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# Living on soup: macropinocytic feeding in amoebae

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**ABSTRACT Macropinocytosis is used by a variety of amoebae for feeding on liquid medium. The amoebae project cups and ruffles from their plasma membrane, driven by actin polymerization, and eventually fuse these back to the membrane, entrapping droplets of medium into internal vesicles. These vesicles are of up to several microns in diameter and are processed through the lysosomal digestive system to extract nutrients. Recognizably the same process is used in metazoan cells for a number of medically important purposes, including the pathological growth of cancer cells. We describe the discovery of macropinocytosis in *Dictyostelium* amoebae, its genetic regulation by the NF1 RasGAP, and the tools available for its investigation. Work on *Dictyostelium* over the last 30 years has identified many genes that may be important for macropinocytosis, which are listed at dictyBase, and give a basis for mechanistic studies. We argue that the actin cytoskeleton is organized for macropinocytosis by a signalling patch of PIP3 and active Ras and Rac, together with their regulatory proteins and effectors, including the protein kinases Akt and SGK. The Scar/WAVE complex is recruited to the periphery of this patch, triggering the formation of a hollow ring of protrusive actin polymerization, and eventually a macropinocytic cup. Major problems to be addressed include: the dynamics sustaining macropinocytic patches and the mechanism of Scar/WAVE recruitment; the mechanisms of cup closure and of membrane fusion; the ecological situations where amoebae feed by macropinocytosis; and the evolutionary relationship between macropinocytosis and growth factor signalling.**

KEY WORDS: *macropinocytosis, Dictyostelium, NF1, PI3-kinase, Ras*

## Introduction

Macropinocytosis is a process of large-scale, non-selective fluid uptake (Swanson, 2008, Buckley and King, 2017, Swanson and Yoshida, 2019). Cells extend thin sheets and cups (circular ruffles) from their plasma membrane into the medium, and eventually close them to entrap a droplet of fluid inside an internal vesicle as shown in Fig. 1A. This macropinosome can be several microns in diameter and is trafficked through the endolysomal system so that its contents are digested and nutrients and other useful molecules extracted. Undigested remnants can be eventually exocytosed back into the medium (Fig. 2A).

Macropinocytosis is a conserved process that probably evolved for feeding in early single cells (King and Kay, 2019). *Dictyostelium* cells still use macropinocytosis to feed on liquid media, but in metazoan cells it now serves a number of purposes, some of great medical significance (Bloomfield and Kay, 2016). Many cancer cells have retained (or regained) the original ability to feed by macropinocytosis, and consume the surrounding protein-rich bodily fluids to help meet their metabolic demands (Commisso *et al.*, 2013). In the immune system, dendritic cells and macrophages use macropinocytosis for sampling antigens from the medium (Sallusto *et al.*, 1995). It is also an entry route for both pathogens (Mercer and Helenius, 2008) and drugs (Desai *et al.*, 2019); and more speculatively uptake of disease agents by macropinocytosis may underlie the spread of neural degeneration in the brain

*Abbreviations used in this paper:* GAP, GTPase activating protein; GEF, GTPase exchange factor PIP3, PI3,4,5P3 (*Dictyostelium* PIP3 is a plasmanyl, not phosphatidyl lipid); NF1, neurofibromatosis 1; PI3-kinase, phosphorylates inositol of PIs on 3 position.

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(Munch *et al.*, 2011).

The extension and closure of macropinocytic cups is driven by the actin cytoskeleton, which forms protrusive rings of F-actin under the plasma membrane. How such rings are shaped is a mystery, but the process requires an intimate cooperation between force-producing and structural proteins of the actin cytoskeleton and a group of signalling proteins, which help coordinate their activity in space and time. Key among these organizers is the signalling cascade of Ras, PI3-kinase (Ras-activated) and the protein kinases Akt and SGK. This cascade is better known for mediating growth factor signalling in metazoa, but it can function cell-autonomously in *Dictyostelium*, possibly reflecting an ancestral function in organizing the actin cytoskeleton.

In this review, we describe the discovery of macropinocytosis in *Dictyostelium* and other amoebae, how it is regulated and can be investigated, the genes and proteins involved and progress towards understanding how macropinocytic cups are shaped. For the equally important question of how macropinocytic vesicles are processed, see (Maniak, 2003) and Vines and King (this issue).

## Discovery of macropinocytic feeding in *Dictyostelium* amoebae

Warren Lewis described macropinocytosis in macrophages and tumour cells from time-lapse movies of early tissue cultures made in the 1930s (Lewis, 1931, Lewis, 1937), thus considerably predating the discovery of clathrin-mediated endocytosis. Macropinocytosis was also described in giant amoebae at about the same time as Lewis, or perhaps even earlier (Edwards, 1925, Mast and Doyle, 1934). However discovery in *Dictyostelium* amoebae was greatly delayed as these avid phagocytes prefer to feed on bacteria and concealed their macropinocytic capacity for many decades.

The first step in the recognition of macropinocytosis in *Dictyostelium* was the isolation in the 1960s of a mutant strain that could grow in liquid medium without bacteria (axenically) (Sussman and Sussman, 1967). To do this, Raquel and Maurice Sussman gradually adapted cells from rich medium containing serum, to simplified media. Though the original axenic strain, Ax1, is now lost, the standard axenic strains Ax2 and probably Ax3/4 were isolated from it (Watts and Ashworth, 1970, Loomis, 1971). These strains can grow in a liquid medium based on glucose, peptone and yeast extract, without further enrichment, and even on defined medium.

Keith Williams and co-workers explored the genetic basis of axenic growth using parasexual genetics (Williams *et al.*, 1974, North and Williams, 1978). It is a recessive trait, implying that it is due to loss-of-function of at least one key gene, and they were able to distinguish three genes that contributed, with *axeB* on chromosome 3 the most important, while mutation of *axeA* and *axeC* improve the growth rate of cells, provided the *axeB* mutation is also present. Williams also confirmed the genetic simplicity of axenic growth by showing that new axenic strains could be obtained from wild-type cells by direct selection for growth in standard axenic medium (Williams, 1976), without the prolonged passaging in rich medium used by the Sussmans.

