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Cosimo Commisso *Editor*

Macropinocytosis

Functions and Mechanisms

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Editor

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Functions and Mechanisms

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Preface

Macropinocytosis: Functions and Mechanisms

Macropinocytosis is an endocytic mechanism of fluid-phase uptake that has a diverse range of functions across biology. The process of macropinocytosis is dependent on the dynamics of the actin cytoskeleton, which drive plasma membrane ruffling. When these membrane ruffles fuse with each other or back-fuse with the plasma membrane, they form a vesicular compartment called a macropinosome. The nascently formed macropinosome non-selectively captures extracellular fluid and any factors that may be present or solubilized within it. The newly formed macropinosome then undergoes a maturation process whereby its cargo can be degraded in a lysosome-dependent fashion, integrated via fusion events into other vesicular compartments or organelles, or recycled back to the cell surface. In this way, macropinocytosis can serve a variety of functions across a plethora of cellular contexts. For example, macropinocytosis has been implicated as a nutrient delivery mechanism in different cell types, in both normal and pathological states. In the immune system, macropinocytosis operates in immune surveillance mechanisms to support antigen presentation. In addition to intrinsic functions, macropinocytosis can also be hijacked. One great example is how the macropinocytosis pathway is subverted by pathogens, such as bacteria and viruses, to enter host cells. In addition, macropinocytosis can also be leveraged for drug delivery as it represents a major internalization pathway for nanoscale therapeutics, such as peptides and nanoparticles. It has become widely appreciated that the regulation of macropinocytosis at a molecular level is highly intricate and extensively context dependent. Considering the different roles that macropinocytosis can play that impact disease, recent efforts are geared toward understanding the signaling outputs that control this uptake mechanism in different settings and identifying molecular targeting strategies that can be employed to dial the process up or down. In this book, the different intrinsic functions of macropinocytosis will be discussed first. In the

second half of the book, the signaling pathways and molecular drivers of macropinocytic uptake are highlighted.

Macropinocytosis: A Diverse Landscape of Functionality

The functions of macropinocytosis are exquisitely dependent on cellular context. To appreciate the uniqueness of macropinocytosis within the broad spectrum of uptake mechanisms, it is crucial to understand the selective aspects of the macropinocytic cellular machinery. In Chap. 1, the discussion is centered on how macropinocytosis contrasts to other endocytic mechanisms, including phagocytosis, pinocytosis, clathrin-mediated endocytosis (CME), and caveolae-mediated endocytosis. The functions of macropinocytosis in different cell types are then explored in broad strokes, focusing on immune surveillance, metabolism, and cell death. In subsequent chapters in this portion of the book, we take a deep dive into specific functions of macropinocytosis. In Chaps. 2 and 3, the role of macropinocytosis as a nutrient acquisition pathway is examined. In cancer, tumor cells use macropinocytosis to obtain extracellular nutrients, in the form of either proteins or necrotic cell debris that can be catabolized to their constituent parts. This process supports proliferation despite the nutrient-deprived conditions of the tumor microenvironment. Chapter 2 focuses on the role of macropinocytosis as a nutrient stress adaptation in cancer and discusses how different nutrient deficiencies can elicit macropinocytic induction on a molecular level. Macropinocytosis as an ancient feeding mechanism was initially described in the soil amoeba *Dictyostelium discoideum*. In Chap. 3, we take a close look at the molecular drivers that control macropinocytosis in amoeba. In addition to bulky nutritious cargo, macropinocytosis can serve as an internalization mechanism for smaller molecules that are solubilized within the extracellular fluid. Chapter 4 describes how extracellular ATP is taken up by tumor cells via macropinocytosis and how this might impact tumor cell properties. Macropinocytosis plays critical roles in fundamental cellular processes, and Chaps. 5 and 6 delineate in detail two of these functions. Chapter 5 examines the physiological links between macropinocytosis and cell migration, while Chapter 6 focuses on the function and regulation of macropinocytosis in immune cells. It is intriguing to note that the “inducible” and “constitutive” forms of macropinocytosis that are operational in the immune system are also present in cancer cells, but with underlying molecular mechanisms that are seemingly divergent.

Regulation of Macropinocytosis by Signaling Molecules

At the molecular level, macropinocytosis is controlled by an intricate web of membrane lipids and proteins that control critical aspects of macropinosome generation ranging from actin cytoskeletal dynamics to macropinosome closure and

maturation. The availability and abundance of several membrane lipids can dictate the extent and rate of macropinocytosis. In Chap. 7, how a subset of phosphoinositides regulates different steps of the macropinocytosis pathway is examined. These membrane lipids, as well as the lipid kinases that regulate their synthesis, play an important role in controlling macropinosome dynamics in a wide range of organisms ranging from amoeba to mammalian cells. The lipid constituents mediating macropinocytic induction must organize and fit within an elaborate network of enzymes, such as GTPases and protein kinases. In Chap. 8, the precise signaling mechanisms that dictate the interactions between lipids and signaling proteins that culminate into macropinosome formation are examined. These pathways, including nutrient sensors such as mTORC1, play important roles in integrating a growth factor-driven response with a functional output. One up and coming signal transduction pathway at the "leading edge" of macropinocytosis research, and the focus of Chap. 9, is the Wnt/GSK3 pathway. Through GSK3 inhibition, Wnt signaling can "unleash" a cell that is poised for macropinocytosis by enhancing actin dynamics, revealing an interesting signaling nodule that normally functions to constitutively restrain macropinocytosis. By far the best studied oncogenic signaling pathway that regulates macropinocytic induction is the Ras signaling pathway. In normal cells, Ras potentiates growth factor signaling occurring through receptor tyrosine kinases (RTKs) to put into motion the mechanical machinery that drives macropinocytosis. In transformed cells harboring oncogenic Ras mutations, Ras signaling is mostly uncoupled from RTKs and the cancer cell becomes primed to constitutively induce macropinocytosis or to integrate nutrient availability signals to induce uptake. In the last two chapters, we dive deep into Ras signal transduction mechanisms. Chapter 10 examines how oncogenic Ras acts through integrins and their binding partners to control macropinocytosis and survival, while Chap. 11 explores how unique oncogenic Ras mutations differentially regulate effector pathways and macropinocytic induction. These insights into oncogenic Ras biology have the potential to impact the design of novel macropinocytosis-based therapies for cancer, and could shed light on future patient stratification approaches for eventual macropinocytosis inhibitors.

Overall, *Macropinocytosis: Functions and Mechanisms* comprises various topics pertaining to *what* macropinocytosis does in a cell and *how* molecules communicate to control this endocytic pathway. This book will help readers to have a better understanding of the physiological relevance of macropinocytosis and, in this way, the field of macropinocytosis research can integrate knowledge from different contexts, both normal and disease.

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Cosimo Commisso

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About the Editor

Cosimo Commisso earned his Ph.D. in 2008 at the University of Toronto in the Department of Molecular Genetics and completed his Postdoctoral Fellowship in Cancer Biology at New York University Langone Medical Center in 2014. He is currently an Associate Professor and Program Director at the Sanford Burnham Prebys Medical Discovery Institute NCI-Designated Cancer Center in La Jolla, California. Dr. Commisso made a seminal contribution to the field of cancer research by identifying the way that pancreatic tumors augment their nutrient supply through boosting a process called macropinocytosis. This "cellular drinking" pathway allows tumors to obtain nutrients from their environment and supports tumor growth. Research in Dr. Commisso's lab is centered on identifying novel drug targets in the macropinocytosis pathway that could be harnessed to develop new therapeutic modalities for cancer.

Part I
Functions of Macropinocytosis

Chapter 1
Functional Diversity of Macropinocytosis

Rajeev Mishra, Yamini Gupta, Garima Ghaley, and Neil A. Bhowmick

Abstract Eukaryotic cells are capable of internalizing different types of cargo by plasma membrane ruffling and forming vesicles in a process known as endocytosis. The most extensively characterized endocytic pathways are clathrin-coated pits, lipid raft/caveolae-mediated endocytosis, phagocytosis, and macropinocytosis. Macropinocytosis is unique among all the endocytic processes due to its nonselective internalization of extracellular fluid, solutes, and membrane in large endocytic vesicles known as macropinosomes with unique susceptibility toward Na +/H+ exchanger inhibitors. Range of cell types capable of macropinocytosis and known to play important role in different physiological processes, which include antigen presentation, nutrient sensing, migration, and signaling. Understanding the physiological function of macropinocytosis will be helpful in filling the gaps in our knowledge and which can be exploited to develop novel therapeutic targets. In this chapter, we discuss the different molecular mechanisms that initiate the process of macropinocytosis with special emphasis on proteins involved and their diversified role in different cell types.

Keywords Endocytosis, Caveolae · Clathrin · Lipid rafts · Macropinocytosis · RAS

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Introduction

Eukaryotic mammalian cells with a membrane-bound nucleus and large subcellular compartments known as organelles and this arrangement of these organelles distinguish eukaryotic cells from prokaryotic cells. Subcellular organelles are structures within a mammalian cell that are essential for various biological processes such as protein synthesis, energy generation, and utilization. Each organelle's membranes and interior spaces "contain a special group of proteins," allowing each structure to perform a specific function. The nucleus, endoplasmic reticulum, Golgi complexes, lysosomes, mitochondria, and an internal cytoskeleton are the major organelles in eukaryotic cells. Cell junctions are specific areas of the cell membrane which contain proteins and glycolipids that facilitate metabolite exchange by forming unique structures between cells. Proteins on the membrane of cell often serve as receptors, binding signaling molecules including hormones, growth factors, and neurotransmitters, all of which are essential for cell growth and regulation. All organisms need to sense or uptake the nutrients from outside environment for sustainable growth and development and therefore cells adopt various mechanisms to uptake nutrients (Chantranupong et al. 2015).

Cells require a continuous supply of nutrient that fulfill energy demand to accomplish the tasks of life and making building blocks to sustain growth and division. While the mammalian cells are surrounded by a variety of nutrients, but to pass across the cell membrane (which act as a barrier between the cell and surrounding environment) there are special uptake mechanisms to internalize the nutrients (Palm 2019; Palm and Thompson 2017). These nutrients come in many forms, which primarily include glucose, amino acids, various macromolecules, and micronutrients. In mammalian cells, internalization of nutrients has been evolved and regulated by various important pathways like receptor-mediated, fluid-phase internalization, nutrient transporters of cell surface, and bulk solute micropinocytosis to acquire these diverse nutrients (Kumari et al. 2010). All these processes are coordinated by highly regulated by unique signaling pathway, for nutrient delivery and utilization. This chapter provides an account of nutrient uptake mechanism and describes endocytic routes with special emphasis on Macropinocytosis and its important function in various cell types.

Nutrient Transporters: A Specialized Protein Pumps to Intake Small Molecules

Since the polar molecules, such as glucose and amino acid, cannot cross the plasma membrane, therefore they require specialized carrier proteins protein called transporter (Vander Heiden et al. 2009). Because these transporters are primarily involved in cell nutrition that is why they are generally known as nutrient transporters, which are tightly linked with extrinsic growth signals. Nutrient transporters such as glucose and amino acid transporters are upregulated by the stimulation of the

phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin (PI3K/Akt/mTOR) pathway to fulfill the nutrient demand by allowing enhanced influx of nutrients through transporters (Hoxhaj and Manning 2020; Magaway et al. 2019; McCracken and Edinger 2013). In the event of unavailability of nutrients, the signal stimulation is lost and therefore transcription of nutrient transporters is inhibited, and existing transporter get endocytosed and degraded in lysosomes and thereby actively removed (Mayer and Grummt 2006; Todkar et al. 2017). Nutrient transporter degradation is primarily regulated by one of the important proteins Rab7, which belongs to larger superfamily of Ras-like GTPases (Ao et al. 2014; Guerra and Bucci 2016). Nutrient transporters are generally categorized into two different types, i.e., influx (facilitates inward nutrient uptake, i.e., from outside into cytoplasm) and efflux transporter (outward movement of molecules, i.e., from cytoplasm to outside the cell). Numerous studies have demonstrated a strong correlation between expression of plasma membrane nutrient transporters, such as the glucose transporters (GLUTs) and different amino acid transporters (ASCT2, LAT1) and metabolic reprograming of the cells, which make these transporters an ideal pharmacologic target in various diseases (McCracken and Edinger 2013; Broer 2018; Scalise et al. 2017; Sniegowski et al. 2021).

