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COMMENTARY

Uses and abuses of macropinocytosis

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ABSTRACT

Macropinocytosis is a means by which eukaryotic cells ingest extracellular liquid and dissolved molecules. It is widely conserved amongst cells that can take on amoeboid form and, therefore, appears to be an ancient feature that can be traced back to an early stage of evolution. Recent advances have highlighted how this endocytic process can be subverted during pathology – certain cancer cells use macropinocytosis to feed on extracellular protein, and many viruses and bacteria use it to enter host cells. Prion and prion-like proteins can also spread and propagate from cell to cell through macropinocytosis. Progress is being made towards using macropinocytosis therapeutically, either to deliver drugs to or cause cell death by inducing catastrophically rapid fluid uptake. Mechanistically, the Ras signalling pathway plays a prominent and conserved activating role in amoebae and in mammals; mutant amoebae with abnormally high Ras activity resemble tumour cells in their increased capacity for growth using nutrients ingested through macropinocytosis. This Commentary takes a functional and evolutionary perspective to highlight progress in understanding and use of macropinocytosis, which is an ancient feeding process used by single-celled phagotrophs that has now been put to varied uses by metazoan cells and is abused in disease states, including infection and cancer.

KEY WORDS: Amoeba, Cancer, Evolution, Macropinocytosis, Therapeutics

Introduction

Macropinocytosis is defined functionally as the uptake of fluid droplets that are visible by light microscopy. It occurs in different forms in a variety of cell types, for example, in amoebae and leukocytes as they ingest extracellular material and in neurons when they perform bulk endocytosis during intense synaptic activity (Amyere et al., 2001; Clayton and Cousin, 2009). Invariably, macropinocytosis is dependent on the actin cytoskeleton. During what we can consider its canonical form, a macropinosome forms from a ring of actin polymerizing beneath the plasma membrane. This ring extends as a circular ruffle and eventually closes through constriction at the top so that membrane fusion produces an internal vesicle containing a sample of the extracellular fluid (Swanson, 2008) (Fig. 1A). Macropinocytosis resembles phagocytosis closely, except that the vesicle does not form around a solid object. The distinction between these two endocytic modes is to some extent arbitrary because large particles, such as bacteria, can be taken up into 'spacious phagosomes' whose membrane is not tightly apposed to the surface of the particle (Alpuche-Aranda et al., 1995; Kerr and Teasdale, 2009) (Fig. 1B). It appears likely that macropinocytosis, neuronal bulk endocytosis, phagocytosis and other large-scale actin-dependent uptake mechanisms are all homologous processes, which are related by their descent from feeding mechanisms in the earliest eukaryotic ancestral cells.

Macropinocytosis was first observed in mammalian tissue culture cells using light microscopy (Lewis, 1931, 1937) and was called simply pinocytosis because these findings predated the discovery of clathrin-dependent endocytosis and other micropinocytic mechanisms (Clark, 1959; Policard and Bessis, 1962; Roth and Porter, 1964; Nichols and Lippincott-Schwartz, 2001; Doherty and McMahon, 2009). Recent work emphasises its roles in several important processes. One theme is the subversion of normal macropinocytotic function. For instance, certain bacteria and viruses use macropinocytosis to invade host cells (Mercer and Helenius, 2012), and cancer cells are able to use this pathway to scavenge extracellular nutrients (Commisso et al., 2013; White, 2013). This Commentary will explore examples of physiological uses and pathological exploitation of macropinocytosis in cancer, neurodegenerative disease and infection, as well as novel approaches for possible therapeutic applications. We also bring to the fore a comparative, evolutionary perspective and, where possible, relate these large-scale endocytic processes to ancestral feeding behaviours.

Constructing a macropinosome

Macropinocytosis is an actin-driven process, accompanied by dramatic rearrangements of filamentous (F-)actin, and is sensitive to inhibitors such as cytochalasin A (Dowrick et al., 1993; Hacker et al., 1997; Lee and Knecht, 2002). Macropinosomes must compete with other F-actin structures, such as pseudopods, for generic cytoskeletal resources and require mechanisms to direct these resources to create their unique spatial structure. The most striking feature of macropinocytosis is the formation of a hollow ring of actin polymerization of up to several microns in diameter under the plasma membrane. This circular ruffle can form through two distinct routes in different cells. In cells with prolific ruffling, a linear ruffle sometimes folds back on itself to circularise (Welliver et al., 2011); alternatively, a circular ruffle can form *de novo* as an expanding ring on the plasma membrane (Bernitt et al., 2015). In either case, outwardly directed actin polymerization must be restricted to the walls of the circular ruffle and suppressed at the centre.