In principle, axenic cells could take up nutrients from their medium either using specific transporters in the plasma membrane, or by large-scale endocytosis followed by intracellular processing to extract nutrients. No evidence for plasma membrane nutrient transporters came from uptake experiments, though this possibility has not been totally excluded. *Dictyostelium* amoebae take

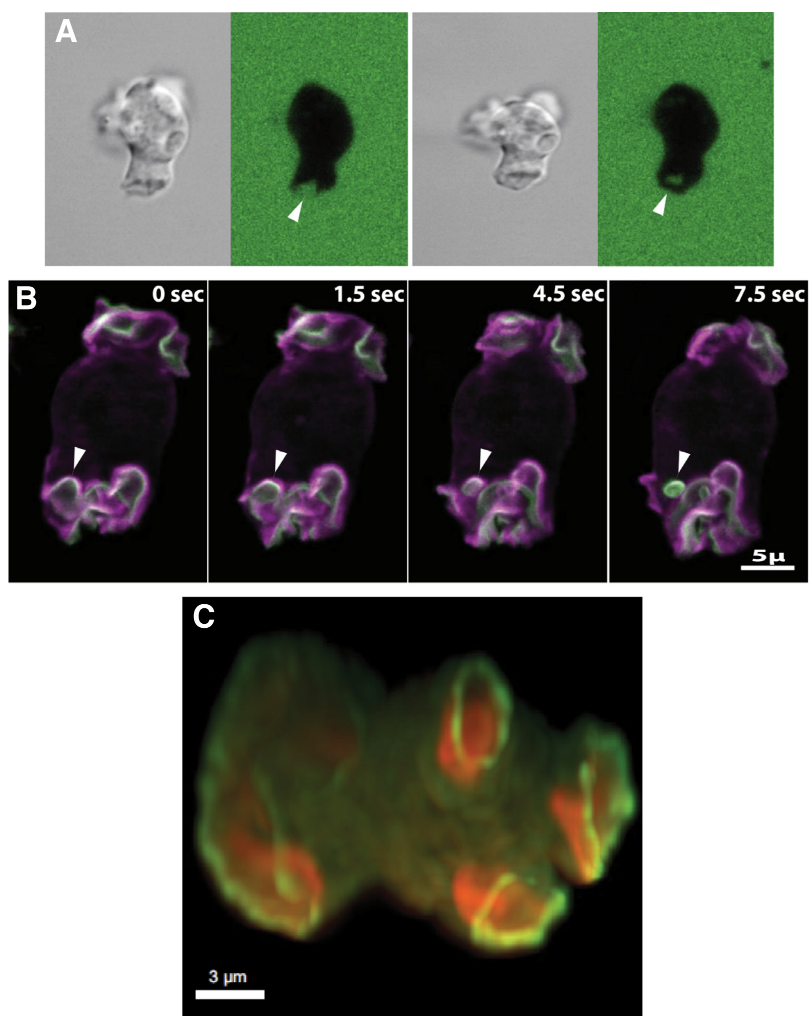


**Fig. 1. Images of macropinocytosis in vegetative *Dictyostelium* amoebae. (A)** *Paired DIC and confocal fluorescent images at successive time points (about 5 sec apart) of a cell bathed in FITC-dextran and showing the closure of a macropinocytic cup to form a macropinosome (arrowed).* **(B)** *Light sheet microscopy images of a single cell expressing reporters for PIP3 – green and F-actin – purple. The closure of a macropinocytic cup is arrowed; once the cup has closed, the F-actin coat is removed, leaving a macropinosome with only the green PIP3 reporter. Microscopy used a custom-built light sheet microscope, taking a full volume every 1.5 sec (JM, unpublished). Images were deskewed and deconvolved and are given as maximum intensity projections from the top. Ax2 cells expressing pPI304 (PH-*pkgE-*GFP; lifeAct-mCherry) (Paschke* et al.*, 2018) were incubated in SUM overnight before microscopy (Williams and Kay, 2018b). See also movie 1.* **(C)** *Rings of the SCAR/WAVE complex (green) surround PIP3 patches (red) in macropinosomes. A 3D reconstruction of an Ax2 cell expressing reporters for the Scar/WAVE complex and PIP3 (HSPC-GFP and PH-CRAC-mRFPmars; pDM767). Imaged using spinning-disc microscopy. Taken from (Veltman* et al.*, 2016).*

up non-physiological substrates such as inulin or FITC-dextran as efficiently as physiological ones and uptake does not saturate at higher concentrations as would be expected if it depended on specific transporters (Lee, 1972, North and Williams, 1978, Thilo and Vogel, 1980, North, 1983). Rather, the medium is taken up in bulk, along with any solutes or small particles that it may contain.

By following an inert tracer such as fluorescent FITC-dextran, the transit of solute through the cell can be tracked to its eventual release back into the medium after 60-90 minutes. Reporters sensitive to pH show that vesicles are acidified to around pH 4.5 starting within minutes of uptake, and then partially neutralized to around pH 6.0 (Brenot *et al.*, 1992, Aubry *et al.*, 1993, Padh *et al.*, 1993, Aubry *et al.*, 1997). Lysosomal enzymes are delivered to these endocytic vesicles (Souza *et al.*, 1997) and endocytosed proteins digested (Padh *et al.*, 1993, Bloomfield *et al.*, 2015). Individual small molecules, such as amino acids and sugars, are presumably extracted from the lysosomes after digestion and used to fuel the cell, though to date the relevant transporters have not been identified, apart from the iron transporter Nramp1 (Buracco *et al.*, 2015) and the FcsA enzyme, which is required for fatty acid utilization (von Lohneysen *et al.*, 2003).

The rate of fluid uptake is around 8 fl/cell/minute in growth medium (Aubry *et al.*, 1997), though less in buffer, which with a cell volume of about 600 fl equates to nearly a cell volume per hour – a remarkable feat, if scaled to a human. With cells about 14 % dry weight (RRK, unpublished) and nutrients in the medium 3.8 % dry weight, the rate of fluid uptake should be sufficient to support the doubling time of 8-9 hours observed in HL5 medium. Wild-type cells take up medium at a much lower rate than axenic cells (Maeda, 1983, Clarke and Kayman, 1987), explaining why they cannot grow in standard liquid medium, and suggesting that the axenic mutations stimulate endocytosis. Thus, the axenic phenotype can be explained as being due to a greatly increased, non-specific uptake of nutrient medium by axenic cells and its subsequent processing in a membrane-bound digestive system.

