestin (Figure 1B) [Eichel and von Zastrow, 2018; Hanyaloglu and von Zastrow, 2008]. Receptor ubiquitination and the interaction of postsynaptic density 95/disc large/zonula occludens-1 (PDZ) motifs in the receptor tail with actin, have a crucial role in determining the

dynamics of GPCR-containing CCPs. Indeed, these CCPs present a slower rate of formation and an increased surface residence time compared with constitutive CCPs, indicating the existence of a direct control of CCP dynamics exerted by the cargo itself and its connection with the actin cytoskeleton [Henry et al., 2012; Puthenveedu and von Zastrow, 2006]. This cargo-dependent control of CCP properties has been proposed to limit the competition between different endocytic cargoes, functionally and molecularly diversifying the CME pathway.

β-Arrestin is not involved in the internalisation of all GPCRs; for instance, protease-activated receptors, PAR1 and PAR4, are internalised via CME involving AP2, but independently of β-arrestin, pointing again to a cargo-dependent specialisation of CME [Arakaki et al., 2018]. An interesting case is the β1-adrenergic receptor (β1AR) that, differently from β2AR, is endocytosis-incompetent [Eichel et al., 2016]. However, upon agonist stimulation, β-arrestin transiently interacts with β1AR at the PM; it then detaches from the receptor tail and is internalised in CCPs from where it signals in the absence of the receptor [Eichel et al., 2018; Eichel et al., 2016; Latorraca et al., 2018]. This modality of CME of the adaptor in the absence of the cargo is peculiar for the β-arrestin/β1AR pair, and future work is needed to understand if it is utilised in other systems.

In line with the existence of different modalities of CME, it has been recently shown that, although AP2 is ubiquitously expressed, its levels are variable in different cellular contexts and this affects the properties of CCPs in terms of dynamics and dimensions [Dambournet et al., 2018]. This result suggests that the relevance and the contribution of AP2 to CME might vary depending on the cellular context. In addition, while AP2 is absolutely required for constitutive endocytosis of the transferrin receptor (TfR) (Figure 1C), for some ligand-induced endocytic processes, AP2 is not essential and other adaptors are responsible for AP2-independent CME (Figure 1D) [Boucrot et al., 2010; Hinrichsen et al., 2003; Huang et al., 2004; Johannessen et al., 2006; Maurer and Cooper, 2006; Motley et al., 2003]. This is the case of the epidermal growth factor receptor (EGFR), for which AP2-dependent (Figure 1C) and -independent (Figure 1D) modalities of CME exist [Pascolutti et al., 2019]. The two subclasses of EGFR-containing CCPs differ in molecular composition, dynamics and function. CCPs lacking AP2 rely on the endocytic adaptors, EPS15, EPS15L1 and epsin, and are smaller and relatively short-lived, compared with canonical AP2-containing pits [Pascolutti et al., 2019]. Interestingly, the effects of AP2 on CCP formation appear to be opposite to those of the adaptor CALM, since the increase in CALM levels has been shown to result in smaller and short-lived pits [Miller et al., 2015].

At the functional level, different AP2 levels produce CCPs with specialised roles in the regulation of EGFR fate and signalling: AP2-containing pits sustain EGFR recycling and signalling, while AP2-lacking CCPs are recycling impaired and target the EGFR to degradation, reducing EGF-dependent signalling and cell migration. Notably, the impact of AP2 depletion on the EGFR-induced transcriptional changes is clearly cell context-dependent, suggesting that the regulation of EGFR signalling/transcriptional outputs by CME is cell specific [Pascolutti et al., 2019]. These data reveal an unexpected level of plasticity in CME influenced by the cargo and cellular needs.

Adaptors with specialised functions in clathrin-dependent versus -independent endocytosis

Although CME accounts for a large fraction of endocytic events, many PM cargoes and receptors are internalised via non-clathrin endocytosis (NCE) [Johannes et al., 2015]. NCE includes different endocytic mechanisms that are heterogeneous in terms of morphology, molecular machinery, adaptors, cargoes and upstream regulatory signals [Johannes et al., 2015; Thottacherry et al., 2019]. These mechanisms are not active in all cell types, but are highly cell context regulated. They can be classified by the presence of some form of coat, as in the case of caveolin- and flotillin-mediated endocytosis [Meister and Tikkanen, 2014; Parton et al., 2020], and by the type of fission [Glebov et al., 2006; Johannes et al., 2015; Thottacherry et al., 2019]. Indeed, they mostly rely on actin for the fission step, in cooperation or not with dynamin action.

NCE mechanisms lacking a proper coat include: (i) endocytosis via clathrin-independent carriers (CLICs) [Kirkham et al., 2005; Sabharanjak et al., 2002], also referred to as GPI-enriched early endosomal compartments (GEEC) [Sabharanjak et al.,

2002]; ii) the so-called fast endophilin-mediated endocytosis (FEME) [Boucrot et al., 2015]; (iii) the EGFR-NCE mechanism [Caldieri et al., 2017]; and (iv) interleukin-2 receptor (IL-2R) endocytosis [Grassart et al., 2008; Lamaze et al., 2001; Sauvonnet et al., 2005].

At the molecular level, NCE mechanisms have been characterised only recently and, in many cases, they share some morphological and molecular features, as well as cargo, suggesting they might not be strictly distinct internalisation mechanisms. How closely they relate to one another and whether they represent variations on a theme in different cell contexts, is still a matter of investigation.