There is increasing evidence that Ras and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) regulate the early events of macropinosome formation. The injection of activated (oncogenic) Ras protein into fibroblasts causes ruffling and macropinocytosis (Bar-Sagi and Feramisco, 1986; see also below). Genetic ablation of certain Ras proteins inhibits macropinocytosis in *Dictyostelium* amoebae (Chubb et al., 2000; Hoeller et al., 2013), and loss of the Ras GTPase-activating protein (RasGAP) NF1 increases the frequency of macropinocytosis and size of macropinosomes in these cells (Bloomfield et al., 2015; see also below). Ras activates class-I phosphatidylinositol 3-kinases (PI3Ks) through their Ras-binding domain, and these enzymes produce the membrane lipid

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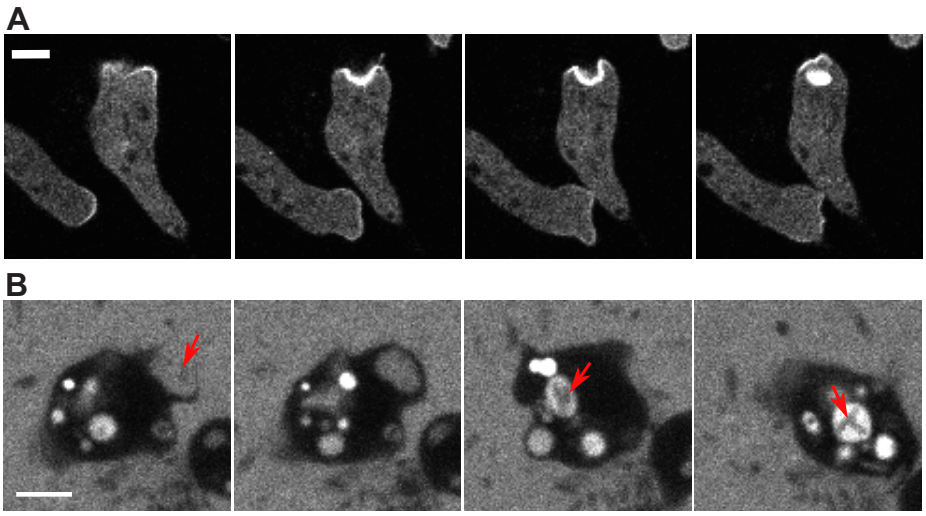

Fig. 1. Macropinocytosis in feeding amoebae. (A) *Dictyostelium discoideum* amoebae, showing a single macropinocytosis event. Active Ras, reported by the GFP-tagged Ras-binding domain of Raf1, was imaged by using confocal microscopy in cells null for the RasGAP NF1 (*axeB*), which produce over-sized macropinosomes. Ras is active at a relatively low level as cells extend pseudopodia for movement, but its activity intensifies as the membrane invaginates during macropinocytosis. Scale bar: 5 µm. (B) *Diderma hemisphaericum* amoebae feeding on bacteria using macropinocytosis. These myxogastrid amoebae were bathed in TRITC–dextran in a suspension of *Escherichia coli* cells. In these conditions, the amoebae do not rely on contact-mediated phagocytosis to feed, but rather extend large circular ruffles outwards to enclose relatively large volumes of liquid, often capturing several bacteria (arrows) into a single macropinosome (often referred to as a 'spacious phagosome' in this context). Scale bar: 5 µm.

PIP3 (Rodriguez-Viciana et al., 1994), which is an ether-linked plasmanyl inositol in *Dictyostelium* (Clark et al., 2014). When PIP3 production is attenuated either with drugs or genetically, macropinocytosis is inhibited to striking extents (Araki et al., 2007, 1996; Buczynski et al., 1997; Hoeller et al., 2013; Zhou et al., 1998). In both mammalian cells and *Dictyostelium*, PIP3 is lost from macropinocytic vesicles shortly after they close, giving rise first to phosphatidylinositol (3,4)-bisphosphate [PI(3,4)P_2], which (in mammalian cells at least) is then converted to phosphatidylinositol 3-phosphate as the vesicle progresses through the endocytic pathway (Araki et al., 2007; Dormann et al., 2004; Maekawa et al., 2014; Swanson, 2014; Welliver and Swanson, 2012; Yoshida et al., 2009; Egami et al., 2014). The evolutionary conservation between mammals and *Dictyostelium* of these Ras and phosphoinositides functions shows that they can be regarded as core features of macropinocytosis, presumably dating back to the last common ancestor of eukaryotes.

A surprising and significant feature of Ras and PIP3 signalling in macropinocytosis is that it is not diffuse throughout the plasma membrane but is restricted to intense patches that form the core of circular ruffles and appear to extend to their lip, but not beyond it. The evidence for this includes the observation of PIP3 patches in the over-sized macropinosomes of axenic strains of *Dictyostelium* (Parent et al., 1998, and see below), which coincide with patches of active Ras (Sasaki et al., 2007; Hoeller et al., 2013), as well as the patches of active Ras and PIP3 that become prominent in circular ruffles (Araki et al., 2007; Welliver and Swanson, 2012). These patches must be maintained despite the expected rapid diffusion of Ras and PIP3 in the plasma membrane, and most likely depend on autocatalytic Ras activation and PIP3 production, possibly combined with the establishment of a diffusion barrier around macropinosomes (Welliver et al., 2011).