"Endocytosis": Intake of Macromolecules

While entry of small molecule requires protein pumps (transporters) to enter cells (Broer 2018), however, macromolecules enters through a special mechanism known as "Endocytosis" where cargo macromolecule being captured and invaginate within membrane-bound carriers that pinch off from the plasma membrane after maturation (Kumari et al. 2010). Endocytosis is not only an intake mechanism of nutrients but also involves in providing protection (immune defense) and maintaining homeostasis (Cossart and Helenius 2014). Even this mechanism is "hijacked" by certain pathogenic bacteria, protozoa, and enveloped and non-enveloped viruses to enter into cells (Manes et al. 2003). Endocytosis occurs through different uptake modes which include phagocytosis, clathrin-mediated endocytosis (CME), caveolae-dependent uptake, and other forms of less selective forms of uptake through small pinocytic vesicles or macropinocytosis (Kumari et al. 2010). These pathways represent emerging fields of study and are yet to be explored.

Phagocytosis

Phagocytosis is an important endocytic process where the surrounding material (i.e., molecule or bacteria) is engulfed into the specialized cells, known as phagocytes such as neutrophils, macrophages, monocytes, and dendritic cells (Rosales and Uribe-Querol 2017). During the process of phagocytosis, molecules bind to specific

protein receptors on the surface of the cell membrane creating a circumferential movement of plasma membrane to internalize the receptor-bound particles into the large vesicle known as "phagosome" (Richards and Endres 2014). These cells can phagocytose the particles even larger than their own surface area and phagosome eventually travels to the lysosome forming phagolysosomes for the final destruction where the enzymes of the lysosome degrade and digest the material. There are several receptors involved in the triggering signaling cascade of phagocytosis by recognizing précised molecular associate patterns associated with microorganisms. These receptors can be divided into non-opsonic or opsonic receptors. Internalization through the opsin receptor is known as "opsonization, and this process requires assistance of antibodies; complement factors C1q, C3b, and C4b; and lectins (McGreal and Gasque 2002). On the other hand, binding of engulfing molecules on non-opsonic receptors is associated with molecular patterns of pathogen commonly known as pathogen-associated molecular patterns (PAMPs), which are present on the surface of the microorganisms. Commonly PAMPs include peptidoglycan (found in bacterial cell walls), flagellin (bacterial flagellar protein; lipopolysaccharide (LPS; on the outer membrane of Gram-negative bacteria), lipopeptides (bacterial expressing molecules), and nucleic acids (viral DNA or RNA) (Lu et al. 2020).

Pinocytosis

The term pinocytosis was coined by Warren H. Lewis in 1931, where he refers to it as the uptake of extracellular fluid by cells in vitro also known as "cell drinking" (Schmid et al. 2014). It is a process that is performed by a broad array of cells involving cellular internalization or endocytosis of fluids and solutes regardless of their size. This process occurs continuously and is a non-specific process, i.e., not molecule specific, unlike receptor-mediated endocytosis. Pinocytosis can be classified into various types of cellular internalization, or endocytic pathways including clathrin-mediated endocytosis, caveolae-dependent uptake, and the CLIC/GEEC (clathrin-independent carrier/glycosylphosphatidylinositol (GPI)-anchored protein-enriched early endosomal compartment) pathway (Mayor et al. 2014). These pathways represent a broad field of study and further research not only explored new mechanistic but also identified a novel target for cell-based therapeutics.

Clathrin-Mediated Endocytosis

Discovered by Roth and Porter in 1964 (Schmid et al. 2014) and one of the most studied and well-characterized type endocytic route. All mammalian cells used this endocytic route to acquire nutrients from surroundings, for example, uptake of iron through transferrin receptor and cholesterol through low-density lipoproteins

receptors (Lodish 2000). This process is primarily driven by the clathrin protein, which is composed of a heavy chain (~190 kDa) and light chain (~25 kDa) forming a trimeric structure known as "triskelion" (Fotin et al. 2004; Royle 2006). Conceptually, CME is a highly regular sequential and partially overlapping step, in which apart from Clathrin, more than 40 other cytosolic proteins (such as dynamin, adapter proteins, and other proteins) are involved in this endocytic process (Popova et al. 2013). CME can be divided into six overlapping steps which include initiation, cargo recruitment, membrane bending, scission, and uncoating (Pastan and Willingham 1985). Briefly, CME is initiated when clathrin protein from cytosolic pool assemble on the inner leaflet of plasma membrane forming a "protein coat assembly" (*Initiation*), which is followed by recruitment of other cytosolic proteins. Clathrin-coated cargo recruitment enables other cargo proteins at the assembly to grow further (*Cargo recruitment*). Plasma membrane starts bending leading to flat membrane into a "clathrin-coated pit" (Mettlen and Danuser 2014). Assembling coat protein at cargo site promotes membrane bending (Pastan and Willingham 1985; Kirchhausen et al. 2014; Hawes et al. 1991; Schmid et al. n.d.), which transforms the flat plasma membrane into a "clathrin-coated pit" *(Membrane bending)*. With the help of BAR domain protein and Dynamin, CCP get pinched off leading to scission of vesicle *(scission)*. Finally, uncoating of vesicle delivers cargo to endosomes or other membrane-bound organelles *(uncoating)* (Mettlen et al. 2018).

Caveolae-Mediated Endocytosis

Caveolae ("little caves") are special flask- or omega-shaped, 50–100 nm wide plasma membrane invaginations found in most of the mammalian cells (Cohen et al. 2004; Filippini 2020). Caveolae were first described by George Palade in 1950 (Williams and Lisanti 2004; Kobayashi and Endoh 2003). Caveolae formation is primarily driven by membrane protein caveolins act as the major structural protein that binds directly to membrane cholesterol. In mammalian cells, three different proteins, i.e., caveolin-1 (CAV-1), caveolin-2 (CAV-2), and caveolin-3 (CAV-3), the first two involved caveolar formations in non-muscle cells and later one in muscle cells (Kobayashi and Endoh 2003). Caveolae are most abundant on endothelial cells (>10% of the plasma membrane area), adipocytes, skeletal muscle cells, and fibroblasts, however, they are totally absent in neurons. Caveolae are generated by the recruitment of soluble cytosolic protein, PTRF-cavin (Polymerase I and transcript release factor, Cavin also know as Cav-p60, Cavin) at the membrane (Kiss and Botos 2009). Four different types of cavin proteins are known to play role in caveolar formation: cavin1 (PTRF: polymerase I and transcript release factor), cavin2 (SDPR: serum deprivation protein response), cavin3 (PRKCDBP: Protein kinase C delta-binding protein), and cavin4 (MURC: muscle-restricted coiled-coil protein) (Kovtun et al. 2015). Knockout mice model of caveolin proven to be an important tool to functional studies of this protein where it has been shown that endothelial cells of these mice are unable to take serum albumin from blood

ascertaining its important role in endothelial cells (Razani et al. 2001). This endocytic process primarily enables albumin uptake, bacterial toxins such as cholera toxin, tetanus toxin, and uncoated polyoma and simian 40 (SV40) virus entry (Norkin and Kuksin 2005).

Macropinocytosis

Macropinocytosis ("cell drinking") process is an actin-dependent nonselective process, where plasma membrane ruffles and allows internalization of a large amount of fluid phase material (typically larger than 250 nm size) into large vesicle known as macropinosomes (Canton 2018; Swanson and King 2019). Macropinocytosis occurs in a variety of cell types, especially in those cells where other endocytic mechanisms such as clathrin- and caveolae-mediated endocytosis is not possible. Macropinocytosis is involved in various types of function in different cell types such as nutrient acquisition in Ras-transformed cancer cells (Commisso et al. 2013), participated in rapid turnover of cell surface integrin's in migratory cells (Gu et al. 2011), calcium-induced F-actin polymerization in growth cone collapse in nerve cell (Kabayama et al. 2009) and bulk membrane retrieval in the synaptic terminal of retinal bipolar cells (Holt et al. 2003). Many key regulators are involved in the regulation of macropinosomes formation such as Arf6, actin, small GTPases, Rac1, and Cdc42, as well as Rab5 demonstrating its link with other endocytic pathways (Kerr and Teasdale 2009).

Functions of Macropinocytosis in Different Cell Types

Macropinocytosis has been implicated as an event of extensive membrane ruffling to form macropinosomes to enclose fluid material or solute. Depending upon the cell type and organism, this process can be stimulated and accordingly classified into two types, i.e., induced (induced by growth factor in different cells) and constitutive (macrophages and dendritic cells), however, both types are driven by a common mechanism of action. Either constitutive or induced, macropinocytosis involves diverse functions as summarized below.

Role in Immune Surveillance

Heavy drinking is one of the important phenomena of macropinocytosis which can be equated up to 25% of the whole cell volume per minute (Freeman et al. 2020). Macropinocytosis plays a crucial role in making innate immune cells capable of providing immune defense. Cells of the innate immunity (dendritic cells and

macrophages) perform macropinocytosis which is directly linked with immune defense. Basically, macropinocytosis allows sampling of microenvironment for soluble antigens and consequently making peptide fragments available for presentation to both class I and class II major histocompatibility complex (MHC) molecules (von Delwig et al. 2006; Lim and Gleeson 2011) to initiate T cell response against a pathogen (17589544). MHC I and MHC II peptide complexes are recognized by CD8+ (cytotoxic) and CD4+ (helper) T cells, respectively. While induced and constitutive macropinocytosis both are playing important role in immune defenses, however, phagocytosis is generally linked with induced macropinocytosis by chemokines like CXC chemokine receptor (CXCR4) during HIV infection of the host cells (Tanaka et al. 2012). Macropinocytosis is not only a prominent feature only in macrophages and dendritic cells but it is also been recognized in a wide range of other immune players such as B and T cells (Garcia-Perez et al. 2012; Charpentier et al. 2020). Macropinocytosis is governed by different stimuli in cell type-specific manner with a unique molecular mechanism to fulfil specialized functions to perform.

Role in Fueling Cancer Cells

Due to the rapid cell division, expansion, and the abnormal vascular microenvironment, cancer cells acquire various metabolic adaptations to access the extracellular nutrients in a nutrient-poor, but protein-rich, microenvironment. Altered metabolic programs impart an additional advantage to cancer cells so that they can bypass nutrition limitations thereby fulfilling enhanced energy demands even in nutrient-deprived environments (Commisso and Debnath 2018). Notably, macropinocytosis is identified as an important scavenging strategy in which cancer cell adapt to produce macropinosomes by plasma membrane ruffling and internalization of extracellular fluid such which include proteins, liquids, and small particles (Commisso and Debnath 2018). The serum albumin uptake through macropinocytosis identified as a rich source of extracellular proteins in tumors (Jayashankar and Edinger 2020), which can help the tumor cell meet its metabolic and biosynthetic needs on multiple levels (Commisso and Debnath 2018). Activation of RAS and PI3K pathways are most common primary drivers for initiating macropinocytosis (Bar-Sagi and Feramisco 1986), however, other molecular drivers such as AMPK, PTEN, IGF1, and Lrp6 also play important role in various types of cancers. After the maturation of macropinosomes, it fuses with the lysosome, and thereby cargo protein is degraded into several amino acids and released (Xiao et al. 2021; Kamphorst et al. 2015). The efficacy of macropinosomes–lysosome fusion varies between cell types and tissue settings, and flux through the macropinocytic route, which determines its nutritional value (Ha et al. 2016).