In addition to intracellular endocytic adaptors, extracellular determinants, such as sugar-binding proteins of the galectin family, can act as endocytic adaptors in NCE, recruiting glycosylated cargoes and glycolipids in PM nanodomains, and are involved in the formation of an extracellular lattice that drives PM bending, invagination and formation of CLIC vesicular intermediates [we refer the reader to a comprehensive review on this topic [Johannes et al., 2016].

Interestingly, some NCE mechanisms utilise the same repertoire of intracellular adaptors as CME, but with specialised roles. This is the case of endophilin-A that is involved in some forms of CLIC/GEEC (CG) endocytosis [Renard et al., 2015] and in FEME [Boucrot et al., 2015] as well as in CME. In CME, endophilin-A is recruited at a later stage of CCP formation and cooperates with dynamin in the fission step [Ferguson et al., 2009; Gad et al., 2000; Milosevic et al., 2011]. Similarly, in the CG mechanism, endophilin-A is required for fission, while it is not involved in the formation of initial membrane invaginations [Johannes et al., 2015). Importantly, endophilin-A covered membrane tubules are prone to fission in concert with the pulling force exerted by the actin cytoskeleton and the microtubule-associated dynein motor protein. This novel mechanism of PM fission, referred to as 'friction-mediated scission', relies on the endophilin-A scaffolding function that creates a lipid barrier on the tubules, thus reducing the lipid flow that, in combination with the pulling force exerted by motor proteins, increases membrane tension and causes the squeezing of the tubules [Simunovic et al., 2017). This mechanism acts additively with the dynamin- and actin-based fission mechanism and is involved in Shiga toxin endocytosis.

Differently from CME and CG endocytosis, endophilin-A is recruited at an early step in FEME [Casamento and Boucrot, 2020). In this case, endophilin-A appears to be involved in the formation of endocytic tubular invaginations at the PM inducing membrane curvature through its BAR domain and engaging the cargo via its SH3 domain [Casamento and Boucrot, 2020). Similarly to CG endocytosis of Shiga toxin, endophilin-A also acts in the membrane scission step in FEME, recruiting dynamin and actin through its SH3 domain [Casamento and Boucrot, 2020). Thus, depending on the cargo and endocytic mechanism, the same adaptor can be exploited by the cell to exert specific functions at different steps of endocytic vesicle formation.

EPS15, EPS15L1 and epsin1 (EPN1) are further examples of adaptors working in multiple endocytic mechanisms. These proteins are involved in CME, in particular, in AP2-independent EGFR-CME that targets the EGFR to degradation (as discussed in Section 1) [Pascolutti et al., 2019]. In this case, they possibly work by binding to FCHo1/2, clathrin and/or other adaptors, and by recruiting the EGFR into the nascent CCPs, although the mechanism of cargo recruitment remains unclear.

The same set of adaptors is also involved in EGFR-NCE [Sigismund et al., 2005]. This endocytic mechanism is selectively activated in the presence of saturating EGF doses, which induce a sharp increase in EGFR ubiquitination [Sigismund et al., 2013]. EPS15/EPS15L1 and EPN1 possess ubiquitin-interacting motifs (UIMs) that recognise the ubiquitinated EGFR and target it for internalisation via NCE [Sigismund et al., 2005]. EGFR-NCE also relies on the formation of membrane contact sites between the endoplasmic reticulum (ER) and the PM that are induced upon EGFR activation and require the ER-tubulation factor, reticulon-3 (RTN3) (Figure 1E) [Caldieri et al., 2017]. Upon high-dose EGF stimulation, EGFR and RTN3 are situated in close proximity [Caldieri et al., 2017], but the precise role of EPS15/EPS15L1/EPN1 and receptor ubiquitination in ER/RTN3 recruitment are yet to be defined. Interestingly, EGFR entering into this trafficking route is targeted to degradation [Sigismund et al., 2008], indicating that the same set of adaptors is used in AP2-independent CME and in NCE to direct the

receptor towards a degradative fate and to downmodulate EGFR signalling.

Endocytic protein family members with specialised roles in physiology and cancer

One important level of regulation of the endocytic process is the functional specialisation of different protein family members and isoforms that has occurred in evolution. Several endocytic genes of the same family have adapted and evolved to carry out specific functions that are often cell context dependent, as exemplified by the clathrin light chains (CLCs) that compose the clathrin triskelia. There are two CLC family members sharing 60% identity, CLCa and CLCb; the former is ubiquitously expressed, while the latter is predominant in brain and neuroendocrine cells [Acton et al., 1993; Brodsky et al., 1991]. CLCb is also up-regulated in pathological conditions such as cancer, for example, aggressive human lung cancers and lung metastases [Chen et al., 2017]. CLCb overexpression appears to selectively accelerate CME and recycling of the EGFR, but not of the TfR, resulting in increased EGF-dependent Akt signalling [Chen et al., 2017].

Similarly, the Myosin VI isoforms (short and long), which are regulated by alternative splicing and differ in their C-terminal region, have divergent functions [Biancospino et al., 2019; He et al., 2016; Wollscheid et al., 2016]. The long isoform is expressed in polarised epithelial cells with well-developed apical microvilli [Au et al., 2007], while the short isoform is up-regulated in non-polarised migratory cells and is involved mainly in migratory dynamics [Wollscheid et al., 2016]. Alternative splicing regulates the presence or absence of a regulatory helix, the α2-linker, which defines a clathrin-binding domain unique to Myosin VI long [He et al., 2016; Wollscheid et al., 2016]. Myosin VI is overexpressed in prostate [Puri et al., 2010) and ovarian cancer [Yoshida et al., 2004]. In the latter, the short isoform is involved in the induction of tumour cell migration [Wollscheid et al., 2016].