Meanwhile, work on the actin cytoskeleton in the Gerisch laboratory identified an actin binding protein – coronin – that, together with F-actin, formed curious circular 'crowns' on the dorsal surface of growing cells (de Hostos *et al.*, 1991). These crowns are in fact macropinocytic cups, and using a GFP-fusion reporter for coronin and TRITC-dextran as a fluid tracer, Maniak and co-workers pulled the different strands of the story together by describing the morphological process of macropinocytosis in *Dictyostelium* for the first time (Hacker *et al.*, 1997). They could observe droplets of fluid being taken into cells by cups extending from the plasma membrane. The sensitivity to actin inhibitors argued that uptake is driven by actin dynamics, which could be observed with an F-actin reporter (Pang *et al.*, 1998). In fact, macropinocytic cups dominate the actin cytoskeleton of vegetative axenic cells, which produce several macropinosomes a minute. Calculations of the volume of fluid taken up by these macropinosomes suggest that they account for around 90% of the fluid uptake of axenic cells, with the tiny vesicles produced by clathrin-mediated endocytosis presumably contributing the residue. However due to surface to volume considerations, clathrin-mediated endocytosis probably accounts for most of the membrane taken up (Aguado-Velasco and Bretscher, 1999).

Finally, to complete the link back to the original axenic strains, Bloomfield and co-workers used genomic sequencing of new axenic strains to identify *axeB*, the primary gene enabling macropinocytic growth (Bloomfield *et al.*, 2015). As expected, disruption of *axeB* massively up-regulates macropinocytosis and fluid uptake in wild-type cells. Unexpectedly, *axeB* encodes the *Dictyostelium* homologue of the RasGAP, NF1, which is a major tumour suppressor and underlies neurofibromatosis, one of the commonest human genetic diseases (Ratner and Miller, 2015). Biochemically, inactivating NF1 would be expected to increase Ras activity, thus providing a strong mechanistic link between Ras signalling and macropinocytosis (see later). Since disruption of *axeB* increases fluid uptake in wild-type cells to the same level as in Ax2, without conferring a similar growth rate in liquid medium, the *axeA* and *axeC* mutations are presumably required for this fluid to be effectively utilized. They remain to be identified.

Also unexpected but confirming an earlier suggestion (North and Williams, 1978), Ax2 and Ax3/4 have the identical NF1 mutation. This shows that they gained their primary axenic mutation from a common ancestor, not independently as originally supposed. As Ax2 is derived from Ax1 (Watts and Ashworth, 1970), it is likely that Ax3 also came from this source, perhaps by cross contamination.

## The toolkit for macropinocytosis research in *Dictyostelium*

Work with *Dictyostelium* has made important contributions to our understanding of macropinocytosis and the organism offers biological advantages for tackling this problem, as well as providing a unique evolutionary standpoint, from which universal features can be discerned. To realize the potential of the *Dictyostelium* model requires several things: good assays for macropinocytosis; physiological control of the process; genetic manipulation; inhibitors for acute perturbation and good microscopy, all of which are now in place.

### *Assays of macropinocytosis*

Since macropinocytosis by axenically growing cells accounts for most of their fluid uptake, it can be simply and reliably assayed by measuring the uptake of various tracers from the bathing medium, as already discussed (Thilo and Vogel, 1980, Aubry *et al.*, 1997, Hacker *et al.*, 1997, Rivero and Maniak, 2006). This is not true in most mammalian cells, where only a small fraction of fluid uptake is by macropinocytosis, and microscopic assays have to be used instead (Commisso *et al.*, 2014). The standard assay, in which fluorescent dextran uptake is measured by fluorimetry, has been adapted to high-throughput flow cytometry, allowing hundreds of samples to be assayed per day (Williams and Kay, 2018b, Williams and Kay, 2018a).

Tracers can give much additional information: fluorescent ones allow macropinosomes to be tracked and their pH measured; quenched proteins that become fluorescent on proteolysis show digestion; radioactive or NMR probes can be used (North, 1983, Brenot *et al.*, 1992); and magnetic particles allow macropinosome isolation from cell lysates for biochemical study (Journet *et al.*, 2012).

### *Physiological regulation*

Axenic cells in liquid medium perform macropinocytosis constitutively. Even isolated amoebae continue macropinocytosis at a high rate (Williams and Kay, 2018b). However, it is not unregulated. Cells grown on bacteria largely shut down macropinocytosis (although macropinocytic cells retain their ability to phagocytose bacteria) and it is only regained when the cells are transferred to liquid medium free of bacteria (Kayman and Clarke, 1983, Williams and Kay, 2018b). Upregulation takes 6-12 hours and involves extensive changes in gene expression, many of which seem likely to adapt cells to their new, sugar-rich axenic diet (Sillo *et al.*, 2008).

A minimal medium of three amino acids and glucose suffices to up-regulate macropinocytosis in most strains, and since upregulation depends on macropinocytosis itself, it is likely that the nutrients are sensed within the macropinocytic pathway, probably at the lysosomes (Williams and Kay, 2018b). Wild-type cells, with intact NF1, can grow in a very rich medium, such as HL5 with added 10% foetal calf serum (Maeda, 1983, Bloomfield *et al.*, 2015), and in these conditions will also upregulate macropinocytosis. Thus, strains with low macropinocytic levels can be maintained on bacteria and then macropinocytosis studied after switching to up-regulation medium for 12-24 hours.

When cells are removed from axenic medium to bacteria, macropinocytosis is shut down over a number of hours. If instead they are transferred to non-nutrient conditions, which initiate development, macropinocytosis is again shut down over a few hours, but persists to some extent into the stages where chemotaxis to cyclic-AMP is studied. In this case the residual macropinocytic cups, which contain intense patches of PIP3, have frequently been mistaken for pseudopods.

If cells are starved at low cell density, which attenuates intercellular signalling, macropinocytosis continues at a high rate for at least 24 hours: shutdown requires an unidentified signal released by cells. The need for this signal can be bypassed by activating PKA either with 8-Br-cyclic-AMP (a cell-permeant cyclic-AMP analogue) or genetically (Williams and Kay, 2018b).

### *Molecular genetics*

The powerful molecular genetic methods available in *Dictyostelium* were developed for cells growing axenically (Kuspa and Loomis, 1992, Veltman *et al.*, 2009, Sekine *et al.*, 2018). They have limitations when applied to macropinocytosis, since mutants with impaired macropinocytosis are difficult to maintain axenically, and their poor growth may allow them to be overgrown by suppressors. To meet this problem, improved methods have been developed for transfecting cells grown on bacteria instead of liquid medium (Paschke *et al.*, 2018, Paschke *et al.*, 2019). As an added bonus these methods are faster and allow wild-type (non-axenic) strains to be manipulated as well.