Extracellular matrix (ECM) proteins are also ingested into tumor cells through macropinocytic absorption, in addition to serum proteins. In pancreatic ductal adenocarcinoma (PDAC), collagen fragments are taken up by cells, which further

break down and release collagen-derived proline, thereby helping cancer cells to survive under nutrient-depleted situations. macropinocytosis has been demonstrated to be a major source of amino acids in KRAS-mutant pancreatic tumors in vitro and in vivo *(*Commisso et al. 2013*)*. Among the six possible single-base missense KRAS mutations, i.e., G12V, G12C, G12A, G12S, and G12R that can occur at G12, only G12D is found to be driving macropinocytosis in PDAC (Hobbs et al. 2020). Limiting amino acid activity or direct mTORC1 inhibition promotes colocalization of macropinosomes–lysosome and breakdown of BSA, which enhances proliferation by boosting macropinocytic flux (Yoshida et al. 2015). Since inhibiting mTOR activity slows down anabolism and hence limits protein synthesis, it may also boost proliferation, to avoid a lethal bioenergy crisis when resources are scarce (Finicle et al. 2018).

Isotopic labelling technologies are useful tools in investigating protein scavenging in cancer cells (Ong et al. 2002). Necrotic cellular debris is labelled with "stable isotope labelling with amino acids in cell culture" (SILAC) as a food supply for unlabeled prostate cancer cells, and then uses mass spectrometry to determine the level of label incorporation into intracellular peptides. This approach permitted the determination that macropinocytosis can provide up to 70% of the biomass and intracellular amino acid pool in these prostate cancer cells by measuring the extent of peptide labelling (Commisso and Debnath 2018). Because increasing tumor necrosis is linked to increased aggressiveness and poor prognosis in people, investigating how macropinocytosis of necrotic debris enhances tumor biomass in prostate and other solid tumors in vivo is an important subject for future research (Commisso and Debnath 2018).

Role in Inducing Methuosis

While macropinocytosis plays a crucial role in cancer cell proliferation and provided amino acid supply from nutrient-deprived environment, it has also harmful to cancer cells as well. When the macropinocytosis balance gets disrupted, the macropinosomes start fusing with each other thereby forming large vacuoles, which ultimately led to tumor cell death and this process is known as "methuosis." Basically, the term methuosis is derived from the Greek word "Methuo," which means drink to intoxication). There are no certain markers present to biomarker identified for this phenomenon making it hard and difficult to mark and stain for any identification. It simply resembles large vacuoles. Methuosis phenotype can be defined as initial acceleration of macropinosomes formation followed by extreme cytoplasmic vacuolization, caused by dysfunctional trafficking of macropinosomes leading to cell death, which is different from morphology of apoptosis. Early stages of methuosis can be identified by late endosomal markers such as LAMP-1 and Rab7 (Donaldson et al. 2009; Racoosin and Swanson 1993). Ras is known as an important driver to induce methuosis which is modulate downstream effector GTPases, Rac1, and Arf6, thereby regulating macropinosome biogenesis and recycling in

glioblastoma cells (Bhanot et al. 2010). Differential sensitivity to methuosis is dependent on the level of macropinocytosis induction as well as on the degree of cell type specificity. Understanding the signaling mechanisms that trigger methuosis, not only benefit to macropinocytic field but also helpful in designing specific molecular targets which can be helpful in providing a cure of glioblastomas, and perhaps other types of rare cancer.

Conclusions

Macropinocytosis is an emerging area of research that can be linked with various pathological disorders. Seminal contribution of commisso Lab to identify macropinocytosis-mediated nutrient uptake in cancerous cells and Li Lab to understand the implication of macropinosomes in membrane recycling during metastasis-associated cancer cell migration (Li et al. 2020) are the important breakthrough for macropinocytosis field. Similarly, involvement of macropinocytosis in uptake and propagation of protein aggregates in neurodegenerative diseases (Zeineddine and Yerbury 2015) and inducing methuosis in glioblastoma cells underlines its diversified function in different cell types making this field an attractive area for novel therapeutics. These tend support the notion that we are just at the beginning to decrypt the diversified roles of macropinocytosis in various pathologies and continuous research in this area holds promise for many exciting discoveries in the coming days.

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Chapter 2
Macropinocytosis and Cancer: From Tumor Stress to Signaling Pathways

Guillem Lambies and Cosimo Commisso

Abstract Macropinocytosis is an evolutionarily conserved endocytic pathway that mediates the nonselective acquisition of extracellular material via large endocytic vesicles known as macropinosomes. In addition to other functions, this uptake pathway supports cancer cell metabolism through the uptake of nutrients. Cells harboring oncogene or tumor suppressor mutations are known to display heightened macropinocytosis, which confers to the cancer cells the ability to survive and proliferate despite the nutrient-scarce conditions of the tumor microenvironment. Thus, macropinocytosis is associated with cancer malignancy. Macropinocytic uptake can be induced in cancer cells by different stress stimuli, acting as an adaptive mechanism for the cells to resist stresses in the tumor milieu. Here, we review the cellular stresses that are known to promote macropinocytosis, as well as the underlying molecular mechanisms that drive this process.

Keywords Macropinocytosis · Cell metabolism · Nutrient uptake · Stress stimuli · Nutrient scarcity · Cancer malignancy

Introduction

Macropinocytosis, also known as "cellular drinking," is a clathrin-independent endocytic pathway that non-selectively internalizes extracellular cargo into large vesicles known as macropinosomes (Bloomfield and Kay 2016; Recouvreux and Commisso 2017; Palm 2019). This process has been observed in different cell types, for instance, in amoebae and *Drosophila* haemocytes, as well as in several mammalian cells including fibroblasts, monocytes, and epithelial cells (Bloomfield and Kay 2016; Palm 2019). Several functional roles have been attributed to macropinocytosis. For instance, this uptake pathway has been largely described to be involved in the immune defense response against foreign agents (Palm 2019; Liu

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and Roche 2015). In addition, some viruses and bacteria use macropinosomes to gain entry and infect the host cell (Mercer and Helenius 2009). Macropinocytosis also plays a key role in the remodeling of the plasma membrane (PM), allowing the reorganization of adhesion factors such as integrins that leads to the modulation of cell migration and signal transduction (Donaldson et al. 2009; Gu et al. 2011).

A recently appreciated role of macropinocytosis is in the regulation of mammalian cellular metabolism via nutrient acquisition, which was actually first observed in the unicellular organism *Dictyostelium discoideum* (Recouvreux and Commisso 2017; Palm 2019). Generally, cells can acquire nutrients from their microenvironment using specific transporters. One such example is the attainment of circulating glucose, which can be transported across the PM by the GLUT transporters *via* a facilitated diffusion mechanism (Navale and Paranjape 2016). However, the building blocks and small molecules required for cell growth and homeostasis are often contained within large macromolecules that cannot be acquired through cell transporters. Finding an alternative way to access these nutrients contained within these macromolecules is crucial for cell homeostasis and viability when the microenvironment is deficient in free nutrients. Macropinocytosis stimulation allows the intracellular obtention of these macromolecules and, as a consequence, access to the nutrients that are contained within them (Recouvreux and Commisso 2017; Palm 2019).

The process of macropinocytosis starts with PM protrusions driven by the actin cytoskeleton, which leads to the formation of cup-shaped structures known as membrane ruffles. These ruffles can then distally fuse with each other or fold back onto the PM, encapsulating the macromolecules from the extracellular space. The closure of these cup-shaped ruffles is what leads to the formation of the macropinosome. Once suspended in the cytosol, macropinosomes can fuse with the lysosomes, and upon compartmental acidification, lysosomal hydrolases become activated, parsing the internalized macromolecules into discrete nutrients (Swanson 2008; Racoosin and Swanson 1993). These nutrients, which can include amino acids, sugars, lipids, and nucleotides, are then used by cells to sustain anabolism and support growth and homeostasis. Overall, macropinocytosis is considered a key process for nutrient obtention by cells, supporting cell growth and survival. In line with its clear role in nutrient acquisition, tumor cells have been observed to exploit macropinocytosis in order to survive the nutrient-deficient conditions of the tumor microenvironment (Recouvreux and Commisso 2017; Davidson and Vander Heiden 2017; Commisso et al. 2013; Commisso and Debnath 2018; Kamphorst et al. 2015; Lee et al. 2019). Tumor cells utilize macropinocytosis as a nutrient stress compensation mechanism, and in some cases, the nutrient stress conditions that they encounter can promote macropinocytosis. In this way, tumor cells can survive and overcome different stresses.

Several stress stimuli have been observed to promote macropinocytosis. Nonetheless, it is important to note that many cells display constitutive macropinocytosis that is independent of a stimulus. Constitutive macropinocytosis has been observed to be a general trait of some malignant cancer cells that harbor oncogenic mutations in H-Ras, v-Src, or K-Ras (Commisso et al. 2013; Schmees et al. 2012; Veithen et al.

1996; Kasahara et al. 2007). In addition, nutrient-independent uptake occurs in non-tumor cell types, such as immature dendritic cells (DCs) and macrophages (Bohdanowicz et al. 2013; Choi et al. 2009). In this system, constitutive macropinocytosis depends on the presence of extracellular calcium (Canton et al. 2016). In cancer, constitutive macropinocytosis is thought to represent a metabolic adaptation that selects for the most fit tumor cells, fueling unrestricted tumor growth (Recouvreux and Commisso 2017). However, malignant cancer cells can also display inducible macropinocytosis, as observed in pancreatic ductal adenocarcinoma (PDAC) (Lee et al. 2019). While the molecular and genetic traits that define inducible versus constitutive macropinocytosis are not fully understood, both forms are thought to have some commonalities in terms of mechanisms that will be elaborated on throughout this chapter. A main difference is that inducible uptake requires a stimulus to promote the macropinocytic cascade, whereas in the case of constitutive uptake a trigger is not required, although it has been observed in some circumstances that a stimulus can further increase the macropinocytic activity in the constitutive system (Lee et al. 2019). However, whether this enhancement causes some relevant additional biological effect relative to basal conditions remains to be elucidated. The most well-known stress stimulus that promotes macropinocytosis is nutrient scarcity, which is a classical feature of tumors that display regions of poor vascularization (Farnsworth et al. 2014). Besides nutrient scarcity, there are also other stress conditions that stimulate macropinocytosis, such as hypoxia or oxidative stress (Kamphorst et al. 2013; Ackerman et al. 2018; Ghoshal et al. 2017; Singla et al. 2018). Specific stimuli and associated mechanisms that promote macropinocytosis under such conditions will be expanded upon below.