In the next two paragraphs, we describe in detail two additional endocytic protein families, the dynamins and the epsins, which further illustrate functional specialisation that is relevant to normal physiology and to cancer.

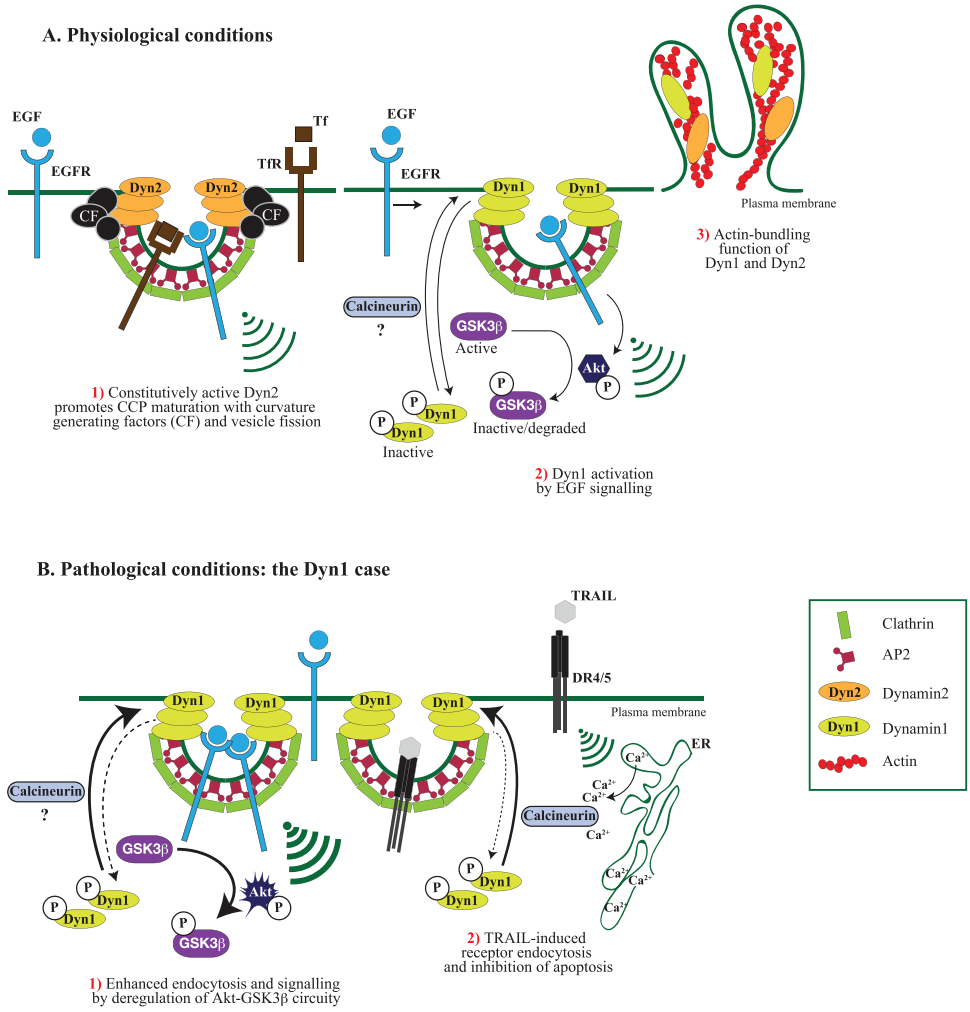
The dynamin family

Specialised functions of dynamins in physiology

Dynamins are a family of GTPase proteins well known for their fundamental role during the late stages of membrane fission, both in CME and NCE [Antonny et al., 2016; Ferreira and Boucrot, 2018; Kaksonen and Roux, 2018]. The first dynamin was discovered in 1989 [Shpetner and Vallee, 1989] and since then many dynamin-related proteins belonging to the dynamin superfamily have been identified [Ramachandran and Schmid, 2018]. In vertebrates, there are three dynamins: dynamin1 (Dyn1), dynamin2 (Dyn2) and dynamin3 (Dyn3) [Ramachandran and Schmid, 2018]. They share similar protein structure [Liu et al., 2011] and are composed of five domains: the N-terminal GTP hydrolysis domain (GTPase), the middle domain, the pleckstrin homology (PH) domain, the GTPase effector domain (GED) and the C-terminal proline-rich domain (PRD) [Ramachandran and Schmid, 2018]. Despite their high structural similarity, they present specific patterns of expression: Dyn1 is highly expressed in neurons, but is also present at substantial levels in all other tissues [Cao et al., 1998; Ferguson et al., 2007; Schmid, 2017]; Dyn2 is ubiquitously expressed in the different tissues [Cao et al., 1998]; Dyn3 is expressed mainly in lung, heart, brain and testis [Cao et al., 1998; Ferguson et al., 2007]. While the best-known role of dynamins is in catalysing membrane scission (Figure 2A) [Chen et al., 2017; Reis et al., 2015; Srinivasan et al., 2018], this differential pattern of expression results in distinct and specialised functions of the different dynamins depending on the cell context.