### *Inhibitors of macropinocytosis*

Suitable inhibitors allow acute inhibition of biological processes such as macropinocytosis (including through essential proteins) without allowing time for extensive compensatory changes; but off-target effects and incomplete inhibition are always a consideration. A limited screen for inhibitors of macropinocytosis is reported in (Williams and Kay, 2018b).

Macropinocytosis is suppressed or significantly impaired by inhibitors of actin dynamics (cytochalasin A, latrunculin B), Arp2/3 (CK666), WASP (wiskostatin), formins (SMIFH2), PI3-kinase (LY294002, TGX221), TORC1/TORC2 (torin 1), Rac (EHT1864) and microtubules (thiabendazole). Blebbistatin and dynasore, which inhibit myosin-II and dynamin respectively, have little effect (Williams and Kay, 2018b, Hacker *et al.*, 1997, Rupper *et al.*, 2001).

The closest to diagnostic inhibitors for macropinocytosis in mammalian cells are amiloride and EIPA (West *et al.*, 1989), which block the plasma membrane Na+/H+ exchanger, but these are without effect in *Dictyostelium*. EGTA also has no effect, ruling out an essential role for extra-cellular calcium (Williams and Kay, 2018b).

Unexpectedly both caffeine (Gonzalez *et al.*, 1990) and cycloheximide (Gonzalez and Satre, 1991, Clotworthy and Traynor, 2006) rapidly inhibit macropinocytosis. The effect of caffeine is probably due to its inhibition of both PI3-kinase and TORC2 (Tariqul Islam *et al.*, 2019). We speculate that the suppression of macropinocytosis by cycloheximide and other inhibitors of protein synthesis may be a defense mechanism designed to limit the uptake of environmental toxins.

### *Microscopy*

Microscopy is fundamental to the study of macropinocytosis and many aspects can be appreciated in detail using confocal, spinning disc and TIRF microscopy. In our experience, the integrated process is difficult to follow in this way because it involves large, dynamic structures (ruffles and cups) that move over a good proportion of the cell surface and require tracking for several minutes. However, lattice light sheet microscopy with its rapid acquisition of cell volumes and reduced photoxicity, is ideally suited to the job and allows macropinocytic cups to be followed in three-dimensional glory from birth to closure (Fig. 1B and movie 1) (Chen *et al.*, 2014, Veltman *et al.*, 2016). The macropinocytic vesicles can then be tracked as they move around the cell. Similar approaches in mammalian cells suggest novel ways of forming and closing macropinocytic cups (Condon *et al.*, 2018).

## Genetics of macropinocytosis

Genetic screens have been key to the dissection of cell-biological processes such as the cell cycle and membrane trafficking. While direct genetic analysis of macropinocytosis in *Dictyostelium* is quite limited, gene knock-out strains have been accumulated for over 30 years and their phenotypes curated in dictyBase (Fey *et al.*, 2013). This database can easily be searched for candidate macropinocytosis genes providing an *in silico* genetic screen: for instance, searching mutant phenotypes for ‘decreased growth rate’ yields 244 strains and the more specific ‘decreased pinocytosis’ yields 46, as of May 2019.

To focus on genes likely to have a mechanistic role in macropinocytosis two further criteria are useful: location of the protein to macropinocytic cups or vesicles, or more rarely, exclusion from these (as for PTEN), implying a direct function within macropinocytosis; and normal (or near-normal) growth of mutants on bacteria, showing that there is not a generalized growth defect. A list of genes likely to be involved in macropinocytosis is given in Table 1, together with information on fluid uptake by mutants and location of the protein.

The genes largely fall into two groups: classical signalling genes, including Ras and PI3-kinase; and the actin cytoskeleton and its proximal regulators, such as Scar/WAVE and formins. Membrane trafficking proteins are not included as beyond the scope of this article, though clathrin is an interesting example. Clathrin mutants are severely impaired in fluid uptake (O’Halloran and Anderson, 1992), which historically led to the suggestion that fluid uptake by axenic cells is via coated pits. However, as it is now clear that the bulk of fluid uptake in axenic cells is through macropinosomes

TABLE 1

**_DICTYOSTELIUM_ PROTEINS GENETICALLY IMPLICATED IN MACROPINOCYTOSIS**

| Protein Class & Dicty name | Location | Strain genotype | Fluid Uptake | Growth in HL5 | Ref |
|---|---|---|---|---|---|
| RasGAP: NF1 (in DdB) | MP | *axeB– (NF1–)* | +++ rel DdB | +++ rel DdB | (Bloomfield *et al.*, 2015) |
| Ras: RasG, RasS (in DdB) | MP (GTP) | *rasG or RasS* activated | ++ rel DdB | | (Williams *et al.*, 2019a) |
| Ras: RasB, RasG, RasS | MP (GTP) | *rasG–, rasS–* | -- | - | (Chubb *et al.*, 2000, Khosla *et al.*, 2000, Williams *et al.*, 2019a) |
| RasGEF: GefF | MP | *gefF:GFP* | -- | -- | (Williams *et al.*, 2019a) |
| RasGAP: IqgC | MP | *iqgC–* | + | | (Marinovic *et al.*, 2019) |
| RasGAP: RasGAP2 DDB_G0282055 | PM MP base | null | -- | - | (Li *et al.*, 2018) |
| PI3-kinase: PI3K1, PI3K2 | MP | *pikA–, pikB–* | --- | -- | (Hoeller *et al.*, 2013, Buczynski *et al.*, 1997) |
| PI3-kinase: PI3K4 | PM | *pikF–* | -- | -- | (Hoeller *et al.*, 2013) |
| PI3-phosphatase: PTEN | PM not MP | *ptenA–* | --- | -- | (Veltman *et al.*, 2016) |
| PI4P5-kinase (makes PIP2): PikI | PM | *pikI–* | --- yeast | --- | (Fets *et al.*, 2014) |
| PI-5-phosphatase (OCRL): Dd5P4 | C | *Dd5P4–* | -- yeast | --- | (Loovers *et al.*, 2007) |
| AKT protein kinase: PKB | MP | *pkbA–, pkgB–* | --- | --- | (Williams *et al.*, 2019b) |
| SGK protein kinase: PKBR1 | PM inc MP | | | | |
| TORC2 protein kinase: various subunits | | *lst8–*, or *piaA-, or ripA-* | -- | | (Williams *et al.*, 2019b) |
| PDK1 protein kinase: PdkA | PM inc MP? | *pdkA–* | --- | --- | (Kamimura and Devreotes, 2010, Williams *et al.*, 2019b) |
| Rap: RapA | | rapA (antisense) | -- | --- | (Kang *et al.*, 2002) |
| RapGEF: GflB | MP | *gflB–* | -- | | (Inaba *et al.*, 2017) |
| RapGAP: RapGAP3 | PM MP base | null | -- | - | (Li *et al.*, 2018) |
| Rac: various | MP (GTP) | *racC–, racE–* | | -- | (Wang *et al.*, 2013) |
| RacGEF: GxcT | | gxcT– | | -- | (Wang *et al.*, 2013) |
| RhoGAP: GacG | ND | *gacG–* | --- | --- | (Williams *et al.*, 2019b)2019b |
| Scar/WAVE: Scar complex | MP ring | scrA– | -- | -- | (Seastone *et al.*, 2001, Veltman *et al.*, 2016) |
| WASP: WASP | MP ring | *wasA–* | - | --- | (Williams, 2017) |
| Formin: ForG | MP | *forG–* | -- | -- | (Junemann *et al.*, 2016) |
| Myosin 1: Myo1B,C | MP ring | *myoB–,C–,D–,E–,F–* | --- | - | (Chen *et al.*, 2012) |
| Myosin 1: Myo1D,E,F | MP | | | | PP |
| Carmil | MP | *carmil–* | -- | - | (Jung *et al.*, 2001) |
| Coronin: CorA | MP | *corA–* | - | | (de Hostos *et al.*, 1991, Ishikawa-Ankerhold *et al.*, 2010), PP |
| Actin interacting protein: Aip1 | MP | *aip–* | - | | (Ishikawa-Ankerhold *et al.*, 2010) |