Nutrient Deprivation as a Driver of Macropinocytosis

A critical microenvironmental stress that promotes macropinocytosis in cancer cells is nutrient deficiency (Commisso et al. 2013). In such nutrient scarcity conditions, tumor cells stimulate this process resulting in the uptake of serum albumin (Fig. 2.1) (Commisso et al. 2013; Kamphorst et al. 2015), which is the most abundant protein in plasma and other physiological fluids (Merlot et al. 2014). The uptake of serum albumin by macropinocytosis is a strategy used for tumor cells to obtain non-biosynthetic sources of amino acids. Moreover, considering that in a physiological setting albumin acts as a carrier protein, its macropinocytic uptake additionally allows for the acquisition of albumin-bound molecules, such as fatty acids (FA) and cholesterol, which are essential molecules for maintaining the integrity of cellular membranes (Commisso and Debnath 2018). Thus, internalizing albumin by macropinocytosis is a very effective strategy for tumor cell survival in nutrient-scarce stress conditions. On the other hand, the uptake of serum proteins may not be the only option for overcoming the stress generated by a nutrient-deprived environment. For example, the internalization of extracellular proteins from the extracellular matrix (ECM), such as fibronectin and collagen, has been described in PDAC

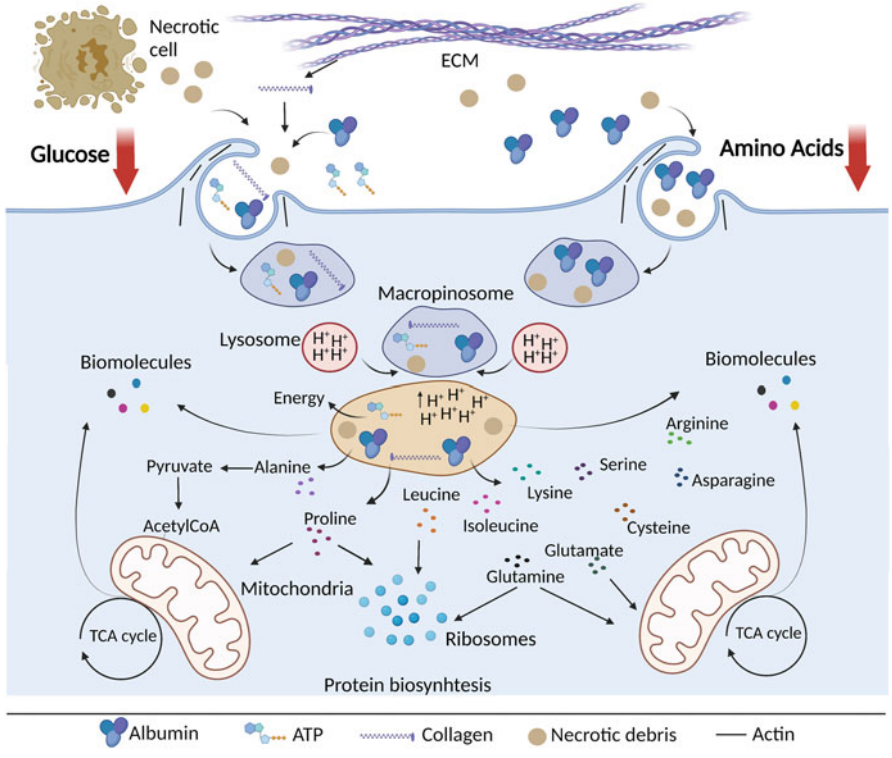

Fig. 2.1 Schematic representation of macropinocytosis as promoted by glucose or amino acid deficiency. In different cell-based systems, the macropinocytic process is stimulated by nutrient stress stimuli, such as the deprivation of microenvironmental glucose or amino acids. (Left) In low glucose conditions, macropinocytosis becomes activated to compensate for the absence of glucose. The uptake of serum albumin permits the acquisition of alanine, which is used by the cell for pyruvate production that drives the TCA cycle, generating intermediates that are essential for the de novo synthesis of biomolecules. The de novo production of biomolecules in these conditions can also be achieved through the uptake of collagen from the ECM. This in turn increases the intracellular levels

(Davidson et al. 2017; Olivares et al. 2017). This indicates that cancer cells, besides obtaining nutrients from the uptake of serum proteins in the tumor interstitial fluid, might also catabolize ECM proteins from their immediate environment to keep up with their high energy demands (Fig. 2.1). Overall, the uptake of extracellular proteins is an effective way to maintain the supply of nutrients to a tumor. Nevertheless, proteins are not the only molecules that can be taken up *via* macropinocytosis. One example is the uptake of extracellular ATP, as was observed in a model of lung cancer (Qian et al. 2014). The addition of extracellular ATP to lung cancer cells exposed to a nutrient stress stimulus increases the levels of intracellular ATP, indicating that extracellular ATP is internalized by cells using a specific mechanism. Since ATP cannot cross the plasma membrane due to its hydrophilic nature, and no specific membrane-associated ATP transporter has been identified, it was speculated that an endocytic process modulates this uptake. It was then determined that, in these conditions, the specific activation of macropinocytosis promotes the uptake of extracellular ATP (Fig. 2.1), underscoring the role of this endocytic pathway as a cell survival mechanism in a nutrient-deprived environment (Qian et al. 2014). In addition, the macropinocytic uptake of ATP has also been observed to increase the metastatic properties of lung cancer cells, since it modulates several signaling events that drive epithelial-to-mesenchymal transition (EMT) (Cao et al. 2019), which further reinforces the protumoral role of macropinocytosis.

In addition to extracellular proteins and ATP, other nutrient-rich cargo can also be taken up by cancer cells *via* macropinocytosis. During tumor growth, poor vascularization in regions of the tumor can lead to oxygen and nutrient depletion that is often accompanied by an increase in cell necrosis (Weis and Cheresh 2011). However, this increase in cell necrosis can counter-intuitively benefit tumor growth and progression. Necrotic debris can serve as a rich source of biomolecules that are required for cancer cell growth, such as proteins, FA, and triglycerides (TAG), and this debris, unlike apoptotic bodies, is small enough to be engulfed by macropinosomes (Kim et al. 2018). Internalization of this necrotic debris by macropinocytosis (Fig. 2.1), a phenomenon recently coined "necrocytosis," has been observed to sustain the growth of PDAC, prostate and breast cancer cells (Kim et al. 2018; Jayashankar and Edinger 2020; King et al. 2020). The advantage

Fig. 2.1 (continued) of proline, which through its conversion to glutamate, replenishes the intermediates of the TCA cycle (anaplerosis). In addition, proline also fuels the protein synthesis machinery. When glucose is limiting, biomolecules can also be obtained in their final form through the uptake of necrotic cell debris (necrocytosis). Furthermore, macropinocytosis mediates the uptake of extracellular ATP to help cells meet their energy demands when glucose is absent. (Right) In the context of amino acid deprivation, the uptake of serum albumin allows for the production of different amino acids that are critical for cell growth and viability, such as glutamine. This process supports anaplerosis and protein synthesis, which is sustained by protein-derived leucine. With amino acid deprivation, necrocytosis might allow for the obtention of biomolecules, in this way compensating for the metabolic stress caused by an amino acid-poor tumor microenvironment

of obtaining nutrients from necrotic debris is that not only would free amino acids be produced to support anabolic pathways, but it would also permit the acquisition of the end-products of these anabolic reactions. For instance, lipid droplets in prostate cancer cells store FA and cholesterol, which are crucial for maintaining tumor growth and progression, and their loss strongly compromises these features (Kim et al. 2018; Yue et al. 2014). In a low glucose and low amino acid tumor microenvironment, lysosomal degradation of these lipid droplets is increased to fuel mitochondrial metabolism. Under these conditions, cells stimulate necrocytosis to reconstitute these lipid droplets using the necrotic debris, allowing prostate cancer cells to survive and maintain their proliferative capacity (Kim et al. 2018).

The relevance of necrocytosis has also been described in other types of cancer, such as breast cancer. In nutrient-depleted conditions, breast cancer cells stimulate macropinocytosis for albumin uptake, but they can also obtain sugars, lipids, and nucleotides through the engulfment of necrotic bodies (Jayashankar and Edinger 2020). The uptake of macromolecules by necrocytosis in this type of cancer is not only important for sustaining tumor growth and progression, but it is also a resistance mechanism against chemotherapeutic agents that are antimetabolites, as observed with the nucleotide synthesis inhibitors 5-FU and gemcitabine (Jayashankar and Edinger 2020). Altogether, the macropinocytosis of cell debris is an effective tool for nutrient acquisition when supplies are low in the tumor microenvironment, and further understanding of how this process is modulated would be beneficial when developing future cancer therapies.

Amino Acid Scarcity and Macropinocytosis

Tumor cells are reliant on high levels of nutrients to support elevated bioenergetic demands, the production of macromolecules, and maintain their growth and survival. Generally, this is achieved through the utilization of molecules in their monomeric form, such as free amino acids and glucose (Palm et al. 2015). In the tumor microenvironment, amino acids are mainly found incorporated into proteins, such as serum albumin, as opposed to their free form. This makes extracellular proteins a rich source of nutrients for cells to use to support their metabolic demands. Thus, in an environment depleted of free amino acids, macropinocytosis becomes critical to sustaining cell growth and survival (Commisso et al. 2013; Palm et al. 2015; Thompson 2011; Nofal et al. 2017). Cancer cells are dependent upon the amino acid glutamine as a vital nutrient to sustain their proliferation and growth. Glutamine metabolism in cancer cells supports anabolic processes and biomass formation, which are indispensable for tumor growth and progression. Glutamine is used to replenish the metabolite intermediates of the tricarboxylic acid cycle (TCA) (Fig. 2.1), which are then used for the generation of the biosynthetic precursors leading to the production of lipids and nucleotides, a process known as anaplerosis (Chen et al. 2018; Daye and Wellen 2012). Therefore, cancer cells,

particularly those expressing oncogenic Ras mutations, exhibit high sensitivity to glutamine deprivation (Commisso et al. 2013; Jayashankar and Edinger 2020).

This increased sensitivity of cancer cells to glutamine deprivation was confirmed in PDAC cells (Commisso et al. 2013). When these cancer cells were cultured in vitro in glutamine-starved conditions, a clear reduction in cell viability was observed, which validates the essential role of glutamine in the maintenance of PDAC cell growth. However, when an exogenous source of albumin was added to the medium, the growth of these cells was rescued, indicating that the presence of extracellular albumin compensates for glutamine deficiency. Importantly, when cells were treated with the Na^+/H^+ exchange inhibitor 5-[N-ethyl-N-isopropyl] amiloride (EIPA), which specifically blocks macropinocytosis, the rescue in cell viability was abolished. This indicates that, under such unfavorable conditions, cancer cells compensate for the lack of glutamine through the uptake of extracellular albumin, and this is achieved *via* macropinocytosis. In vivo studies further validated these data, since macropinocytosis inhibition in PDAC tumors showed a clear tumor growth reduction, indicating that macropinocytosis supports tumor growth (Commisso et al. 2013). These data were consistent with the notion that PDAC cells use macropinocytosis to supply glutamine when glutamine is limiting. Underscoring this concept was the observation that glutamine is the most depleted amino acid in human pancreatic tumors relative to adjacent benign tissue (Kamphorst et al. 2015). Thus, macropinocytosis allows tumors to meet their glutamine demands, in this way, sustaining tumor growth and progression.

The uptake of serum albumin *via* macropinocytosis by tumor cells was later directly demonstrated in vivo (Davidson et al. 2017). This was studied in PDAC tumors, where endogenous albumin was exchanged with a heavy isotope-labeled form of albumin in *PDX-Cre; K-Ras$^{G12D/+}$; p53$^{loxP/loxP}$* (KPC) mice, a mouse model that spontaneously develops PDAC (Commisso et al. 2013; Davidson et al. 2017; Westphalen and Olive 2012). In this study, it was observed that KPC mice showed an increased accumulation of labeled albumin in PDAC tumor tissue compared to normal pancreas tissue, indicating that albumin uptake is higher in tumors. Similar to this, the presence of labeled albumin-derived amino acids was also increased in the tumors, indicating that the catabolism of serum albumin to produce free amino acids is a selective feature of tumors and not normal tissue. Supporting previous studies, it was then observed that the uptake of serum albumin by tumor cells in this model is achieved through macropinocytosis, since a clear decrease in free amino acids was observed when tumor-bearing mice were exposed to EIPA (Davidson et al. 2017). Altogether, these in vivo approaches further corroborate the role of macropinocytosis in supporting PDAC progression.