Dyn1 plays a major role in vesicle recycling in pre-synaptic events [Ferguson et al., 2007], while it is not usually involved in the regulation of constitutive CME fission events in other cellular contexts. Its function in neurons is highly regulated through phosphorylation/dephosphorylation reactions that link its activation to calcium influx during neurotransmission [Clayton et al., 2010; Graham et al., 2007]. In agreement with its neuronal role, *Dnm1* knockout (KO) mice show postnatal defects during neuronal transmission indicating that this function is tightly linked to Dyn1 expression in a non-redundant manner [Ferguson et al., 2007]. For these reasons, Dyn1 was considered to play a role only in neuronal cells. However, it has since

Figure 2 | Differential roles of Dyn1 versus Dyn2 in CME in non-neuronal cells. (**A**) Physiological conditions. (1) Dyn2 is constitutively active and promotes both CCP maturation and fission. (2) In addition to its well-characterised role in neurons, Dyn1 is also involved in CME in non-neuronal cells, but its function needs to be activated through dephosphorylation. In particular, EGF-dependent activation of Akt leads to GSK3β phosphorylation and degradation, resulting in Dyn1 dephosphorylation (possibly by calcineurin phosphatase) and activation in EGFR CME. Dyn1-mediated EGFR CCPs have faster kinetics and promote signalling. (3) Dyn1 and Dyn2 have been shown to act as actin-bundling proteins involved in the formation of F-actin enriched structures in the cell. It is still unknown whether this function is linked to the endocytic role of dynamins. (**B**) In pathological conditions, Dyn1 is hyperactivated through different mechanisms: (1) hyperactivation of Akt and hyperphosphorylation and degradation of GSK3β leads to aberrant Dyn1 activation and deregulated EGFR endocytosis and signalling; (2) TRAIL-dependent calcium release from the ER leads to calcineurin-mediated dephosphorylation of Dyn1, leading to increased death receptor 4/5 (DR4/5) endocytosis and inhibition of apoptosis.



been shown that Dyn1 is expressed at substantial levels also in non-neuronal cells, but that it is kept in an inactive state by GSK3β-dependent phosphorylation that negatively regulates its activity (Figure 2A) [Clayton et al., 2010; Mettlen et al., 2018; Reis et al., 2015]. This inhibition can be released by EGF stimulation, a condition that leads to Dyn1 activation in ligand-induced EGFR-CME. Dyn1 is also activated in cancer cells where GSK3β function is inhibited [Chen et al., 2017; Srinivasan et al., 2018].

Dyn2 is responsible for ubiquitous and constitutive endocytic events, which is reflected in the fact that *Dnm2* KO mice are embryonic lethal [Ferguson et al., 2007; Ferguson et al., 2009; Liu et al., 2008; Reis et al., 2015; Srinivasan et al., 2018]. Differently from Dyn1, Dyn2 is constitutively active and seems not to be regulated by phosphorylation [Srinivasan et al., 2018]. The two dynamins also present different biochemical properties: Dyn1 is a powerful membrane curvature generator and it is also able to catalyse fission, while Dyn2 is thought to act as a curvature sensor, working in concert with other curvature-generating factors to induce fission [Neumann and Schmid, 2013]. Dyn1 and Dyn2 also appear to act at different cellular locations during the vesicle invagination in non-neuronal contexts [Altschuler et al., 1998; Bhave et al., 2020; Srinivasan et al., 2018]. Indeed, in MDCK cells, it was shown by overexpression of a dominant-negative Dyn1 mutant that Dyn1 regulates receptor-mediated endocytosis at the apical surface, while Dyn2 is located at the baso-lateral surface [Altschuler et al., 1998].

Although dynamins are best known for their role in catalysing membrane scission, increasing evidence suggests that both Dyn1 and Dyn2 can also regulate the early stage of CCP maturation and cargo loading (Figure 2A) [Chen et al., 2017; Reis et al., 2015; Srinivasan et al., 2018]. However, in this early step of CME in non-neuronal cells, they appear to act in a non-redundant fashion due to differences in their protein domains and in their regulation by phosphorylation, with Dyn1 requiring dephosphorylation for activation. Thus, Dyn2 is likely involved in a wide range of endocytic events, while Dyn1 could be activated in specific cases [Bhave et al., 2020; Srinivasan et al., 2018].

Dyn3, on the other hand, appears to have partially overlapping functions with the other dynamins, particularly with Dyn1 [Antonny et al., 2016; Ramachandran and Schmid, 2018]. Indeed, Dyn3 can fully restore rapid synaptic vesicle recycling in Dyn1-null hippocampal neurons, at variance with Dyn2. However, the *Dnm1*/*Dnm3* double KO mouse model shows a worsening of the phenotype, compared with the *Dnm1* only KO [Raimondi et al., 2011]. Detailed analysis showed that Dyn3 – differently from Dyn1 – is implicated in the post-synaptic response, indicating that cooperative functions of Dyn1 and Dyn3 could be critical for the correct transmission of the electrical stimulus in neurons [Raimondi et al., 2011].