Genes are selected based on their mutant phenotype (reduced or increased fluid uptake or growth in liquid medium) and specific location of the protein to macropinocytic cups (or in the case of PTEN, exclusion). All parental strains are axenic with NF1 deleted, except for the wild-type DdB, which has intact NF1 and therefore very little fluid uptake, and no ability to grow in HL5. MP, macropinocytic cup/macropinosome; PM, plasma membrane; C, cytoplasm; rel DdB, relative to DdB parental strain; yeast, uptake of yeast; PP, Peggy Paschke, unpublished.

Fluid uptake of mutant relative to parental strain: - small reduction (75-100% of parental); -- moderate reduction (25-50%); --- severe (<25%)

Growth in HL5 relative to parental strain (MGT ~ 9hr): - small reduction (MGT 9-15 hr); -- moderate (MGT 15-25 h); --- severe (MGT >25 h). Increased growth (relative to DdB): +++

and since no direct role for clathrin in macropinocytosis has been discovered, it is likely that the mutant defect is due to an indirect effect on membrane trafficking.

The genetics reveals a surprising distinction between on the one hand macropinocytosis and phagocytosis of large particles (yeast sized), and on the other, phagocytosis of small particles (bacteria sized). Wild-type cells with intact NF1 are poor at phagocytosing large particles, just as they are poor at macropinocytosis (Bloomfield *et al.*, 2015). They gain both abilities with the disruption of NF1, without much effect on the phagocytosis of bacteria. However mutation of PI3-kinases removes this ability to drink and eat large particles, while leaving phagocytosis of bacteria intact. It appears that phagocytosis of large particles and macropinocytosis may depend on strong Ras activation and PIP3 in a way that uptake of bacteria does not.

## The macropinocytic signalling patch

The most striking set of macropinocytic genes are those encoding proteins of the signalling axis of Ras, PI3-kinase and the protein kinases Akt and SGK, together with their associated regulatory proteins, including RasGEFs and RasGAPs, PTEN, PDK1 and TORC2 (Fig. 2B). These proteins cooperate to form a 'signalling patch' in the plasma membrane that appears to play a key role in organizing the actin cytoskeleton to form macropinocytic cups.

### *PI3-kinase (Ras-activated)*

The first observations suggesting the existence of a signalling patch in macropinocytic cups came serendipitously from work on chemotaxis in the Devreotes laboratory (Parent *et al.*, 1998), and were followed up in detail by later workers (Rupper *et al.*, 2001, Dormann *et al.*, 2004). A reporter using the PH domain of CRAC revealed intense patches of PIP3 in the plasma membrane of vegetative axenic cells. These lie at the heart of macropinocytic cups and are present from the birth of a cup right through to when it closes. PIP3 lingers for a short period on newly closed vesicles and then is lost once the F-actin coat has been removed, and as further trafficking begins. A second phosphoinositide, PI3,4P2, appears as at about the time cups seal (Dormann *et al.*, 2004). It may be produced from PIP3 by the 5-phosphatase, OCRL, and