PIP3 can recruit effector molecules, which typically contain plextrin homology (PH)-domains that bind to it, to the membrane, and these are likely to have important roles in shaping macropinosomes. Notably, the protein kinase Akt (encoded by *pkbA*) and a set of myosin-I motor proteins are recruited by PIP3 to macropinosomes in *Dictyostelium*, and their genetic deletion strongly impairs macropinocytosis (Rupper et al., 2001; Chen et al., 2012). In *Dictyostelium*, myosin-IB, which does not bind to PIP3, is also recruited to the macropinosome rim, where it forms a striking 'bull's eye' pattern with the PIP3-binding proteins (Brzeska et al., 2016). Because Ras activity can control the size of macropinosomes, it is tempting to speculate that the Ras and/or PIP3 patches play a direct organizational role in macropinosome formation by delimiting a membrane region that is marked as active and operative for macropinocytosis (Fig. 2). One attractive possibility is that these signals drive actin polymerization at their periphery and suppress it in their body, as is observed in the related basal PIP3 patches of *Dictyostelium* (Gerisch, 2010).

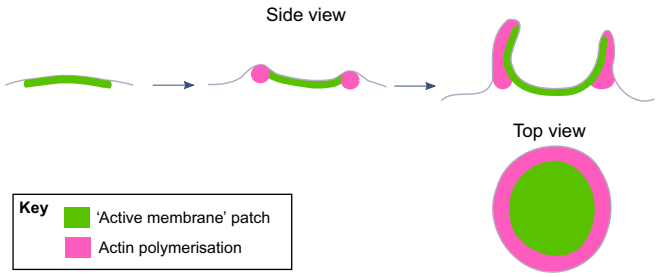

Fig. 2. A model outlining the formation of 'canonical' macropinosomes. In *Dictyostelium,* the initiating event during the formation of a macropinosome appears to be the establishment of a region of the plasma membrane that is 'activated' or primed for macropinocytosis. These regions are marked by high levels of Ras activity and intense accumulation of PIP3; the events preceding intensified Ras activity remain unclear. Actin polymerisation is stimulated around this region, causing a circular ruffle to extend outwards, which will eventually close to form a macropinosome. From a viewpoint above the membrane, the 'active membrane' region and outer ring of newly polymerised F-actin form a 'bull's eye' pattern. In some cell types, it is possible that ring formation occurs before the signal inside it becomes intensified.

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The macropinocytic cup that extends from the cell surface eventually constricts and closes, with membrane fusion producing an intracellular vesicle containing a droplet of medium. In some cases, the macropinosome rim appears to contract in a coordinated manner, as if powered by a contractile ring (Swanson et al., 1999), but in other less-regularly-shaped macropinosomes, such a contractile ring is not so readily apparent. If closure of a macropinosome occurs after contraction of the rim, membrane fusion might only occur at a narrow pore and use similar molecular machinery as in clathrin-mediated endocytosis. However, if fusion occurs when a membrane flap folds back onto the plasma membrane, a different mechanism might have to be envisioned.

Macropinocytosis in life and death – roles for Ras

Recent work in amoebae and mammalian cells has revealed the importance of macropinocytosis in cellular feeding (Fig. 3). The association of the target of rapamycin complex 1 (TORC1) with lysosomes (Zoncu et al., 2011) is likely to reflect ancestral modes of nutrient sensing in phagotrophic predators. In phagotrophic cells, the food supply is ingested in bulk and concentrated in endosomes, which fuse with lysosomes so that their contents can be degraded. A constant flow of prey-derived solutes from these degradative organelles through transporters into the cytosol is maintained during the growth of such phagotrophic cells as long as feeding can continue. Metazoan cells appear to retain a version of this ancient system (Goberdhan et al., 2005; Nicklin et al., 2009; Wang et al., 2015; Rebsamen et al., 2015), and it has been recently shown that delivery of amino acids to lysosomes is required for fast activation of TORC1 in response to platelet-derived growth factor and macrophage colony-stimulating factor, with a parallel cytosolic signal through the Akt protein kinase also being necessary (Yoshida et al., 2015). Growth factors stimulate anabolic metabolism, but their efficiency in doing so is modulated by the supply of amino acids available from macroautophagy and other cell-autonomous sources, as well as by nutrients delivered from the vasculature. This is likely to be especially important during cancer, when these metabolic responses are crucial (Tsun and Possemato, 2015). A recent report suggests that cancer cells can even boost their ATP levels directly by ingesting it through macropinocytosis when they are in microenvironments in which extracellular ATP is abundant (Qian et al., 2014).