Dynamins are also known to bundle actin filaments and have been implicated in the formation of F-actin enriched cellular structures, such as podosomes, invadopodia, lamellipodia and actin comet tails [Baldassarre et al., 2003; McNiven et al., 2004; Ochoa et al., 2000; Yamada et al., 2013]. The recently elucidated mechanism underlying this function revealed that dynamin acts as a multifilament actin-bundling protein by direct binding to actin through its C-terminal PRD, regulating both the dynamics and strength of the actin cytoskeletal network (Figure 2A) [Zhang et al., 2020]. This function was demonstrated for both Dyn1 and Dyn2 and it is retained in lower organisms, such as *Drosophila*, where dynamin plays a crucial role in propelling invasive protrusions during myoblast fusion [Zhang et al., 2020]. Thus, the actin-bundling ability of dynamins appears to be evolutionarily conserved and a common feature of all family members. However, further studies are needed to understand whether this function is exploited by the different dynamins in specific processes and/or cell contexts.

Specialised functions of dynamins in tumorigenesis

Given the central role of dynamins in the vast majority of endocytic events, it is not surprising that their deregulation is associated with cancer. Indeed, numerous studies have implicated dynamins in tumorigenesis, cancer progression, chemoresistance and metastasis [Haferlach et al., 2010; Meng, 2017]. Considering that the three dynamins have very diverse roles in physiology, it is expected that they have similarly different non-overlapping functions also in cancer.

Dyn1 has been found to be overexpressed in acute myeloid leukaemia, lung adenocarcinoma, colon adenocarcinoma and hepatocellular carcinoma [Tian et al., 2020]. In particular, in hepatocellular carcinoma, high levels of Dyn1 act as an independent prognostic factor of poor overall survival, although this finding needs to be confirmed in larger patient cohorts [Tian et al., 2020]. In addition to protein overexpression, Dyn1 up-regulation in cancer can also be achieved by aberrant Akt/GSK3β signalling. In non-small cell lung cancer cell lines, Dyn1 is

hyperactivated as a result of increased Akt-mediated phosphorylation and inactivation of GSK3β, the kinase that phosphorylates Dyn1 and negatively regulates it. This leads to an increase in unphosphorylated, active Dyn1 resulting in increased CCP formation and accelerated CME culminating in dysregulated endocytic dynamics (Figure 2B) [Chen et al., 2017; Reis et al., 2015]. Moreover, it was also proposed that Dyn1 overexpression or activation through its calcium/calcineurin-dependent dephosphorylation can increase death receptor endocytosis in cancer cells induced by TNF-related apoptosis-inducing ligand (TRAIL), conferring resistance to apoptosis and inducing migration (Figure 2B) [Reis et al., 2017].

Dyn2 is also overexpressed in different types of cancer [Meng, 2017], such as prostate cancer [Xu et al., 2014], pancreatic cancer [Burton et al., 2020; Eppinga et al., 2012], cervical cancer [Lee et al., 2016], hepatocellular carcinoma [Gong et al., 2015]), glioblastoma [Feng et al., 2012], non-small cell lung cancer [Yamada et al., 2016] and adult T-cell acute lymphoblastic leukaemia [Ge et al., 2016]. In particular, high levels of Dyn2 have been shown to correlate with tumour volume in prostate cancer [Xu et al., 2014] and in cervical cancer [Lee et al., 2016], and with mortality in pancreatic ductal adenocarcinoma [Burton et al., 2020]. Mechanistically, Dyn2 deregulation causes increased EGFR-dependent signalling [Gong et al., 2015; Sousa et al., 2012] or deregulation of focal adhesion kinase [Ezratty et al., 2009], leading to augmented motility and migration. In the pancreas, Dyn2 is able to interact with α-activin4 and induce tumour invasion [Burton et al., 2020].

Dyn3, differently from the other two dynamins, has been less well characterised in cancer, probably because of its more restricted expression. Nevertheless, some studies have pointed to a tumour suppressor role of Dyn3, in particular in hepatocellular carcinoma, where it induces p53 expression and subsequent activation [Zhang et al., 2016]. Moreover, Dyn3 was found to be down-regulated in hepatocellular tumours, corroborating the idea that it could serve as a negative regulator of tumorigenesis [Inokawa et al., 2013], in contrast to Dyn1 and Dyn2 that seem instead to positively control tumorigenesis and invasion.

The epsin family

Specialised functions of epsins in physiology

Epsins belong to a family of endocytic adaptor proteins composed of 3 paralogs: EPN1, EPN2 and EPN3 [Rosenthal et al., 1999; Spradling et al., 2001]. The first epsin to be discovered was EPN1 in 1998, when it was described as an EPS15-interacting protein, since it was able to bind to the EH domain of EPS15, another endocytic adaptor protein [Chen et al., 1998]. Epsins are known to be involved in the first steps of endocytosis [De Camilli et al., 2002; Horvath et al., 2007; Sen et al., 2012], in particular there is evidence that they have a specific role in cargo selection and coat assembly [De Camilli et al., 2002; Horvath et al., 2007; Kaksonen and Roux, 2018; McMahon and Boucrot, 2011]. Importantly, the vast majority of epsin functions discovered so far are linked to EPN1 because it was discovered first and is ubiquitously expressed.