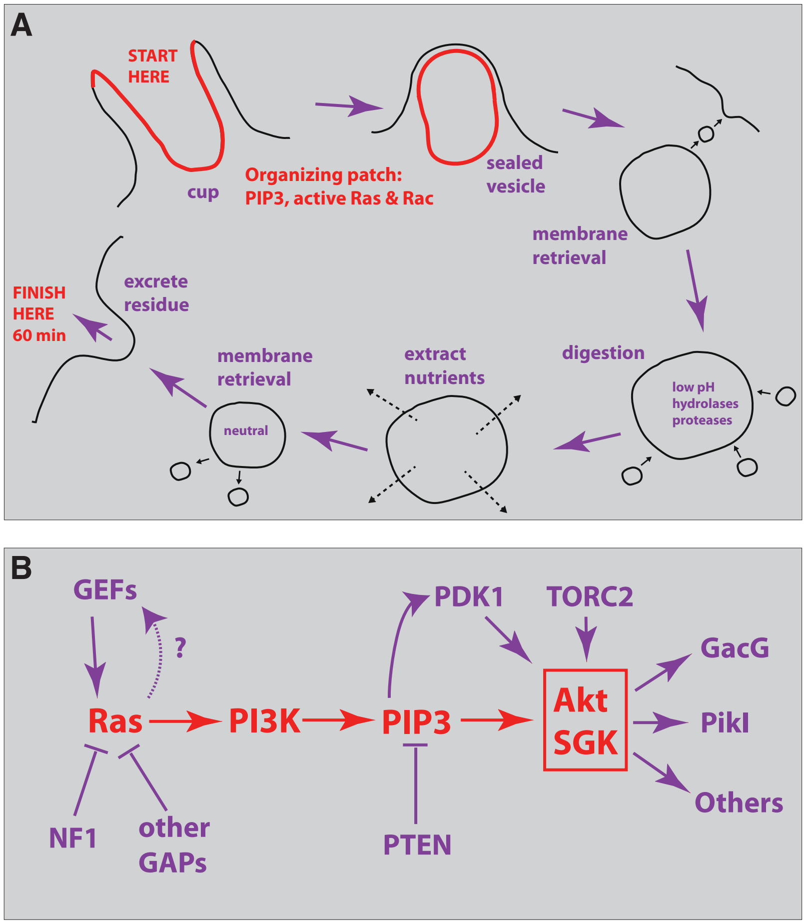


**Fig. 2. Organization of macropinocytic feeding. (A)** *The pathway of nutrient acquisition. Macropinocytosis starts with the formation of a macropinocytic cup (also know as a circular ruffle or crown) whose extension is driven by actin polymerization and which is organized by a 'patch' of PIP3, active Ras, Rap and Rac. This cup seals to form a macropinosome, which loses its F-actin coat and whose identity is altered by the loss of PIP3 and active Ras, gain of PI3,4P2 and changes in other effectors not discussed here. The macropinosome is then trafficked through the endo-lysosomal digestive system to digest and extract nutrients and sustain cell growth.* **(B)** *Signalling within the macropinocytic cup. The cup contains a localized domain, or 'patch' of signaling along the Ras, PI3-kinase and Akt/SGK axis, which is shown together with key regulators. All proteins named are genetically important for macropinocytosis. This axis does not require receptor activation, but is sustained by positive feedback, including a proposed Ras auto-activation loop, and limited by a linked inhibitory process. Effectors of this axis include targets of the protein kinases Akt and SGK, but also proteins directly regulated by the small G-proteins and additional PIP3-binding proteins.*

required for cup closure (Loovers *et al*., 2007).

At around the same time, the first *Dictyostelium* 'class-1' PI3-kinases were cloned in the Firtel laboratory. Like mammalian Class 1 enzymes they have a Ras-binding domain (RBD) allowing activation by Ras, but differ in that no regulatory subunit has been described (Funamoto *et al*., 2002). PI3K1, and possibly the other PI3-kinases, can be targeted to plasma membrane by the Ras-binding domain and also by sequences of the N-terminus that appear to bind F-actin (Hoeller *et al*., 2013).

A double mutant of PI3K1 and PI3K2 grows poorly in axenic medium and when investigated in detail was found to be defective in macropinocytosis (Buczynski *et al*., 1997). A full analysis of the five 'Class 1' PI3-kinases confirms their importance, and suggests that PI3K4 may have an additional unique role (Hoeller *et al*., 2013). Excessive production of PIP3 caused by deletion of the PIP3 phosphatase, PTEN, is very deleterious for macropinocytosis (Veltman *et al*., 2016), indicating that PIP3 levels have to be closely controlled.

Similarly in mammalian cells work with PI3-kinase inhibitors and PIP3 reporters shows that PIP3 is concentrated in macropinocytic cups and is essential for their closure (Swanson, 2008). Although mammalian and *Dictyostelium* phosphoinosites differ in chemical detail - *Dictyostelium* phosphoinositides are ether lipids (Clark *et al*., 2014) - their function in macropinocytosis is conserved.

With the importance of PIP3 in macropinocytosis established, attention turned to the upstream regulators of PI3-kinase and the downstream effectors.

### *Upstream of PIP3: Ras and its regulators*

In mammalian cells, Ras was implicated in macropinocytosis in early studies of growth factor signalling, where introduction of activated (oncogenic) Ras protein caused cell ruffling and macroinocytosis (Bar-Sagi and Feramisco, 1986). These gain of function experiments do not replicate in axenic *Dictyostelium* cells, presumably because Ras is already partially activated by loss of NF1. However, expression of activated Ras in wild-type cells, with intact NF1, does stimulate macropinocytosis (Williams *et al*., 2019a). Active Ras forms patches in macropinosomes coincident with PIP3 (Sasaki *et al*., 2007, Veltman *et al*., 2016).

*Dictyostelium* has a plethora of Ras genes but the key Ras proteins for macropinocytosis appear to be RasG and RasS (Chubb *et al*., 2000, Bolourani *et al*., 2006, Williams *et al*., 2019a). However, a double mutant still takes up fluid at about 35% of the parental rate, indicating that at least one more Ras protein is involved, which is likely RasB (Junemann *et al*., 2016).

Ras is activated by GEF and inactivated by GAP proteins. The genome encodes more than 20 RasGEFs of which GefB is implicated in macropinocytosis by null mutants having a severe defect in fluid uptake in the Ax3 genetic background (Wilkins *et al*., 2000) and a lesser one in Ax2 (Williams *et al*., 2019a). GefF is implicated in macropinocytosis by an insertional mutant that also has strongly reduced fluid uptake, though the gene has not so far been knocked out (Williams *et al*., 2019a).

As already described, the RasGAP NF1 controls overall Ras patch size and number (Bloomfield *et al*., 2015). NF1 is recruited to macropinocytic cups and leaves macropinosomes shortly after they are sealed, but even in its absence Ras activity remains closely regulated, implying that other RasGAPs have important roles. IqgC is an active RasGAP, related to IQGAPs and is strongly recruited to macropinosomes. However, null mutants have a surprisingly mild phenotype: the rate of fluid uptake is unchanged, but more fluid accumulates on longer incubation, suggesting that subsequent trafficking of macropinosomes is somehow affected (Marinovic *et al*., 2019). It may be that the full phenotype will only be revealed by a knock-out in non-axenic cells, with intact NF1. RasGAP2 (DDB_G0282055) is recruited towards the base of macropinocytic cups as they close and on the newly formed vesicles; it binds PI3,4P2 as well as PIP3. Mutants have impaired fluid uptake and axenic growth (Li *et al.*, 2018).

Rap proteins are closely related to Ras proteins and RapA is an essential protein in *Dictyostelium* whose depletion by anti-sense inhibition causes a strong macropinocytic defect (Kang *et al.*, 2002). Mutants of GefIB, a GEF for RapA form arrested and extended macropinocytic cups, and so have a severe macropinocytic defect (Inaba *et al.*, 2017). The RapGAP, RapGAP3, like RasGAP2, is recruited to macropinocytic cups as they close, binds PI3,4P2 as well as PIP3 and is required for efficient fluid uptake (Li *et al.*, 2018). These proteins link Rap into macropinocytosis, and though it is likely that active Rap forms patches in macropinocytic cups, this has not yet been investigated in detail.