Mutations in Ras are common drivers in a variety of cancers (Stephen et al., 2014). These small G-proteins activate a signalling cascade, including class-1 PI3Ks and the Raf-extracellular signal-regulated kinase (ERK) pathway. One consequence of Ras activation is macropinocytosis (Bar-Sagi and Feramisco, 1986; Amyere et al., 2000; Porat-Shliom et al., 2008). This effect appeared to be an interesting curiosity until recently when it was shown that activating mutations in K-Ras promote tumour cell feeding through macropinocytosis (Commisso et al., 2013). The polypeptides taken up from extracellular fluid, which is normally rich in protein, are digested in the endocytic pathway, and allow the growth and survival of cells that are otherwise limited by the availability of essential and non-essential amino acids (Commisso et al., 2013; Palm et al., 2015). TORC1 plays a complex role in regulating these responses –extracellular amino acids promote growth through TORC1-mediated signals, whereas amino acids that have been derived from extracellular protein and have been digested in lysosomes suppress the proliferation of K-Ras-mutant cells in which TORC1 is active (Palm et al., 2015) (Fig. 3).

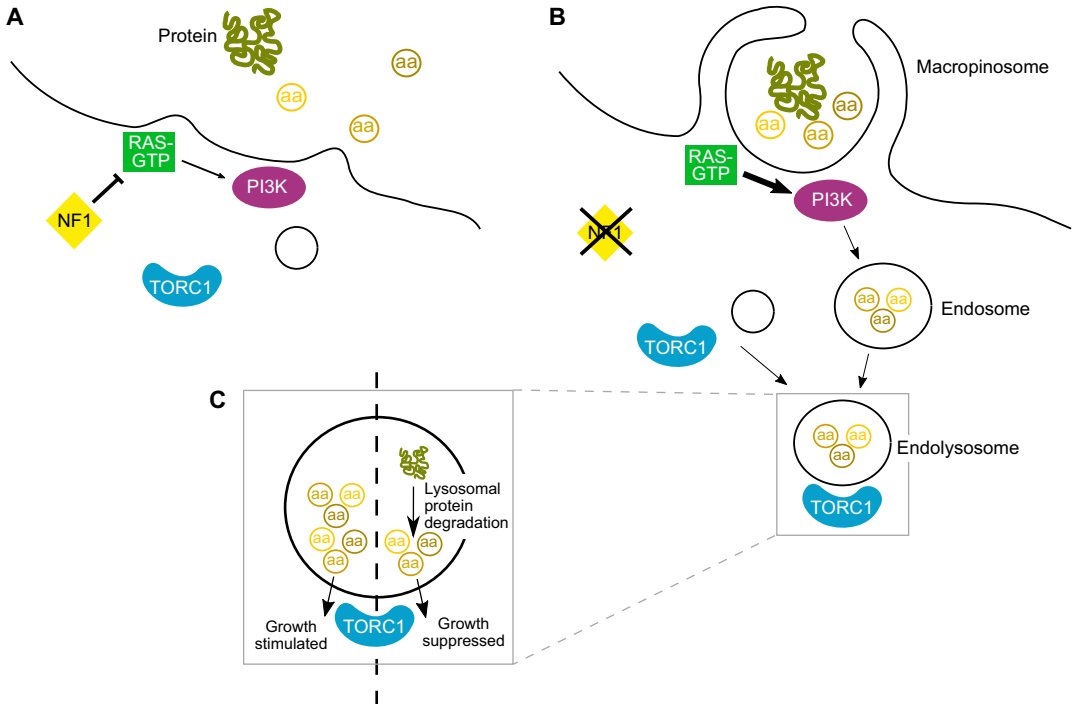

Fig. 3. The Ras–PI3K–TORC1 pathway during macropinocytotic nutrient uptake. (A) In *Dictyostelium* cells, Ras activity at nascent macropinosomes is negatively regulated by the GTPase-activating protein NF1. NF1 activity restrains Ras-mediated activation of PI3K, thereby limiting the intensification of these signals that drives macropinocytosis. (B) In NF1 mutants, Ras activity is not globally increased, but rather specifically during macropinocytosis (and phagocytosis, not illustrated here). (C) TORC1 controls the response of cells to nutrients that are taken in by macropinocytosis. In mammalian cells, the free amino acids ('aa') acquired from endolysosomes stimulate cell growth through TORC1 signalling. However, when extracellular protein is broken down into amino acids in endolysosomes – i.e. after it has been ingested through macropinocytosis – proliferation is not supported unless TORC1 signalling is suppressed; this limits inappropriate growth when extracellular amino acids are unavailable, for instance in poorly vascularised tumours.

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The roles of oncogenic Ras in tumour cells are strikingly parallel to the effects of Ras in the feeding behaviour of *Dictyostelium* amoebae. Wild isolates of *Dictyostelium* have only a low rate of macropinocytosis that is insufficient to support growth in standard laboratory media. However, most of the widely used strains carry mutations that promote growth in these media. When the key mutation was finally identified, it was found to reside in the RasGAP and tumour suppressor gene *NF1* (Bloomfield et al., 2015). Loss of NF1 results in increased Ras activity at sites of large-scale endocytosis, resulting in enhanced ruffling, increased rates of macropinocytosis and larger macropinosomes (Fig. 3). Therefore, loss of NF1 in an amoeba has results that are analogous to those upon the activation of Ras in tumour cells, which also feed more actively than normal cells and so evade growth arrest. *Dictyostelium* NF1 mutants also have enhanced phagocytic capacity and are able to ingest larger particles than wild-type amoebae. These protists feed in essentially the same way as their early eukaryotic ancestors, and phylogenomic analysis suggests that NF1 was present in the last common ancestor of all eukaryotes, implying that this regulator of cell growth evolved first to control feeding processes.