Although the macropinocytic uptake of serum albumin is essential to provide protein-derived glutamine to tumor cells, other amino acids are also obtained through this process. In a model in which mouse embryonic fibroblasts (MEFs) harboring oncogenic K-Ras mutations were subjected to environments presenting different amino acid deficiencies, cells displayed reduced cell growth and viability, which was especially pronounced when essential amino acids (EAA) were depleted. When albumin was supplemented to media deficient in EAAs, it strongly rescued

cell growth in these starved cells. This rescue effect of albumin included the selective depletion of the EAA leucine(Palm et al. 2015), which is the EAA predominantly found in serum albumin. Leucine is required for maintaining cell growth and survival since, among other functions, it is used as a substrate for protein synthesis (Anthony et al. 2001). Similar rescue effects of albumin were observed when other individual EAAs (isoleucine, arginine, or lysine) were depleted from the media. Importantly, no rescue was observed when macropinocytosis was pharmacologically inhibited, indicating that this increase in cell survival is due to the macropinocytic uptake of albumin, which in turn supplies the cell with protein-derived leucine and other EAAs. All these indicate that cells stimulate macropinocytosis for EAA acquisition when these are low in the microenvironment (Fig. 2.1).

Do amino acid deficiencies lead to macropinocytic stimulation? The answer is yes, but the ability for amino acid deprivation to regulate macropinocytosis might be context dependent. In some situations, macropinocytosis is constitutive and independent of the environmental amino acid content (Commisso et al. 2013; Lee et al. 2019). However, the ability of amino acid depletion to stimulate macropinocytosis seems to be most relevant in settings where cancer cells display low baseline levels of macropinocytic activity when nutrients are plentiful (Lee et al. 2019). The promotion of macropinocytosis by glutamine starvation has been remarkably observed in PDAC (Commisso et al. 2013; Lee et al. 2019). In tumors derived from PDAC cells displaying low basal levels of macropinocytosis, in vivo macropinocytic rates were enhanced in tumor cores, which are the tumor regions most deficient in nutrients. It was determined that glutamine was the specific amino acid deficiency that promotes macropinocytosis in PDAC cells. When cells were deprived of glutamine, an increase in protein scavenging was observed, and this was not reversed when other amino acids were added to the system (Lee et al. 2019). On the other hand, when the downstream glutamine metabolites glutamate and α-ketoglutarate were added to the glutamine-deprived environment, the effects on macropinocytosis were rescued. This indicates the ability of PDAC cells to stimulate macropinocytosis specifically under conditions where glutamine is limiting, and this was further supported when glutamine metabolism was inhibited by the glutamine analog 6-Diazo-5-oxo-L-norleucine (6-DON), since it induced macropinocytosis to a similar extent as glutamine deprivation. Moreover, NEAA starvation in an environment containing glutamine did not induce macropinocytosis, further indicating the specific role of glutamine in this process (Lee et al. 2019).

In addition to glutamine, the acquisition of protein-derived proline *via* macropinocytosis might also be a key feature for sustaining cell growth (Olivares et al. 2017). Similar to glutamine, proline metabolism generates glutamate as a metabolic intermediate (Liu et al. 2012). PDAC cells have been observed to increase the intracellular levels of proline through the macropinocytic uptake of collagen from the ECM (Fig. 2.1) (Olivares et al. 2017), since proline represents approximately 10% of the amino acid composing the collagen molecule (Karna et al. 2020). However, with glutamine starvation, collagen uptake for proline production is likely to be predominantly regulated by a macropinocytosis-independent process, since when macropinocytosis was inhibited in PDAC cells starved of glutamine, collagen

internalization was not prevented (Olivares et al. 2017). Instead, collagen might be internalized into the cell via endocytosis through the collagen receptor uPARAP/Endo180, a process often enhanced in tissues with active ECM remodeling (Melander et al. 2015). This suggests that in PDAC cells exposed to glutamine-starved conditions, different endocytic pathways can contribute to collagen uptake. Whether this occurs in vivo remains to be elucidated.

Macropinocytosis in the Setting of Glucose Deprivation

A low glucose environment has also emerged as a macropinocytic promoter in different systems (Olivares et al. 2017; Qian et al. 2014; Gwinn et al. 2008; Hodakoski et al. 2019). As previously mentioned, intermediate metabolites that drive nutrient biosynthesis are obtained by glutamine anaplerosis, but they can also be obtained by glucose metabolism. Thus, cancer cells enhance the uptake of glucose from the environment to increase their glycolytic rate for metabolic intermediate production and for the production of ATP (Daye and Wellen 2012). Nevertheless, poor glucose availability has been detected in the microenvironment of several tumors (Urasaki et al. 2012; Gullino et al. 1967), invoking the need of an alternative way to support tumor growth. Stimulation of extracellular protein uptake by macropinocytosis might compensate for the lack of glucose in some systems, as it has been reported to in non-small cell lung cancer (NSCLC) (Hodakoski et al. 2019). In this model, alanine was important (Fig. 2.1), since the conversion of alanine to pyruvate by alanine transferase-2 promotes the generation of glycolytic intermediates, this way compensating for the absence of glucose. On the other hand, one of the main consequences of a glucose-deprived microenvironment is a reduction in the intracellular ATP content. Therefore, when glucose is reduced, cells are forced to obtain ATP through an alternative mechanism, possibly through the macropinocytic uptake of ATP (Fig. 2.1), as previously mentioned (Qian et al. 2014). Thus, in some settings, nutrient stress promoted by glucose starvation might lead to macropinocytosis stimulation for ATP obtention, in this way, compensating for the low cellular glycolytic rates.

Stress caused by glucose deprivation has also been observed to promote macropinocytosis in some cells. Assessments in MEFs deficient for the PTEN phosphatase have shown that glucose starvation alone is sufficient to trigger macropinocytosis induction, but depletion of amino acids is not (Kim et al. 2018). In this setting, glucose starvation is able to directly activate the macropinocytic modulator AMPK, and the cooperation between PTEN deficiency and AMPK activation has emerged as a key event for macropinocytosis stimulation (Kim et al. 2018). In other systems, such as PDAC, glucose starvation is the specific stimulus that promotes the macropinocytic uptake of collagen from the ECM for protein-derived proline production (Fig. 2.1), supplying in this context the nutrients required for cell growth. This is in contrast to glutamine deficient conditions, since in PDAC cells deprived of glutamine the uptake of collagen is achieved independent of

macropinocytosis (Olivares et al. 2017). Interestingly, glucose starvation has not been observed to substantially promote macropinocytosis in PDAC tumor cells nor in pancreatic cancer-associated fibroblasts (CAFs) (Lee et al. 2019; Zhang et al. 2021), both settings where glutamine deprivation is a very strong macropinocytosis inductor. Altogether, these observations show that nutrient stress stimuli can display different effects, suggesting that multiple factors are involved in determining how and when a stress stimulus promotes macropinocytosis. Identification of these multiple factors will be critical to better understand how macropinocytosis is regulated.

Macropinocytosis and Oxidative Stress

The generation of reactive oxygen species (ROS) has been observed to modulate the uptake of antigens via macropinocytosis in DCs (Singla et al. 2018). ROS are often generated as a byproduct of several biochemical reactions that take place in different cellular compartments, including the mitochondria and peroxisomes (Balaban et al. 2005; Schrader and Fahimi 2004), and they oxidize sugars, lipids, nucleotides, and proteins (Pizzimenti et al. 2010). This alters the integrity of these molecules, promoting cell stress and damage. In addition, it has also been observed that there are families of enzymes that generate ROS as the primary enzymatic function, not as a byproduct, and that this is mainly observed in a context of host defense response (Ma 2013). In these cases, the production of ROS is a benefit, since reduced ROS production correlates with a reduced leukocyte response. Besides the role of ROS in the modulation of host defense responses, ROS also regulate several processes, such as the activation of kinases, cell senescence and growth, among others (Djordjevic et al. 2005; Colavitti and Finkel 2005). One of the most well-known family of enzymes that generates ROS as a final product are the NADPH oxidases (NOX) (Bedard and Krause 2007). Interestingly, NOX2 has been recently identified as a modulator of antigen uptake in DCs by macropinocytosis, actively participating in the host cell defense response (Singla et al. 2018). In conclusion, ROS has emerged as a potent inductor of macropinocytosis in DC.

In the context that we have described above, the production of ROS is not linked to a cell stress stimulus, since it is rather produced as a cellular messenger. Whether oxidative stress can promote macropinocytosis in cancer has largely remained unknown. Nevertheless, a recent study has demonstrated that this type of stress stimulus can actually activate the macropinocytic program (Su et al. 2021). To survive nutrient scarcity, several types of cancers often display high levels of autophagy, a conserved pathway that controls cellular homeostasis by promoting the lysosomal degradation of damaged cell compartments (Mizushima and Komatsu 2011; Karsli-Uzunbas et al. 2014). Degradation of the associated intracellular macromolecules leads to the release of amino acids and different biomolecules that

are used by tumor cells to support cell growth (Karsli-Uzunbas et al. 2014; Onodera and Ohsumi 2005). Thus, autophagy has emerged as an interesting target for anticancer therapies. Nevertheless, clinical inhibition of autophagy has shown low efficacy in cancer treatment (Su et al. 2021; Karasic et al. 2019), which suggests that autophagy contribution to tumor growth and progression might be redundant or compensated for through other mechanisms. In PDAC, it has been recently observed that, when autophagy is inhibited, cells compensate for this loss through the promotion of macropinocytosis (Su et al. 2021). In this way, tumor cells bypass autophagy and obtain their nutrients from the degradation of extracellular proteins. This switch from autophagy to macropinocytosis is mediated by the nuclear factor erythroid-2 related factor 2 (NRF2), which promotes the transcription and expression of several pro-macropinocytic factors, thus activating the macropinocytic program (Fig. 2.2) (Su et al. 2021). In oxidative stress, NRF2 is the master transcription factor that becomes activated. This transcription factor associates with DNA regions termed Antioxidant Response Elements (AREs), which are specifically located in the promoters of genes that encode detoxification enzymes, such as glutathione *S*-transferase A2 (GSTA2) and NADPH quinone oxidoreductase 1 (NQO1) (Li et al. 2019; Ma 2013; Nguyen et al. 2009). Induction of these enzymes leads to the detoxification and elimination of ROS, alleviating in this way the cellular damage caused by ROS. Due to the relevance of NRF2 in activating the macropinocytic program when autophagy is inhibited, it was posited that any stimulus that mediates NRF2 activation can lead to macropinocytosis promotion. This hypothesis was tested and validated when oxidative stress and, as a consequence, NRF2 activation, were induced in PDAC cells using H_2O_2. NRF2 activation in this context caused an increase in macropinocytic activity, which establishes oxidative stress as a stress stimulus that can promote macropinocytosis.

Although the NRF2 study has provided the first evidence that oxidative stress can directly induce macropinocytosis, a relationship between these two features had already been established in other systems, such as in lung cancer (Seguin et al. 2017). In this model, it was observed that lung cancer cells harboring *KRAS* mutations modulate anchorage-independent cell survival through the promotion of macropinocytosis and the maintenance of redox homeostasis (Seguin et al. 2017). Disrupting K-Ras function in lung cancer leads to a reduced macropinocytic capability and to increased production of ROS. These cellular consequences might be due to impaired activation of NRF2, which is known to be activated by oncogenic K-Ras (Fig. 2.2) (Tao et al. 2019). It is conceivable that in this particular system, NRF2 promotes the expression of detoxification enzymes to eliminate reactive species and parallelly activates the macropinocytic cascade to replace the molecules that have been damaged by ROS. This model further links ROS production to macropinocytosis and future work is required to better elucidate the role of NRF2 in the activation of the macropinocytic program.