#### *Downstream of PIP3: Akt and SGK protein kinases*

PIP3 produced by PI3-kinases serves as a recruitment platform in the plasma membrane for proteins with PIP3-binding domains, of which the genome encodes a considerable number. The classic PIP3 effector is the protein kinase Akt (PKB in *Dictyostelium* (Meili *et al.*, 1999)). Full activation of this enzyme requires binding to PIP3 and phosphorylation by two further protein kinases: TORC2 and PDK1 (Pearce *et al.*, 2010, Kamimura and Devreotes, 2010, Liao *et al.*, 2010). PDK1 is itself a PIP3-binding protein, thus conferring PIP3-dependence on Akt through two routes.

PKBR1 has a similar substrate specificity and activation mechanism (by TORC2 and PDK1) to PKB, but lacks a PH-domain and is instead constitutively targeted to the plasma membrane by myristoylation (Meili *et al.*, 2000). PKBR1 is often described as a variant Akt, but is actually more closely related to the SGK group of protein kinases (Goldberg *et al.*, 2006, Pearce *et al.*, 2010) and we will treat it as such.

Single mutants of PKB or PKBR1 have relatively minor defects in fluid uptake, but this is nearly abolished in a double mutant (Williams *et al.*, 2019b). The PIP3-dependent PDK1, PdkA, is similarly important with very little fluid uptake when it is knocked out, while TORC2 mutants also have severe defects in fluid uptake. Thus the Akt and SGK protein kinases are central, though redundant players in macropinocytosis. The situation is less clear in mammalian cells, where Akt inhibitors affect macropinocytosis in some cells but not others, and the role of SGK remains to be tested (King and Kay, 2019).

PKB/PKBR1 targets have been identified through phosphoproteomics (Kamimura *et al.*, 2008, Williams *et al.*, 2019b) and include the PI4P5-kinase, PikI and the RhoGAP, GacG. PikI makes most of the cellular PI4,5P2 and is required for efficient growth in liquid medium and phagocytosis of yeast, a proxy measure of macropinocytosis (Fets *et al.*, 2014). GacG is required for virtually all fluid uptake, and mutant cells make excessive pseudopods instead of macropinosomes, moving nearly twice as fast as their parent (Williams *et al.*, 2019b, Nichols *et al.*, 2019). It is thus possible that GacG controls the deployment of the actin cytoskeleton between macropinocytotic cups and pseudopods, which are considered to be in competition, both in *Dictyostelium* (Veltman *et al.*, 2014) and dendritic cells.

Rac is also presumably downstream of Ras and PIP3 in this system. Active Rac forms patches roughly coincident with active Ras and PIP3, and inhibition with EHT1864 nearly abolishes fluid uptake (Veltman *et al.*, 2016, Williams and Kay, 2018b), while expression of constitutively active forms of Rac1 causes excessive macropinocytic cup formation but decreased fluid uptake, presumably because closure of the cups requires Rac inactivation (Dumontier *et al.*, 2000), as in macrophages (Fujii *et al.*, 2013). The multiple genes in the Rac/Rho family, and their multiple GEFs and GAPs make genetic dissection of their functions difficult. However, single knock-out mutants of RacC and RacE and the GEF GxcT have severe growth defects in liquid medium, but whether this is due to a macropinocytosis defect is not known (Wang *et al.*, 2013).

## Cytoskeletal proteins

The cytoskeleton provides the building blocks for macropinocytosis, but may be under-represented in genetic screens, either because the proteins have highly redundant functions such as actin binding proteins and defects are only seen in multiple knock-outs (Rivero *et al.*, 1999), or are genetically essential, like the Arp2/3 complex (Langridge and Kay, 2007).

#### *Initiators of actin polymerization*

The most important initiators of actin polymerization in macropinocytic cups are the Arp2/3 complex and certain formins. The Arp2/3 complex initiates the formation of dendritic F-actin and localizes to cups (Insall *et al.*, 2001). It is activated by the Scar/WAVE complex, which is required for efficient macropinocytosis (Seastone *et al.*, 2001, Veltman *et al.*, 2016). WASP, an alternative activator of the Arp2/3 complex, is also required for efficient macropinocytosis (Williams, 2017), and seems able to substitute for Scar/WAVE when this is deleted (Davidson *et al.*, 2018).

Formins trigger the formation of linear F-actin polymers and may be responsible for the F-actin spikes often found in macropinocytic cups. ForG has a Ras-binding domain and is strongly recruited to macropinocytic cups and in its absence fluid uptake is much reduced (Junemann *et al.*, 2016). Mutant cells make abundant F-actin projections of uncertain provenance, instead of fruitful macropinocytic cups.

#### *Myosins*

Macropinocytic cups viewed by lattice light-sheet microscopy sometimes appear to close by a purse-string contraction of the rim of the cup. This behaviour might indicate the presence of a contractile ring of myosin-II. However, such a ring has not been reported with GFP fusions and myosin-II null mutants grow in axenic medium, showing only a modest defect in fluid uptake (Williams, 2017); nor does the myosin-II inhibitor blebbistatin inhibit fluid uptake (Williams and Kay, 2018b). The role of myosin-II is not clear, and requires further investigation.

In contrast, myosin-1 proteins are recruited to macropinocytic cups. One group bind PIP3 (myosin 1D, 1E and 1F) and are strongly recruited to the body of macropinocytic cups, whereas myosin 1B is recruited towards the edge, so that together they form a striking bull's eye pattern (Chen *et al.*, 2012, Brzeska *et al.*, 2016). Deletion of the PIP3-binding myosin-1 proteins or myosin 1B singly has only a minor effect on growth in liquid medium or fluid uptake (Jung *et al.*, 1996, Chen *et al.*, 2012), whereas multiple knock-outs have a progressively more severe effect on fluid uptake, culminating in a quintuple knock-out (of myosin 1B, C, D, E, F; PP, unpublished) where fluid uptake reduced to 25% of the parental Ax2 in HL5. Thus myosin-1 proteins have a significant collective role in macropinocytosis, though exactly what this is remains to be discerned.