From a structural perspective, epsins share a similar protein structure [De Camilli et al., 2002; Horvath et al., 2007; Sen et al., 2012]. They possess an epsin N-terminal homology (ENTH) domain necessary to bind PtdIns(4,5)P_2 at the PM, two or three UIMs known to bind to ubiquitinated cargoes, and the aspartate-proline-tryptophan (DPW) and asparagine-proline-phenylalanine (NPF) motifs that interact with the clathrin machinery. Since the protein structure of the three isoforms is similar, redundant functions have been observed in endocytosis, regulation of the actin cytoskeleton during CME, and development [Chen et al., 2009; Messa et al., 2014]. In agreement, while the single *Epn1* or *Epn2* KO mice are viable and lack any observed phenotypes, the double *Epn1/Epn2* KO is embryonic lethal [Chen et al., 2009] indicating that the two proteins can compensate each other and play redundant functions.

EPN1/EPN2-related functions include roles in the EGFR pathway [Hawryluk et al., 2006; Kazazic et al., 2009; Sigismund et al., 2005], the Notch pathway [Langridge and Struhl, 2017; Tian et al., 2004; Wang and Struhl, 2004] and the Wnt pathway [Chang et al., 2015]. However, there is now increasing evidence indicating that the different epsins have specialised and cell context-specific functions [Ko et al., 2010; Schiano Lomoriello et al., 2020; Sen et al., 2012; Settembre et al., 2011]. In particular, while EPN1 and EPN2 have been shown to

be ubiquitously expressed, EPN3 expression is restricted mainly to gastric parietal cells [Ko et al., 2010] and migrating keratinocytes [Spradling et al., 2001], indicative of a diverse and specific function of this family member.

One example of specialised functions of epsins is the involvement of EPN1 and EPN2 in the regulation of the vascular endothelial growth factor (VEGF) pathway [Mao et al., 2020; Pasula et al., 2012; Rahman et al., 2016; Tessneer et al., 2014; Wu et al., 2018] and the Notch pathway [Langridge and Struhl, 2017; Tian et al., 2004; Wang and Struhl, 2004]. The VEGF pathway regulates endothelial cell proliferation and migration to induce angiogenesis [Kerbel, 2008; Lobov et al., 2007]. VEGF binds its receptor, VEGFR2, and induces its ubiquitination with subsequent internalisation and downmodulation. EPN1 and EPN2 are implicated in this internalisation step [Pasula et al., 2012] and their ablation causes sustained VEGF/VEGFR2 pathway activation with alteration in angiogenesis (Figure 3A) [Pasula et al., 2012; Rahman et al., 2016; Tessneer et al., 2014]. Linked to these findings, the *Epn1*/*Epn2* double KO is lethal at the embryonic stage between E9.5 and E10 due to vasculature defects [Chen et al., 2009]. However, the endothelial-specific *Epn1*/*Epn2* double KO mouse model does not display the same developmental defects as the constitutive double KO mice, suggesting that the function of EPN1 and EPN2 is not limited to the VEGF pathway in endothelial cells [Pasula et al., 2012].

Interestingly, EPN1 and EPN2 play a critical role in the Notch pathway that is also involved in vasculature formation (Figure 3A) [Chen et al., 2009]. This finding is in agreement with the function of epsins in lower organisms, such as *Drosophila* and *C. elegans* [Overstreet et al., 2004; Tian et al., 2004; Wang and Struhl, 2004]. Importantly, the developmental defects observed in Epn1/Epn2 double KO mouse embryos are recapitulated by a global impairment of Notch signalling, indicating that EPN1 and EPN2 functions are linked to Notch activation. Nevertheless, housekeeping forms of CME were not impaired in cells derived from these double KO embryos [Chen et al., 2009]. These findings support a role of EPN1 and EPN2 as specialised endocytic adaptors, with a critical role in the regulation of VEGF and Notch signalling in mammals.