As well as supporting the growth and survival of certain malignant cells, macropinocytosis has paradoxically been implicated in a form of cell death called methuosis, which affects cells from cancers of the brain and again involves Ras. This non-apoptotic form of cell death is driven by the accumulation of macropinosomes that fail to fuse with lysosomes or recycle to the cell surface, leading to extreme vacuolisation of the cytoplasm (Fig. 4). Methuosis was first described in glioblastoma cells that overexpressed oncogenic Ras, which stimulates excessive macropinocytosis (Overmeyer et al., 2008). A similar form of cell death occurs in medulloblastoma cells that have been hyperstimulated with nerve growth factor (Li et al., 2010). Screens have identified small molecules that can induce this form of death in malignant cells, offering hope for new treatments of glioblastoma (Overmeyer et al., 2011; Kitambi et al., 2014). The precise cause of cell death is not clear in some instances of methuosis (Maltese and Overmeyer, 2014), but in acute drug-induced vacuolisation, the cytoplasmic swelling causes the plasma membrane to rupture (Overmeyer et al., 2011; Kitambi et al., 2014).

The observed Ras-mediated endocytosis, which precedes death in methuosis, invites comparison with oncogene-induced senescence (Fig. 4). Senescence is a physiological process that occurs in certain differentiated vertebrate cells (Muñoz-Espín and Serrano, 2014) and involves dramatic changes to their endocytic cycle, often including vacuolisation of the cytoplasm. Replicative senescence occurs in cells that have passed through many cell cycles after their differentiation, and a similar program is executed in some cell types that have been transformed with oncogenes such as Ras. Senescence is often linked with changes in autophagy, which differ between mammalian cell types – basal autophagy is required for stem cells to avoid senescence (García-Prat et al., 2016), whereas autophagy is induced during oncogene-induced senescence in fibroblasts (Young et al., 2009). Recently, macropinocytosis that is driven by oncogenic Ras and is dependent on PI3K has been identified as the source of enhanced endocytic activity and vacuolisation in melanoma cells (Alonso-Curbelo et al., 2015). These cells evade senescence by maintaining high levels and activity of Rab7, a small G-protein that functions on late endosomes to promote their clearance through fusion with lysosomes. Abnormally aggressive feeding behaviour by tumour cells probably always depends on compensatory changes that affect downstream membrane trafficking and fluid clearance pathways.

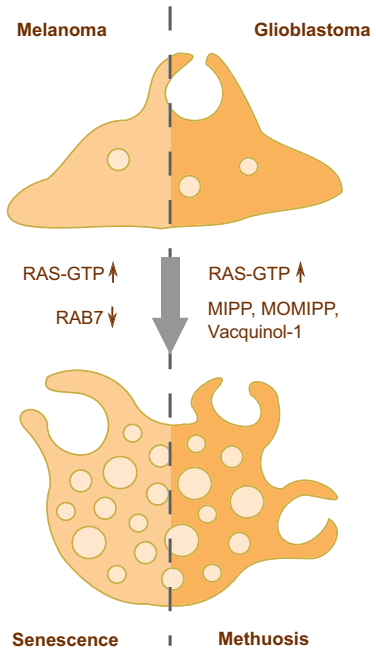

Fig. 4. Comparison between a form of senescence found in melanoma cells and methuotic cell death. Senescence in melanoma cells can be induced by oncogenic Ras signalling that drives an accumulation of macropinosomes in the cytoplasm, causing a characteristic vacuolisation. These cells avoid senescence by maintaining high levels and activity of the late-endosomal Rab protein Rab7. Similarly, methuosis of glioblastoma cells can be induced by oncogenic Ras, which induces macropinocytosis so strongly that cell death occurs very quickly. Several drugs (MIPP, MOMIPP and Vacquinol-1) have been developed that stimulate this process, potentially providing a new means to target these aggressive cancers. The exact relationship between methuosis and oncogene-induced senescence remains unclear, but it is possible that they reflect the same underlying process, with variation existing between different cell types.

Although it is likely that these mechanisms vary in different cell types, they nevertheless could prove to be therapeutic targets. The overlapping characteristics of senescence and methuosis suggest that altered endocytic properties of malignant (and perhaps premalignant) cells might make them vulnerable to well-designed interventions.