***Actin binding proteins***

Coronin and Aip are actin-binding proteins that promote F-actin turnover and are strikingly recruited to macropinocytic cups. Deletion impairs fluid uptake, perhaps due to a delay in removing the F-actin coat from newly-formed macropinosomes (de Hostos *et al.*, 1991, Konzok *et al.*, 1999, Ishikawa-Ankerhold *et al.*, 2010).

Carmil is a multi-domain protein that binds to capping protein and also myosin 1B and the Arp2/3 complex. It is recruited to macropinocytic cups, and its deletion was originally reported to impair fluid uptake by about 50% (Jung *et al.*, 2001). However, a fresh mutant in the Ax2 background is without an uptake defect in our hands (Williams, 2017).

## Ras/PIP3 signalling patches as templates for circular ruffles

In the canonical form of macropinocytosis in both *Dictyostelium* and macrophages, a circular ruffle forms in the plasma membrane, extends outwards forming a cup and then closes producing a macropinosome. To produce the circular ruffle, actin needs to be persuaded to polymerize in a hollow ring of up to several microns in diameter. How can this spatial organisation be achieved?

A possible mechanism is suggested by the observation that PIP3 patches are surrounded by a necklace of the Scar/WAVE complex (Fig. 1C). Since Scar/WAVE activates the Arp2/3 complex, which triggers actin polymerization, this ringed recruitment could trigger a hollow ring of actin polymerization and act as a template for a circular ruffle. Ringed recruitment of Scar/WAVE to PIP3 patches also occurs in phagosomal cups, basal waves and at cell-cell contacts in streams: it seems to be an empirical rule of cytoskeletal organization in *Dictyostelium* (Veltman *et al.*, 2016). In each case the PIP3 patch should specify a ring of actin polymerization and the formation of a cupped structure, though this is frustrated by the substratum in basal waves.

Since both Ras and PIP3 diffuse freely in the plasma membrane, unusual kinetics must be required to sustain patches. Most likely, these involve both positive feedback and a restraining, inhibitory process to limit its extent, as has been studied theoretically in reaction-diffusion schemes. Genetics can provide information about the logic of this process.

When Ras is activated by deletion of NF1, the Ras patches become larger as do the PIP3 patches and the spatial correspondence between them is maintained (Bloomfield *et al.*, 2015). However, when PIP3 patches are abolished by deletion of PI3-kinases, Ras patches persist, though they are smaller than in control cells. Similarly, they persist but are smaller in PKB-/PKBR1- double mutant cells (Williams *et al.*, 2019b). When PTEN is deleted, though PIP3 patches greatly expand to occupy much of the plasma membrane, the Ras patches remain smaller and more discrete (Veltman *et al.*, 2016). To a first approximation, it appears that active Ras patches can exist independently of PIP3, while PIP3 patches slavishly follow Ras patches. This suggests that the postulated positive feedback loop does not require PIP3 and is centered on Ras, but there is a secondary loop through which PIP3 and Akt/SGK can produce larger active Ras paches.

Another factor maintaining patches may be a diffusion barrier around them, as reported in mammalian cells (Welliver *et al.*, 2011). The edges of PIP3 patches will also be sharpened by exclusion of the PTEN phosphatase, whose activity outside patches reduces PIP3 levels in the rest of the plasma membrane.

In summary, we propose that Ras/PIP3 patches trigger actin polymerization around their periphery and so template rings of protrusive actin under the plasma membrane, forming circular ruffles. Patches are sustained by at least one positive feedback loop centered on Ras. The recruitment process that attracts Scar/WAVE to the edge of patches remains to be discovered.

## Macropinocytosis in other amoebae

Macropinocytosis appears widespread among amoebae (Edwards, 1925, Mast and Doyle, 1934, King and Kay, 2019). Some of the earliest descriptions were from giant amoebae and included a form where the amoebae make tubular invaginations from which macropinosomes pinch off (Chapman-Andresen, 1977). The dictyostelids, *Dictyostelium purpureum* and *Polysphondylium pallidum* can both grow axenically in liquid medium and are therefore assumed to be macropinocytic. Macropinocytosis has been clearly demonstrated in *Acanthamoeba castellanii* (Ostap *et al.*, 2003) and the pathogenic *Entamoeba histolytica*, which may use it to feed on host cell debris (Meza and Clarke, 2004).

## Questions and prospects

We currently know enough about macropinocytosis to speculate about underlying mechanisms and relationships, but not so much that these speculations are redundant. Questions and speculations include the following.

The attraction of Scar/WAVE to the edge of active Ras/PIP3 patches suggests a mechanism for organizing actin polymerization into hollow rings and thus to form the walls of macropinocytic cups. Does this recruitment reflect a ‘Goldilocks zone’ for Scar/WAVE binding, perhaps due to the interplay between an activator of binding and an inhibitor? How does the patch maintain itself against the tendency of the components to diffuse away in the plasma membrane?

All PIP3 patches examined seem to have the same organization of Scar/WAVE recruitment to their periphery (Veltman *et al.*, 2016). Does this rule have exceptions or do all patches tend to make circular ruffles? Pseudopods generally do not have PIP3 patches and are not hollow centered: they appear to be distinct structures. Are the PIP3 patches described in much of the *Dictyostelium* chemotaxis literature in fact abortive macropinocytic cups, not pseudopods?

How do macropinocytic cups close and the membranes fuse? Is there a purse-string mechanism to constrict the rim of the cup, or can large flaps of membrane fuse together due to the action of a fusogenic protein? If so, what is this protein?

Wild-type cells, with intact NF1 can only grow on liquid medium if it is reinforced with additional proteins and even then their fluid uptake is less than axenic cells (Williams and Kay, 2018b). Where in Nature would they encounter such a rich medium, and are there circumstances where they can increase fluid uptake to higher levels?

What is the evolutionary relationship between macropinocytosis and growth factor signalling implied by their common usage of the Ras/PIP3 signalling axis? It has been speculated that the evolutionary origin of this axis lies in the organization of feeding structures in single celled organisms, and only later, with the evolution of multicellular organisms, did these feeding structures fall under the control of growth factors (King and Kay, 2019). What, therefore, is the evolutionary history of macropinocytosis and growth factor signalling?

Macropinocytosis research has a long history. For much of the time it has been quietly pursued by just a handful of pioneers. This is changing, as the medical importance of macropinocytosis in cancer, immunology, drug delivery and neurodegeneration is appreciated, along with the intrinsic fascination of the cell biology. With the first dedicated conference held only in 2018, the field is poised for exciting progress, to which work with amoebae can continue to make an important contribution.

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