Macropinocytosis and Hypoxia

Hypoxia is defined as a non-physiological oxygen tension phenomenon that, in the context of cancer, is associated with tumor malignancy (Muz et al. 2015). Similar to the cellular stress triggered by ROS, most studies, until recently, have indirectly linked hypoxia to the macropinocytic process. Different studies have largely suggested that disruption of the cellular homeostasis that takes place during hypoxia might be in part compensated for through the activation of macropinocytosis. For example, the biosynthesis of some biomolecules, mainly FA, is compromised in hypoxia and cells might compensate for this by internalizing albumin-bound lipids via macropinocytosis (Kamphorst et al. 2013; Ackerman et al. 2018). Direct macropinocytosis stimulation by hypoxia has been recently described in PDAC and, similar to oxidative stress, this process is executed in a NRF2-dependent manner (Balaban et al. 2005). How is NRF2 linked to hypoxia? The response to hypoxia by tumor cells is primarily modulated by the transcription factors Hypoxia Inducible Factors 1 and 2 (HIF-1 and HIF-2) (Keith et al. 2011), although other pathways that facilitate tumor progression can become activated in hypoxic conditions, such as NFKappaB (Koong et al. 1994). In normoxia, HIF transcription factors are hydroxylated on proline and asparagine residues by prolyl-hydroxylases (PDH) that use O_2 and α-ketoglutarate as substrates. HIF hydroxylation induces association with the Von Hippel–Lindau (pVHL) complex, which promotes the recruitment of an E3-ligase that targets HIF for proteasomal degradation (Semenza 2013). In hypoxia, HIF hydroxylation is prevented and this mediates its stabilization and subsequent association to Hypoxic Response Elements (HRE) that are located in the promoters of several target genes involved in cell survival, angiogenesis, invasion, and metastasis (Lee et al. 2020), promoting their transcription and thus facilitating tumor growth and progression. NRF2 is one of the main factors known to mediate HIF stabilization (Toth et al. 2017; Kuper et al. 2021; Oh et al. 2016; Hawkins et al. 2016). It was observed in colorectal cancer that the detoxification enzyme NQO1, whose transcription is activated by NRF2, physically interacts with HIF-1 and this interaction reduces the association of HIF-1 with PDH, promoting HIF-1 stabilization (Oh et al. 2016). On the other hand, in induced pluripotent stem cells, it has been postulated that NRF2 increases the levels of HIF through the activation of thioredoxin (Hawkins et al. 2016). Altogether, these studies show that NRF2 activation facilitates the stabilization of HIF transcription factors, modulating the hypoxic response.

In hypoxia, one of the most remarkable consequences of HIF activation in cancer is the reprogramming from oxidative to glycolytic metabolism, a phenomenon known as the Warburg effect (Semenza 2013). Mechanistically, HIF associates with Pyruvate Kinase M2, which functions as a coactivator promoting HIF transactivation of specific targets. These targets include the glucose transporter GLUT1, which increases the uptake of glucose, and lactate dehydrogenase A (LHDA), which controls lactate biosynthesis. Additionally, HIF also affects expression of pyruvate dehydrogenase kinase 1 (PDK1), suppressing pyruvate

dehydrogenase (PDH), blocking the synthesis of Acetyl-CoA and inhibiting oxygen consumption (Luo et al. 2011). As previously mentioned, this metabolic reprogramming impairs the synthesis of FA, since Acetyl-CoA is necessary for their de novo synthesis (Kamphorst et al. 2013). In addition, FA biosynthesis requires the action of stearoyl-CoA desaturase-1 (SCD1), an enzyme often overexpressed in tumors which generates monounsaturated FA in an oxygen-dependent manner (Hess et al. 2010). The lack of Acetyl-CoA and the inhibition of SCD1 in hypoxia force tumor cells to obtain FA from an alternative source, such as macropinocytosis. It has been postulated that Ras-transformed cells are able to overcome the lack of FA biosynthesis in hypoxic conditions through the direct uptake of serum lipids with a single fatty acid tail, known as lysolipids (Kamphorst et al. 2013). The direct scavenging of these lysolipids might be considered an alternate route for FA obtention in hypoxia, making Ras-transformed cells resistant to PDH and SDC1 inhibition (Kamphorst et al. 2013). Thus, it is not surprising that, in hypoxic conditions, Ras-transformed cells activate macropinocytosis, likely via NRF2, to mediate uptake of lysolipids as an alternative way for FA obtention. This would further underscore the protumorigenic role of macropinocytosis, further highlighting its attractiveness as a target for cancer treatment.

Signaling Pathways That Regulate Macropinocytosis in the Context of Tumor Stress

Deciphering the molecular mechanisms that modulate macropinocytosis is crucial for understanding how macropinocytosis functions in the context of a tumor microenvironment with different stresses. In this section, we will discuss the most relevant factors that orchestrate the macropinocytosis pathway in cancer, paying particular attention to the specific contextual roles that these factors play in the regulation of this biological process.

Growth Factors and Cytokines

Ras-mutant tumor cells with low basal levels of macropinocytosis (inducible cells) require a nutrient stress-driven stimulus to activate this process. One way for cells to induce macropinocytic uptake is through growth factors, such as Epidermal Growth Factor (EGF), which associates with and activates its receptor EGFR (Fig. 2.2), instigating several intracellular signaling events that orchestrate the macropinocytic process (Araki et al. 2007; West et al. 1989). Macropinocytosis induced by EGF/EGFR has been described in several different systems. For instance, it was observed that when inducible PDAC cells are exposed to a glutamine-poor environment, EGF ligands are upregulated and activate EGFR, setting into motion the

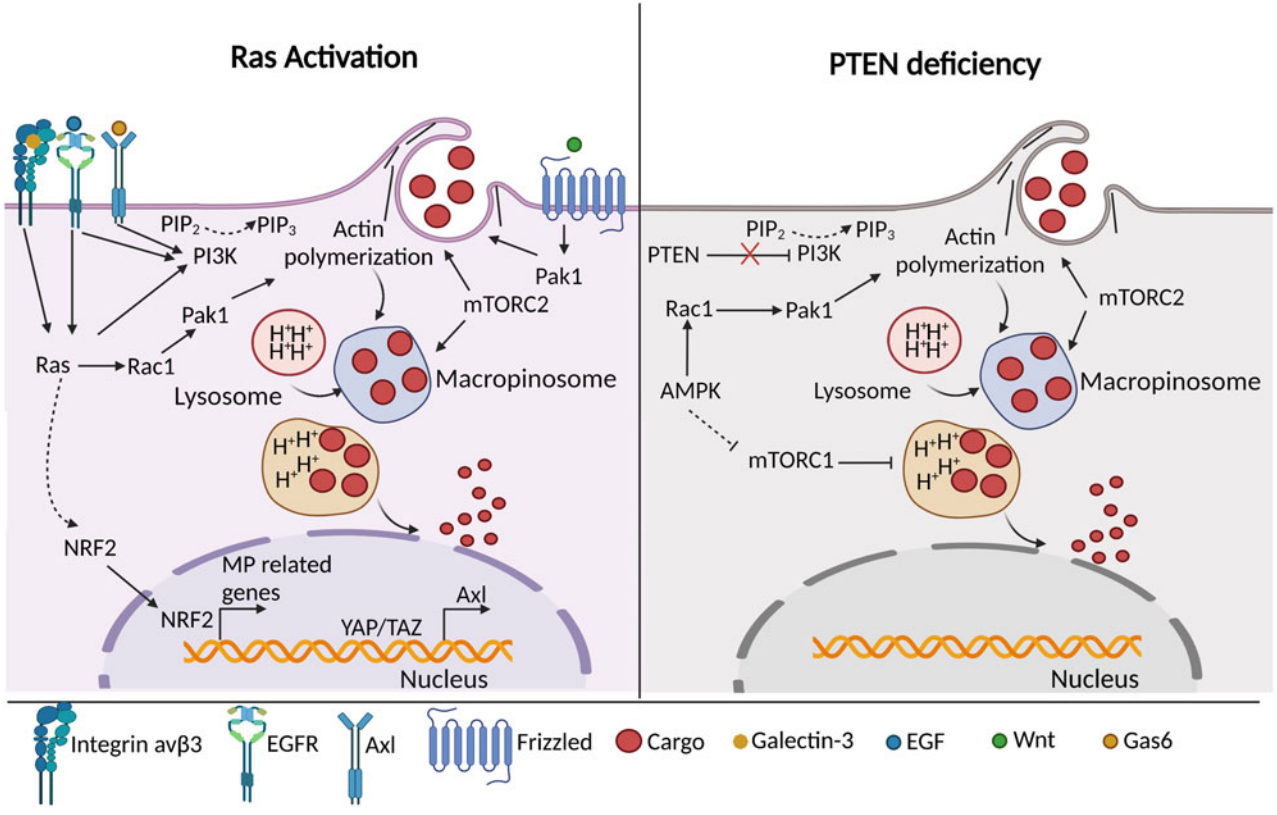

Fig. 2.2 Mechanisms modulating macropinocytosis in the context of Ras activation or PTEN deficiency. Activation of Ras by growth factors such as EGF, or through its recruitment to the plasma membrane by the integrin avβ3-Galectin-3 complex, lead to macropinocytosis stimulation via downstream effectors. Activation of PI3K by Ras mediates the conversion of PIP_2 to PIP_3, a key step for promoting the formation and closure of the macropinosome. In this context, PI3K can also be activated independently of Ras by growth factors (EGF) or Gas6, which activates the Axl receptor whose transcription is modulated by YAP

events that regulate the macropinocytic cascade (Lee et al. 2019). In addition, EGF activates macropinocytosis in PDAC cells to mediate the uptake of exosomes (Nakase et al. 2015), and in breast cancer cells, EGF mediates the macropinocytic uptake of the E-Cadherin–catenin complex (Bryant et al. 2007). Besides EGF, other growth factors and cytokines have been described to activate macropinocytosis. For instance, the Growth Arrest-specific Protein 6 (Gas6) that binds and activates the Axl receptor (Fig. 2.2) and promotes macropinocytosis in PDAC (Hess et al. 2010), the Platelet Derived Growth Factor (PDGF) (Wennstrom et al. 1994), the Macrophage Colony Stimulated Factor (M-CSF) that stimulates macropinocytosis in bone marrow-derived macrophages (BMM) (Racoosin and Swanson 1989), and also the chemokine CXCL12, which drives macropinocytosis in hepatocarcinoma cells (Cepeda et al. 2015). All these different factors have the potential to activate different signaling pathways that lead to the promotion of macropinocytosis. In all likelihood, most of the downstream aspects of these pathways might be active in the context of constitutive macropinocytosis; although, in these systems, they might escape the modulation by the growth factors and cytokines.

Ras, Rac, and PI3K

The small GTPase Ras has been identified as one of the most relevant modulators of macropinocytosis in several cancer types, including lung, pancreas, bladder, and breast (Commisso et al. 2013; Jayashankar and Edinger 2020; Seguin et al. 2017). There are three Ras isoforms encoded for by three different genes, the Kirsten rat sarcoma viral oncogene homolog (*KRAS*), neuroblastoma RAS viral oncogene homolog (*NRAS*), and Harvey rat sarcoma viral oncogene homolog (*HRAS*) (Hobbs et al. 2016). Oncogenic alterations in *KRAS* are the most typically found Ras mutations in cancer and these are predominant in lung, pancreas, and colorectal cancer. N-Ras mutations are found in melanoma and acute leukemias, while mutations in H-Ras are predominant in head and neck squamous cell and bladder carcinomas (Waters and Der 2018). Oncogenic *KRAS* mutations are nearly universal in PDAC and *KRAS* has been shown to be required for both the constitutive and

Fig. 2.2 (continued) and TAZ, inducing macropinocytosis . Rac1 GTPase also becomes active, which in turn activates Pak1 to promote actin polymerization. Alternatively, in the context of oncogenic Ras, Pak1 can also be activated by the Wnt pathway. In Ras-mutant cells, macropinocytosis might also be mediated through the activation of NRF2, which stimulates the transcription of macropinocytosis-related genes. Besides Ras activation, the loss of PTEN phosphatase, which counteracts PI3K function, has emerged as a strong macropinocytic promoter. In PTEN-deficient conditions, AMPK controls macropinocytosis through the activation of Rac1, and might partially suppress mTORC1, which would further enhance uptake. In contrast to mTORC1, activation of mTORC2 in PTEN-deficient and Ras activation conditions positively modulates the macropinocytic cascade

inducible forms of macropinocytosis (Commisso et al. 2013; Lee et al. 2019). It was observed in tumors derived from a subset of PDAC cell lines that cells located in the non-peripheral regions of the tumors, which mostly consist of the nutrient-deprived tumor cores, show enhanced levels of macropinocytosis relative to the tumor peripheries (Lee et al. 2019). The inducible versus constitutive macropinocytosis phenotype did not correlate with a particular *KRAS* mutation, indicating that multiple factors and mechanisms are involved in determining uptake status. Clearer though is the fact that an oncogenic *KRAS* mutation is required to drive either form of macropinocytosis and that *KRAS* wild-type PDAC cells do not engage in macropinocytic induction.