Using macropinocytosis as a route for delivery of macromolecules

Given its function in bulk uptake and transport, macropinocytosis could be utilised to deliver therapeutic cargoes into cells. Small molecules can enter mammalian cells through several potential routes, whose relative importance is often controversial due to the diversity of cell types and experimental setups used. Short interfering RNAs (siRNAs) are a convenient tool for modulating gene function in the laboratory and can be used to silence genes involved in disease. Lipid nanoparticles loaded with siRNAs enter human cells through macropinocytosis, as well as through clathrin-coated pits and, intriguingly, both entry routes can be used in the same cells. An initial, small-scale entry by clathrin-mediated endocytosis is required to induce a later phase of bulk macropinocytotic uptake, which accounts for most of the siRNA taken in (Gilleron et al., 2013). Recently developed lipoprotein vehicles that target hepatocytes with a very high efficiency also enter the cells through macropinocytosis (Dong et al., 2014). However, once nanoparticles enter endosomes, their escape into the

cytoplasm is limited by recycling pathways that transport endosomal content back to the cell surface (Sahay et al., 2013); reducing activity of the recycling machinery should thus help to improve delivery and gene silencing. Nucleic acid aptamers can also enter cells by using macropinocytosis, in some cases stimulating their own uptake (Reyes-Reyes et al., 2010).

Ultimately, the efficient targeting of drugs, whether small molecules or macromolecules, to specific cells and tissues will require a detailed mechanistic understanding of uptake routes. Cell-penetrating peptides are also used widely for drug delivery, but their major route of entry into cells has not always been clear. One new class of peptides, C-end rule motif peptides (CendRs), has been characterised in detail and shown to enter cells using macropinocytosis (Pang et al., 2014, 2015). The sequence of CendRs is derived from a C-terminal motif of proteins that bind to Neuropilin receptors (of which there are two isoforms, neuropilin 1 and neuropilin 2). Neuropilins are important during angiogenesis and axon guidance, where they act as receptors for a number of extracellular polypeptide signals (Guo and Vander Kooi, 2015). Uptake of CendR peptides depends both on Neuropilins and the cytoplasmic protein GIPC1, and involves the extension of lamellopodia and their inward folding. This mode of macropinocytosis is inhibited by the TORC1 nutrient-sensing pathway, and, therefore stimulated by nutrient limitation (Pang et al., 2014). Its physiological role might be in homeostatic responses to nutrient deprivation. A recent study has shown that TORC1 inhibits the utilisation of extracellular protein that has been ingested through macropinocytosis, suggesting that this growth-regulatory complex is intimately connected with the control of macropinocytosis in the context of nutrient acquisition (Palm et al., 2015). Further investigations into the normal developmental and homeostatic roles of CendR-mediated macropinocytosis in vertebrate cells, and its effects on vascular permeability (Pang et al., 2015), could thus not only lead to its use as a therapeutic tool but could also help to improve our understanding of its evolutionary relationships with other uptake mechanisms.

Improvements to delivery protocols allow efficient uptake even in the absence of cell-penetrating peptides. For example, a combination of hypertonic salt treatment, which by itself induces macropinocytosis, and a zwitterionic transduction molecule such as γ-amino-butyric acid, which promotes leakage of endosome contents, can potently stimulate uptake of extracellular macromolecules (D'Astolfo et al., 2015). This approach allows the delivery of proteins and small RNAs and appears to be applicable to a wide range of cultured vertebrate cell types. Increase in osmolarity also promotes macropinocytosis in various amoebae (Brandt, 1958; Chapman-Andresen, 1958; Bowers and Olszewski, 1972; Hacker et al., 1997), although it is not clear whether a common mechanism is involved.

Contributions of macropinocytosis to transmission of prion-like proteins

Prions were initially defined in part by their ability to transmit from organism to organism, and the pathology within an organism depends on cell-to-cell propagation. Macropinocytosis is now thought to be an important route for spread of prions and prion-like proteins (Zeineddine and Yerbury, 2015). Misfolded forms of the Cu-Zn superoxide dismutase protein SOD1 are linked to the neurodegenerative disorder amyotrophic lateral sclerosis (ALS), both in familial forms of the disease in which SOD1 mutations are frequent, and in sporadic ALS. Aggregates of mutant SOD1 can enter cells through macropinocytosis, which is dependent on Rac1-stimulated ruffling (Zeineddine et al., 2015), and trigger the misfolding of soluble mutant SOD1 present there (Münch et al., 2011). Correctly folded mutant and wild-type SOD1 can enter neuronal cells in a similar manner (Sundaramoorthy et al., 2013), and cell-to-cell transmission of misfolded wild-type SOD1 has recently been shown to occur either through macropinocytosis of SOD1 aggregates or through uptake of protein contained within exosomes (Grad et al., 2014).