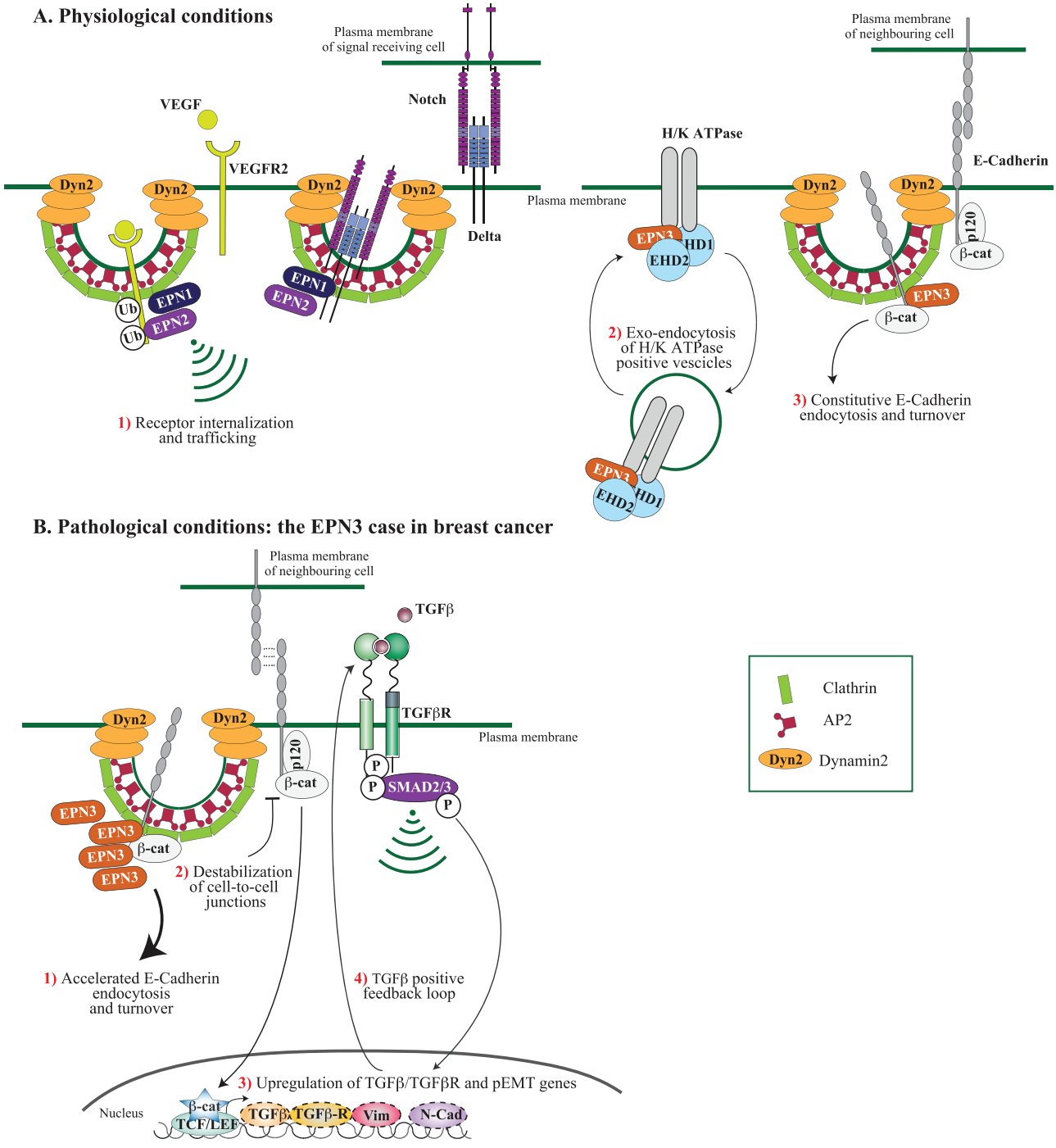
In contrast to EPN1 and EPN2, owing to its low level and restricted pattern of expression, EPN3 has been poorly characterised and only a few studies have pointed to possible EPN3 functions. In particular, it was shown that EPN3 is involved in the exo-endocytosis of H/K ATPase positive vesicles in mouse gastric parietal cells through the interplay with EHD1 and EHD2 (Figure 3A). This study was conducted in an *Epn3* KO mouse model [Ko et al., 2010], however, the lack of phenotype in the mouse suggests overlapping functions with the other epsins. EPN3 is also up-regulated in keratinocytes in cutaneous wounds and is involved in their migration in response to specific factors [Coon et al., 2011; Spradling et al., 2001]. These observations point to a more restricted and tightly regulated role of EPN3 compared with other epsins. Indeed, recent studies showed that unlike EPN1 and EPN2, EPN3 is not involved in EGFR and TfR endocytosis, but instead has a specific role in the physiological regulation of E-cadherin endocytosis and turnover in mammary epithelial cells, pointing again to a selective and diversified role of this family member (Figure 3A) [Schiano Lomoriello et al., 2020].

Specialised functions of epsins in tumorigenesis

Alterations in epsin family genes have been associated with different cancer types, in particular, glioma [Dong et al., 2018; Wang et al., 2018), lung cancer [Hellwig et al., 2016], breast cancer [Hellwig et al., 2016; Schiano Lomoriello et al., 2020], fibrosarcoma [Coon et al., 2011], skin [Spradling et al., 2001], gastric [Ko et al., 2010] and colon cancers [Chang et al., 2015].

EPN1 and EPN2 alterations have been shown to play a major role in tumour angiogenesis in line with their function in the VEGF pathway; lack of EPN1 and EPN2 in mouse models of lung carcinoma and melanoma resulted in vascular defects and markedly retarded tumour growth [Pasula et al., 2012]. In recent years, much effort has been made to develop a therapeutic strategy to inhibit the EPN1 and EPN2 interaction with the VEGFR2, with preliminary but promising results in glioma treatment through the application of a chemically synthesised chimeric peptide that caused impaired angiogenesis, retarded tumour growth and increased survival rates in several

Figure 3 | Specialised roles of epsin family members in endocytosis. (**A**) Physiological conditions. (1) EPN1 and EPN2 have been shown to be involved in the endocytosis of VEGFR2 and the Notch ligand, Delta (in the signal sending cell), both *in vitro* and *in vivo*. (2) EPN3 is involved in the endo-exocytic cycles of H/K ATPase together with EHD1 and EHD2 in gastric parietal cells. (3) EPN3 is involved in the constitutive endocytosis and turnover of E-cadherin in breast epithelial cells. (**B**) In pathological conditions and, in particular, in breast cancer cells, EPN3 is overexpressed and/or amplified, determining an acceleration of E-cadherin endocytosis and turnover (1). This leads to cell-to-cell junction destabilisation (2) and β-catenin nuclear translocation, finally causing the activation of a partial EMT (pEMT) transcriptional program (3), which involves the up-regulation of vimentin (Vim) and N-cadherin (N-Cad). TGFβ ligands and receptors are also up-regulated determining the activation of a TGFβ-dependent positive feedback loop (4) which further sustains the EMT phenotype.



tumour models [Dong et al., 2018; Dong et al., 2017; Dong et al., 2015].