To promote macropinocytosis, oncogenic Ras activates downstream signaling pathways, such as Rac1, a Rho GTPase that is known to modulate the actin cytoskeleton, as well as PI3K (Fig. 2.2) (Recouvreux and Commisso 2017). The activation of Rac1 is one of the best described mechanisms for promoting macropinocytosis (Ridley et al. 1992). Rac1 activation is sufficient to induce macropinocytosis, as was observed when a constitutively active form of Rac1 was expressed in fibroblasts (Kasahara et al. 2007). In addition, Rac1 deactivation may be important for proper maturation of the macropinosome (Fujii et al. 2013). Hence, Rac1 cycling between its active and inactive form is a key feature for the successful stimulation of macropinocytosis (Fujii et al. 2013). Rac1 promotes the polymerization of actin filaments through the activation of p21-activated kinase 1 (Pak1) at regions of the plasma membrane displaying membrane ruffles (Fig. 2.2), which are critical to the formation of the macropinosome (Dharmawardhane et al. 2000). Moreover, Pak1 also contributes to macropinosome closure and release from the plasma membrane, mediating the scission of the macropinosome cup through the activation of ctBP1/BARS (Liberali et al. 2008). Additionally, Pak1 can also work as an indirect Rac1 activator through the activation of Diacylglycerol kinase ζ (DGKζ) enzyme that promotes the formation of phosphatidic acid (PA) from diacylglycerol (DAG). PA derived from DAG inactivates the Rac1 inhibitor RhoGDI, promoting the activation of Rac1 (Ard et al. 2015). Thus, PA plays an important role in macropinocytosis. Although DGKζ and PA are strong macropinocytosis enhancers, they have also been reported to inhibit Ras signaling, since they block the activity of the Ras activator RasGRP (Topham and Prescott 2001). Thus, it could be that PA and DGKζ play a dual role in macropinocytosis modulation. In the regulation of macropinocytosis, Ras also activates phosphoinositide 3-kinase (PI3K), which mediates the formation of PtdIns(3,4,5)P_3 (PIP_3) (Fig. 2.2). PIP3 actively participates in macropinocytosis, either in actin reorganization for membrane ruffle generation (Araki et al. 2007; Wennstrom et al. 1994) or in macropinosome closure (Araki et al. 1996). The relevance of PI3K in macropinocytosis has been further demonstrated in PDAC harboring $KRAS^{G12R}$ mutations. This mutation in KRAS impairs the binding and activation of PI3K and, in this context, PI3K becomes activated in a Ras-independent manner. In this way, cells harboring such a mutation, despite partially impaired K-Ras activation, are able to sustain macropinocytosis through the direct activation of PI3K (Hobbs et al. 2020). The K-Ras-independent PI3K activation in macropinocytosis promotion has been validated in other models. A

recent study has presented that, in PDAC, PI3K is directly activated by the Axl receptor when associated to the Gas6 ligand (Fig. 2.2) (King et al. 2020). Overexpression of Axl receptor in PDAC promotes enhanced macropinocytic activity and this is modulated by the Axl transcriptional activators Yap and Taz (Fig. 2.2) (King et al. 2020). Thus, Yap and Taz have emerged as novel macropinocytic regulators, activating the PI3K pathway through the overexpression of Axl.

In addition to the well-documented roles of Rac1 and PI3K in driving macropinocytosis in Ras-mutant cells, other factors have also been identified as critical to this process. Lung cancer cells that display oncogenic *KRAS* mutations are able to survive and grow using macropinocytosis only when integrin αvβ3 is expressed at the cell surface and clustered with Galectin-3, forming a complex that permits recruitment of activated K-Ras to the plasma membrane (Fig. 2.2) (Seguin et al. 2017). Disruption of this complex through Galectin-3 inhibition results in impaired macropinocytosis, and this presents Galectin-3 as a key modulator. Also in the context of oncogenic Ras, the canonical Wnt pathway has emerged as a crucial macropinocytic regulator, since it promotes the activation of Pak1 (Fig. 2.2) (Redelman-Sidi et al. 2018). In summary, different players co-operate with oncogenic K-Ras to orchestrate the macropinocytic cascade and further identification of all these factors will be important if we are to clinically target this uptake pathway.

mTOR

The mechanistic target of rapamycin (mTOR) is a conserved serine threonine kinase known to modulate cell growth via the stimulation of anabolic processes, including de novo synthesis of proteins, through the modulation of Ribosomal S6 kinase (S6K) and eIF4E binding protein (Ma and Blenis 2009). Additionally, mTOR also regulates lipid biosynthesis, purine and pyrimidine biogenesis, and autophagy (Yoshida et al. 2018; Saxton and Sabatini 2017). mTOR can form two different multiprotein complexes: complex 1 (mTORC1) and complex 2 (mTORC2). Both complexes contain mTOR as a core kinase and the subunits DEPTOR and mLST8. Where the complexes differ is that mTORC1 contains the specific protein subunits Raptor and PRAS, whereas mTORC2 contains Rictor, mSIN1, and PROTOR (Yoshida et al. 2018).

mTORC1 has been described as a positive modulator of cell growth, promoting protein biosynthesis through the activation of its downstream targets in a nutrient-rich environment. Thus, mTORC1 has largely been considered an attractive therapeutical target for cancer treatment. Nevertheless, it was observed in murine KRAS-mutant PDAC tumors treated with the mTORC1 inhibitor rapamycin that tumor growth was reduced in the peripheral tumor regions, which are those regions with a high degree of vascularization, but, in regions with poor vascularization, rapamycin treatment enhanced proliferation (Palm et al. 2015). Tumor cores and other poorly vascularized regions are characteristically nutrient-poor and it is in those regions where macropinocytosis is specifically promoted in order to support

tumor growth (Lee et al. 2019). In a nutrient replete environment, mTORC1 becomes activated and localizes to lysosomal membranes inhibiting protein catabolism and activating the downstream targets S6K and 4E-BP1, which in turn activates 5′ cap-dependent translation, promoting protein biosynthesis (Palm et al. 2015; Ma and Blenis 2009; Wang and Proud 2006). Thus, inhibiting mTORC1 in these conditions causes a reduction in cell growth. On the other hand, when nutrients are limiting, mTORC1 inactivation might have an opposing role, as it was observed in MEFs harboring oncogenic K-Ras mutations (Palm et al. 2015). When these cells were subjected to an environment depleted of leucine and other EAA, and albumin was added as an extracellular nutrient source, inhibition of mTORC1 enhanced cell proliferation. These observations suggest that mTORC1 negatively modulates cell growth under conditions where macropinocytosis is elevated (Palm et al. 2015). So, the maximal acquisition of nutrients by macropinocytosis seems to require mTORC1 inactivation (Palm et al. 2015; Nofal et al. 2017; Yoshida et al. 2018; Jewell and Guan 2013). More recently, it has been postulated that the extent of mTORC1 inhibition required to promote the acquisition of nutrients from macropinocytosis is partial. Data suggest that, when PDAC cells are cultured in low amino acid medium where albumin has been supplemented as a nutrient source, the optimal cell growth conditions are those where mTORC1 is partially inhibited (Nofal et al. 2017). This conclusion was based on the observation that treatment with high doses of the mTOR inhibitor Torin1 under such conditions reduces cell proliferation, indicating that some mTORC1 activity is required for optimal cell growth when cells are engaged in protein scavenging (Nofal et al. 2017). This study postulated that the main cellular objective of mTORC1 inhibition by low nutrients is to prevent protein synthesis, since attempting to synthesize proteins when amino acids are not available would result in a nutrient imbalance and loss of cell fitness. Thus, partial mTORC1 inhibition could be a survival mechanism under conditions where cell growth is dependent on macropinocytosis, as it would balance amino acid demand to supply. Despite the possible importance of partial mTORC1 inhibition in maintaining cancer cell survival, macropinocytosis itself has emerged as a pathway that activates mTORC1 (Yoshida et al. 2018). Amino acids obtained from the hydrolysis of macropinocytosed protein are known to activate Ragulator, which leads to mTORC1 activation (Yoshida et al. 2015, 2018). This suggests that mTORC1 needs to be repressed to maintain amino acid balance, but when intracellular amino acid levels are restored, this would promote mTORC1 activation to sustain cell growth.

Recently, a putative role for mTORC2 in modulating protein scavenging in PDAC has been suggested (Michalopoulou et al. 2020). mTORC2 is known to regulate the actin cytoskeleton, glucose metabolism, and cell survival (Yoshida et al. 2018; Michalopoulou et al. 2020). In this model, while the ablation of Raptor leads to an increase in protein catabolism of scavenged proteins, validating previous studies, the ablation of Rictor shows the opposite effect. Furthermore, loss of Rictor not only causes deficient protein catabolism but also a reduction in macropinocytosis, which suggests that mTORC2 modulates both macropinocytosis and lysosomal processing of the internalized protein, while mTORC1 is specifically

involved in the regulation of lysosome-dependent protein catabolism (Fig. 2.2) (Palm et al. 2015; Nofal et al. 2017; Michalopoulou et al. 2020). mTORC1 and mTORC2 may play opposing roles and, because of that, a therapy directed to mTORC2 inhibition rather than mTORC1 could be more promising for cancer.

PTEN and AMPK

The PTEN phosphatase is a well-known antagonist of PI3K function, since it dephosphorylates PIP_3 to PtdIns(4,5)*P* (PIP_2) (Fig. 2.2) (Fruman and Rommel 2014). In prostate cancer, PTEN is the main tumor suppressor gene altered, and its deficiency supports the promotion of macropinocytosis (Kim et al. 2018). Using MEFs deficient for PTEN, it was observed that these cells displayed macropinocytosis in an environment depleted of glucose and amino acids. Interestingly, in these cells, glucose starvation is able to stimulate macropinocytosis but amino acid depletion is not. In this context, the participation of AMPK, which becomes activated in glucose-starved conditions, plays a crucial role in activating Rac1 (Fig. 2.2) (Kim et al. 2018). This observation was confirmed in prostate cancer cells deficient for PTEN, since AMPK was required for macropinocytosis promotion. Prostate cancer cells mutant for PTEN and with activated AMPK, display constitutive macropinocytosis for necrocytosis. Thus, targeting AMPK here might prevent macropinocytosis and, as a consequence, should be considered when designing new therapeutic interventions for prostate cancer. In addition to the cooperation between PTEN deficiency and AMPK activity for macropinocytosis stimulation in prostate cancer cells, the roles of these two proteins in macropinocytosis modulation have also been observed in other in vitro models.

A key role for PTEN deficiency in supporting macropinocytosis has been described in MEFs expressing oncogenic K-Ras^{G12D}. In these cells, as well as in PDAC cells, proliferation was sustained in low amino acid conditions through the induction of macropinocytosis when PTEN was depleted (Agani and Jiang 2013; Palm et al. 2017). In PDAC, it has been observed that KPC cells devoid of PTEN perform macropinocytosis to sustain proliferation independently of the extracellular amounts of amino acids. Under enriched amino acid conditions, mTORC2 is activated to modulate cell proliferation through the stimulation of protein synthesis. When mTORC2 is inhibited, cell growth is affected, but, in the presence of albumin, KPC cells lacking PTEN display rescue of this effect *via* macropinocytosis (Michalopoulou et al. 2020). The main cellular objective in promoting macropinocytosis under such conditions may be due to its capability of carrying out similar mechanisms for cell growth modulation as mTORC2. The key target in this system is Akt, which is phosphorylated by mTORC2 at Ser473 (Cybulski and Hall 2009; Sarbassov et al. 2005), increasing cell growth and survival. When mTORC2 is inhibited, macropinocytosis promotes Akt phosphorylation, making cells resistant to mTORC2 loss (Michalopoulou et al. 2020). Although the mechanisms by which macropinocytosis regulates Akt phosphorylation when mTORC2 is

inhibited remain to be elucidated, these observations further indicate the relevance of PTEN loss for sustaining macropinocytosis.