A number of neurodegenerative disorders involving the accumulation of amyloid protein fibrils are now thought to develop through prion-like propagation of these fibrils between cells. For instance, the amyloid α-synuclein (the causative agent of Parkinson's disease) and huntingtin-derived aggregates, which are implicated in Huntington's disease (Zeineddine et al., 2015), both stimulate ruffling and macropinocytosis. Misfolded tau protein is linked to a number of neurological diseases and can also enter cells through macropinocytosis or the related bulk endocytosis process that occurs at synapses (Wu et al., 2013). In some cases, fibrils stimulate fluid uptake by specific binding to heparan sulphate proteoglycans at the cell surface (Holmes et al., 2013). Amyloid precursor protein (APP), the source of the peptide comprising most of the amyloid plaques associated with Alzheimer's disease, can also enter neuronal cells through macropinocytosis. Following its uptake, APP is rapidly trafficked to lysosomes in an ARF6-dependent manner, where it can be degraded into pathological peptide forms (Tang et al., 2015).

Neurons are specialised to perform extensive endo- and exocytosis at synapses, including activity-dependent bulk endocytosis (ADBE) and ultrafast endocytosis, which are used to recover the large amounts of membrane added to the surface as a result of synaptic transmission (Clayton et al., 2008; Watanabe et al., 2013a,b). ADBE also occurs in neurosecretory chromaffin cells (Gormal et al., 2015). The physiological roles of macropinocytosis other than ADBE in neurons remain unclear, although as it occurs at growth cones, it is likely to be important in neurons during development and after injury (Kolpak et al., 2009; Kabayama et al., 2011). The processing of extracellular protein in lysosomes suggests that ancient digestive functions are still used in these highly specialised cells, and further knowledge of how growth factors and other extracellular signals modulate these behaviours would be valuable. Similarly, further characterisation of underlying mechanisms, beyond the use of inhibitors that can affect more than one form of large-scale endocytosis, will enable the precise delineation of which uptake route is used in each instance.

Macropinocytosis in immunity and infection

The best-studied roles of macropinocytosis are immunological, both in normal immune-cell function and as a host process that is subverted by invading pathogens. Antigen-processing cells use macropinocytosis, as well as phagocytosis, to sample potential antigens for presentation to T lymphocytes (Liu and Roche, 2015). Immature dendritic cells perform constitutive macropinocytosis and pass ingested polypeptides to endolysosomes for digestion so that potential antigens can be presented to helper T cells. Immature dendritic cells can also store unprocessed material in late endosomes and recycle it back to the cell exterior for detection by B cells once they reach lymph nodes (Roux et al., 2012). A subset of dendritic cells also cross-present peptides that have been derived from material they have gained through macropinocytosis to cytotoxic T cells (Schuette and Burgdorf, 2014); this cross-presentation is important to establish immunity against many viruses as well as against cancerous cells.

Recently, it has been shown that cell motility and macropinocytosis are mutually incompatible (or at least in competition with each other) in dendritic cells and amoebae. Major histocompatibility complex II (MHC-II)-dependent rearrangements of myosin II, which are required for efficient macropinocytosis at the cell anterior in dendritic cells, are incompatible with cell migration (Chabaud et al., 2015). This is similar to the situation in amoebae, where chemotaxis towards folic acid is impaired when high-frequency macropinocytosis occurs, again highlighting the broad similarities that could extend back to the common ancestors of Metazoa and amoebae (Veltman et al., 2014). As large-scale endocytosis depends on many of the same components of the actin cytoskeleton as those required for pseudopodial protrusion during cell migration, it appears that it is difficult for both processes to occur simultaneously, perhaps because different configurations of cortical actin are required in endocytic and migratory pseudopodia.

Macrophages mainly use phagocytosis to process antigens, but inflammatory signals stimulate the use of macropinocytosis to enable a more rapid clearance of certain pathogenic bacteria, which are ingested in bulk without engaging phagocytic receptors (BoseDasgupta and Pieters, 2014). The main role of macrophages it to clear apoptotic 'corpses' by using efferocytosis, a process that is related to both macropinocytosis and phagocytosis. This clearance mechanism limits inflammation and can also result in the destruction of pathogens that trigger apoptosis (Martin et al., 2014). Efferocytosis relies on the presence on the surface of dead and dying cells of molecules (often referred to as 'eat-me' signals) that are not present on healthy cells, notably phosphatidylserine. Perhaps inevitably, these signals have been subverted by pathogens, including viruses, to promote their entry into host cells (reviewed in Amara and Mercer, 2015). For example, when *Listeria* bacteria exit infected cells, they generate phosphatidylserine-containing vesicles, which promote their subsequent uptake into macrophages (Czuczman et al., 2014). Pathogenic bacteria, such as *Salmonella*, can also induce macropinocytosis in order to invade macrophages, dendritic cells and B cells (Rosales-Reyes et al., 2012). There, the bacteria remain within 'spacious phagosomes', which they reconfigure to avoid fusion with lysosomes. Entry through this route involves a number of host proteins, including Arf signalling and the SCAR/WAVE complex (Davidson et al., 2015).