At the protein level, EPN3 is up-regulated in lung [Hellwig et al., 2016], breast [Hellwig et al., 2016; Schiano Lomoriello et al., 2020] and skin cancer [Spradling et al., 2001]. In breast and lung cancers, high levels of EPN3 correlate with metastasis and poor clinical outcome [Hellwig et al., 2016; Schiano Lomoriello et al., 2020]. In contrast, *EPN3* mRNA expression was found to be down-regulated in human gastric cancer tissues from The Cancer Genome Atlas, and, thus, it was speculated that EPN3 could act as a p53-dependent pro-apoptotic anti-tumoral factor in primary gastric cancers [Mori et al., 2017].

Mechanistically, EPN3 was proposed to regulate epithelial-to-mesenchymal transition (EMT) in glioblastoma patients, since higher levels correlated with migratory abilities and the up-regulation of mesenchymal markers [Wang et al., 2018]. More recent evidence has unveiled the molecular mechanism underlying the contribution of EPN3 overexpression to breast cancer. In physiological conditions, EPN3 works as an adaptor in the internalisation of E-cadherin, thereby controlling its physiological turnover at the PM [Schiano Lomoriello et al., 2020]. In breast cancer, EPN3 overexpression amplifies this physiological mechanism, inducing a higher rate of E-cadherin internalisation and the detachment of β-catenin from cell–cell junctions (Figure 3B). These events result, on the one hand, in the destabilisation of cell–cell junctions, and on the other hand, in the translocation of β-catenin into the nucleus, where it interacts with transcription factors responsible for the activation of EMT genes, including transforming growth factor β (TGFβ) ligands and receptors. This leads to the establishment of a TGFβ-dependent autocrine loop that sustains EMT. Thus, EPN3 synergises with the TGFβ pathway to establish an EMT phenotype and the invasive potential of cancer cells [Schiano Lomoriello et al., 2020].

This endocytic-based circuitry leads to the establishment of a so-called 'partial EMT' phenotype, that is, a partial transition state in which cells acquire mesenchymal traits while still presenting epithelial features, thus rendering cells more 'plastic' and prone to metastasize to distant sites [Aiello et al., 2018; Nieto et al., 2016; Pastushenko and Blanpain, 2019]. The partial EMT phenotype results in the emergence of cancer stem cell features and in the acquisition of a pro-invasive behaviour. In agreement, in human breast cancer biopsies, EPN3 is up-regulated in infiltrating areas, where cells undergoing *in situ*-to-invasive transition are located [Schiano Lomoriello et al., 2020].

Thus, EPN3, with its low level of expression in normal tissues, overexpression in cancer and involvement in cancer stem cell and invasive phenotypes, makes it a promising candidate drug target. Despite its high level of structural similarity with EPN1 and EPN2, particularly in the ENTH domain, more divergent regions exist at the C-terminus of these proteins that could be responsible for their diversified roles [Madhivanan et al., 2020]. If verified, these regions in EPN3 could be targeted by selective drugs to interfere with its function in E-cadherin endocytosis, possibly reducing the tumorigenic and invasive potential of EPN3-overexpressing cancer cells.

Conclusions and perspectives

The process of internalisation of macromolecules was originally discovered and described in 1883 by the developmental biologist Ilya Metchnikoff, who coined the term 'phagocytosis'. From that moment, many different types of internalisation mechanisms have been described [Schmid et al., 2014], such as CME [Pearse, 1975; Roth and Porter, 1964] and several non-clathrin internalisation mechanisms [Caldieri et al., 2017; Hansen et al., 1991; Howes et al., 2010; Kirkham et al., 2005; Lamaze et al., 2001; Moya et al., 1985; Renard et al., 2015; Yamada, 1955]. Historically, endocytosis was described as the key cellular mechanism to internalise nutrients and other types of molecules [Sigismund et al., 2012]. After more than half a century from the first observation of CME, it is now clear that endocytosis is much more than a simple cellular 'feeding' mechanism. Indeed, we are only just starting to appreciate the full importance of this process in the cellular masterplan and its relevance to diseases, such as cancer.

The endocytic process has been linked to a variety of cellular functions, from receptor signalling and turnover to cell proliferation and migration, from tissue morphogenesis and mechanics to polarity and stem cell regulation [Sigismund et al., 2012; Sigismund and Scita, 2018]. It was proposed that endocytosis could represent the driving force that caused eukaryotic cell appearance [de Duve, 2007]. Given

these premises, it is not surprising that endocytosis has emerged as a highly sophisticated and regulated process, with many different families of endocytic adaptors with specialised functions. The interest in the discovery and characterisation of the multitude of endocytic mechanisms and their regulators/adaptors is high and growing rapidly.

However, there are still many fundamental questions about endocytosis that need to be answered: (i) What is the evolutionary importance of the different endocytic proteins and mechanisms, when did endocytosis first appear and how did specialised functions develop during evolution? (ii) How are the various endocytic mechanisms and their specialised functions integrated to regulate cellular behaviour in different cellular contexts? (iii) How are endocytic mechanisms aberrantly regulated in tumour growth and progression, and can they be specifically targeted to treat cancer?

The characterisation of the different specialised functions of the endocytic machinery will lead to a more comprehensive understanding of its role in physiology and in pathological contexts.

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Conflict of interest statement

The authors have declared no conflict of interest.

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