Viruses have evolved a variety of strategies to enter eukaryotic cells (Mercer et al., 2010b), and recent findings that the large nucleocytoplasmic DNA virus *Marseillevirus* can enter host amoebae by using phagocytosis and macropinocytosis again suggests that many of these mechanisms stem from co-option of ancestral feeding behaviours (Arantes et al., 2016). In mammalian cells, the flexibility of viral strategies means that different strains of the same poxvirus can use multiple forms of macropinocytosis for invasion, entering through macropinosomes that are initiated by either ruffles or blebs (Mercer et al., 2010a). Ebola virus also stimulates macropinocytosis for cell entry (Saeed et al., 2010; Nanbo et al., 2010) and, recently, the two-pore membrane channels involved in maturation of the resulting macropinosomes have been identified as potential drug targets to combat Ebola and the related *Marburgvirus* (Sakurai et al., 2015). The lentivirus human immunodeficiency virus strain 1 (HIV-1) provides an interesting example of the complexity of viral interactions with the host endocytic mechanisms. HIV-1 can enter macrophages and endothelial cells through macropinocytosis (Maréchal et al., 2001; Liu et al., 2002), but its transmission to T cells, which are permissive for its replication, can be boosted by a process called cross enhancement (or cross infection), whereby virions are first collected by dendritic cells. Rather than entering these antigen-processing cells, HIV-1 virions remain attached to the surface of actin-rich dendrites, from where they travel to T lymphocytes when they make contact with the dendritic cells (Ménager and Littman, 2016). Disrupting dendrite structure promotes macropinocytosis into dendritic cells and so prevents cross-enhancement. This provides an example of how the normal physiological cellular use of macropinocytosis contrasts with the pathological use put to it by many other viruses as a cell entry strategy.

Perspectives and conclusions

The formation of circular ruffles in organisms as diverse as Metazoa, amoebae and *Naegleria* (Boschek et al., 1981; John et al., 1984; Hacker et al., 1997) suggests that the network of core components necessary for macropinocytosis is extremely ancient. The ability of these diverse cells to perform very similar forms of phagocytosis reinforces this idea. The common ancestor of all of these cells was almost certainly a bacterivorous protist that relied mostly on cilia for directed movement, suggesting that pseudopodia evolved first as feeding structures that were later co-opted for cellular propulsion (Cavalier-Smith, 2013). In that biological context, the parallel evolution of phagocytosis and macropinocytosis would have enabled the development of a range of feeding strategies for ingesting bacteria, viruses and other eukaryotes, as well as dissolved and colloidal macromolecules. Amoebae retain forms of these feeding modes and can use macropinocytosis as well as phagocytosis to feed on bacteria (Fig. 1). In Metazoa, complex multicellularity allows food to be efficiently digested extracellularly, reducing the need for endocytosis of complex food sources. At the same time, multicellularity permits the division of labour that enabled specialisations of different cell types, which give rise to new uses for these ancient endocytic processes, for instance in antigen processing and in neuronal communication.

Investigations into macropinocytosis in cancer cells and feeding processes in social amoebae independently converged on the Ras pathway as a crucial controlling factor (Bar-Sagi and Feramisco, 1986; Chubb et al., 2000; Commisso et al., 2013; Bloomfield et al., 2015). This conservation of function again suggests an ancient role for these signalling components that act upstream of PI3Ks and regulate the actin cytoskeleton. Their central roles during amoeboid feeding suggest that the initial function of Ras and PI3K might have been in defining active areas of plasma membrane and driving the formation of pseudopodia. It will be interesting to explore how Ras and its regulators, such as NF1, act in metazoan processes that evolved from endocytic feeding behaviours but that are no longer connected with feeding or growth. For example, is Ras activity important in all of the uptake processes discussed above? The use of Ras-binding-domain reporters confirm localised Ras activity at circular ruffles in macrophages, as well as in social amoebae (Sasaki et al., 2007; Welliver and Swanson, 2012; Bloomfield et al., 2015). The Ras subfamily in metazoans includes many poorly-characterised proteins, so a close examination of family members beyond the 'canonical' Ras proteins could lead to new insights into bulk endocytic mechanisms, such as the recently defined function of the Ras subfamily small G-protein TC21 in trogocytosis – a form of phagocytosis involving the transfer of small portions of a target cell into the phagocyte (Martínez-Martín et al., 2011). In *Dictyostelium*, NF1 appears to function specifically as a regulator of Ras signalling during macropinocytosis and phagocytosis, which raises the possibility that it has a conserved function in controlling endocytosis in metazoan phagocytic cells.

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As a cellular process, macropinocytosis has been viewed predominately within the context of membrane trafficking and signal transduction. It is also valuable to take a broader view in which macropinocytosis is seen as having been derived from an ancient feeding process used by the common ancestor of Metazoa and amoebae, but that has now been adapted for a variety of purposes. Although it is likely that core components are conserved, cells as diverse as free-living amoebae, dendritic cells and neurons are certain to perform large-scale bulk endocytosis in considerably different ways. A comparative approach – defining the core processes and cataloguing the specializations – will eventually give a rounded picture of macropinocytosis and its related processes. The recent renaissance of macropinocytosis has revealed surprising insights, and we can face the future expecting many more interesting developments.

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