In addition to prostate cancer cells, there are other contexts where AMPK function is associated with macropinocytosis. For instance, AMPK modulates the macropinocytic uptake of some viruses such as the Zaire Ebolavirus (EBOV) and the Vaccinia virus (VV) (Kondratowicz et al. 2013; Moser et al. 2010). On the other hand, a recent study has described a clear and relevant role for AMPK in supporting glutamine depletion-induced macropinocytosis in the cancer-associated fibroblasts (CAFs) found in PDAC tumors (Zhang et al. 2021). It was previously established that in glutamine starvation conditions, macropinocytosis in PDAC cells is promoted by EGF ligands that induce uptake through Pak1 activation (Lee et al. 2019). Not surprisingly, this is not the case in CAFs, since EGF failed to promote macropinocytosis in these stromal cells. In pancreatic CAFs, it was observed that stress induced by glutamine starvation promotes an increase in the cytosolic levels of Ca^{2+} and this results in the activation of CaMKK2, a kinase known to activate AMPK by phosphorylation of the Thr172 residue (Garcia and Shaw 2017). In this system, activated AMPK promotes Rac1 activity through the activation of the Rac1-GEF ARGHEF2, which turns on the macropinocytic cascade (Zhang et al. 2021). The selective modulation of ARGHEF2 expression and activation by glutamine starvation determines the specificity of macropinocytosis in CAFs since, in the same model, starvation of glucose that promotes AMPK phosphorylation and activation does not induce macropinocytosis. This macropinocytic process orchestrated by AMPK is indispensable for supporting tumor growth and progression, as PDAC tumors established with fibroblasts devoid of CaMKK2, ARGHEF2, or AMPK displayed diminished growth rates. These findings show a novel role for stromal macropinocytosis in supporting tumor growth and progression in PDAC, and further establish AMPK as a crucial modulator of macropinocytosis (Zhang et al. 2021). AMPK may also be linked to the macropinocytic uptake of extracellular ATP. As previously discussed, macropinocytosis mediates the uptake of extracellular ATP when the ATP intracellular levels are low. Supplying ATP demands by the cell *via* the uptake of extracellular ATP leads to AMPK activation (Qian et al. 2014), which suggests that an AMPK feed-forward loop might actively participate in the macropinocytic uptake of extracellular ATP. Complicating the situation, AMPK is an inhibitor of mTORC1 (Fig. 2.2) (Gwinn et al. 2008; Inoki et al. 2003; Shaw 2009). It is possible that for the maintenance of partial mTORC1 inhibition, AMPK may need to be activated, ensuring in this way an optimal macropinocytic capacity. In addition, AMPK can also be activated by stress stimuli such as ROS (Choi 2018; Hinchy et al. 2018). The association between ROS and AMPK further presents AMPK as a macropinocytic mediator and ROS as a stress stimulus that can promote macropinocytosis.

Concluding Remarks

The stimulation of macropinocytosis by tumor stresses has become a very productive and interesting area of study. Due to its function in supporting tumor growth and progression (Commisso et al. 2013; Kim et al. 2018; Jayashankar and Edinger 2020), macropinocytosis has arisen as a promising target for cancer treatment, either as a strategy to starve tumor cells of critical nutrients, or to improve the effectiveness of chemotherapies. However, many questions in the field remain unanswered. For example, what are the determinants of constitutive versus stress-inducible macropinocytic uptake? The differentiation between these two phenotypes is likely not directly linked to oncogene mutations, since, at least in PDAC, tumor cells with oncogenic *KRAS* mutations can display both forms of uptake. One possible factor that may play a role in determining whether a tumor cell exhibits inducible or constitutive macropinocytosis is spatial geography within the tumor. It is conceivable that metabolic stresses drive different selective pressures within different intratumoral regions, causing the presence of both inducible and constitutive cells within a single tumor. It should be noted though, that the two forms of uptake are not necessarily binary since even constitutive cells can increase their macropinocytic capacity with certain metabolic perturbations. Importantly, since the many different tumor stresses that lead to macropinocytosis stimulation are often co-occurring within a tumor, it is not clear how these various stress signals intertwine or collaborate to drive tumor progression. Even within the setting of only considering nutrient stress, it is not evident how glutamine and glucose depletion coinciding in tumors might affect macropinocytosis as a metabolic read-out. Lastly, the drivers of context dependency remain largely unaddressed. What underlying pathobiological characteristics cause macropinocytosis in prostate cells to differ mechanistically from the uptake occurring in PDAC? Overall, elucidating the functional contexts, as well as the specific mechanisms that control macropinocytosis, will be critical to building a better understood framework that aims to develop novel therapeutic modalities to target macropinocytosis in cancer.

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Chapter 3
The Amoebal Model for Macropinocytosis

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Abstract Macropinocytosis is a relatively unexplored form of large-scale endocytosis driven by the actin cytoskeleton. *Dictyostelium* amoebae form macropinosomes from cups extended from the plasma membrane, then digest their contents and absorb the nutrients in the endo-lysosomal system. They use macropinocytosis for feeding, maintaining a high rate of fluid uptake that makes assay and experimentation easy. Mutants collected over the years identify cytoskeletal and signalling proteins required for macropinocytosis. Cups are organized around plasma membrane domains of intense PIP3, Ras and Rac signalling, proper formation of which also depends on the RasGAPs NF1 and RGBARG, PTEN, the PIP3-regulated protein kinases Akt and SGK and their activators PDK1 and TORC2, Rho proteins, plus other components yet to be identified. This PIP3 domain directs dendritic actin polymerization to the extending lip of macropinocytic cups by recruiting a ring of the SCAR/WAVE complex around itself and thus activating the Arp2/3 complex. The dynamics of PIP3 domains are proposed to shape macropinocytic cups from start to finish. The role of the Ras-PI3-kinase module in organizing feeding structures in unicellular organisms most likely predates its adoption into growth factor signalling, suggesting an evolutionary origin for growth factor signalling.

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Introduction

Macropinocytosis is an endocytic process driven by the actin cytoskeleton (Lewis 1931; Swanson 2008; Buckley and King 2017; King and Kay 2019; Stow et al. 2020; Lin et al. 2020). Cells take up droplets of medium into micron-sized vesicles, using cups and ruffles projected from the plasma membrane. Macropinocytosis remains poorly understood compared to many other cell-biological processes, but interest is increasing due to its medical importance in disease and drug delivery (Bloomfield and Kay 2016) and to the unique features of cytoskeletal organization and membrane trafficking involved.

Many advances in molecular biology have come from the use of well-chosen model organisms: the cell cycle in yeast, programed cell death in nematodes and the Wnt signalling pathway and homeotic genes in Drosophila are obvious examples. The contribution from these organisms rested on a combination of their biology, which made it cheap, rapid, and efficient to investigate the problem of interest, and their leading genetic technologies. All this also depended on the underlying assumption that the process was evolutionarily conserved, though of course this was often in doubt at the time.

Macropinocytosis does not exist in yeast, so far as is known, and is relatively hard to investigate in nematode or fly coelomocytes: a different model organism is therefore required. In fact, studies in amoebae have paralleled work on mammalian cells, right back to the discovery of macropinocytosis about 100 years ago. Initial work used giant amoebae (Edwards 1925; Chapman-Andresen 1984), but then switched to the social amoeba *Dictyostelium discoideum*, as this was developed as a model for cell and developmental biology (Kessin 2001). Here we introduce *Dictyostelium* amoebae as a model system for macropinocytosis, describe how key actors in macropinocytosis have been identified in this organism, and suggest a mechanism for forming macropinocytic cups.

The *Dictyostelium* Model for Macropinocytosis

Dictyostelium is a soil dwelling amoeba, initially famous for a life cycle that spans unicellular and multicellular forms (Raper 1940). The amoebae grow separately by feeding on bacteria, but when food runs out, gather together to make a multicellular "slug" and eventually a stalked fruiting body. It is haploid with a well-annotated genome of around 12,000 genes, many homologous to animal genes (Eichinger et al. 2005). Equally some families, such as receptor tyrosine kinases, are entirely missing, while others are greatly expanded, including the actin and Ras families. The actin cytoskeleton and cell signalling have been studied intensely, and over the last

30 years many genes have been knocked-out by homologous recombination, creating a resource of characterized mutants, including many that affect macropinocytosis. These can be browsed at the organismal database, dictyBase (Basu et al. 2013).

Macropinocytosis was not immediately recognized in *Dictyostelium* because it is an avid phagocyte and prefers to feed on bacteria when these are available. Around 1970, however, mutant strains that grow in liquid medium were isolated and adopted for laboratory use (Sussman and Sussman 1967). These axenic strains (such as Ax2, Ax3, and Ax4) grow because they take up fluid several times faster than their wild-type parents (Clarke and Kayman 1987). However, it was only in 1997 that work on the cytoskeleton led to macropinocytosis being recognized as the route for this fluid uptake (Fig. 3.1) (Hacker et al. 1997). Coronin—an actin-binding protein discovered in *Dictyostelium*—forms curious crowns on the dorsal surface of growing cells (de Hostos et al. 1991), which, using GFP-fusions to detect coronin or F-actin, could be seen closing to take in droplets of medium, showing that they are macropinocytic cups (Hacker et al. 1997; Lee and Knecht 2002). More recently the mutation driving the increased fluid uptake was identified as loss of the RasGAP, NF1 (neurofibromatosis-1), a conserved tumor suppressor, whose biochemical function is to inactivate Ras (Bloomfield et al. 2015).

In suitable medium, axenic strains form one or a few macropinosomes per minute and can take up their own volume in 1–2 h (Thilo and Vogel 1980; Aubry et al. 1997). There is no evidence for selectivity: tracers are just taken up proportional to their concentration in the medium. Macropinocytosis accounts for around 90–95% of fluid uptake, based on morphometry or from the residual uptake remaining in macropinocytosis mutants (Hacker et al. 1997; Williams et al. 2019), allowing total fluid uptake to be used as a direct measure of macropinocytosis. This is easily measured using fluorescent dextran tracers and flow cytometry (Thilo and Vogel 1980; Williams and Kay 2018). The intracellular pathway can also be probed by uptake of reporters for pH, proteolytic digestion and using colloidal magnetic particles that allow macropinosomes to be isolated for proteomic studies (Aubry et al. 1997; Journet et al. 2012).

Unlike in most mammalian cells, macropinocytosis in *Dictyostelium* is constitutive and does not require receptor stimulation, occurring in isolated cells in buffer (Williams and Kay 2018). It is suppressed by food bacteria, and by starvation during the developmental program (Clarke and Kayman 1987). It increases in nutrient media, provided bacteria are absent, with a mixture of just three amino acids and glucose sufficing for this effect. These nutrients appear to be detected within the endocytic system itself, presumably at the lysosomes (Williams and Kay 2018). The transition between high and low macropinocytic states takes several hours and is accompanied by wide-ranging changes in gene expression (Sillo et al. 2008).

Once a macropinosome is internalized, it loses its F-actin coat and transits through the endocytic system. It is rapidly acidified (Aubry et al. 1997), lysosomal enzymes are added and any contents digested, so that the resulting small molecules can be absorbed to nourish the cell. The vesicles are neutralization and undigested contents (such as fluorescent dextran) start being exocytosed after 60–90 min.

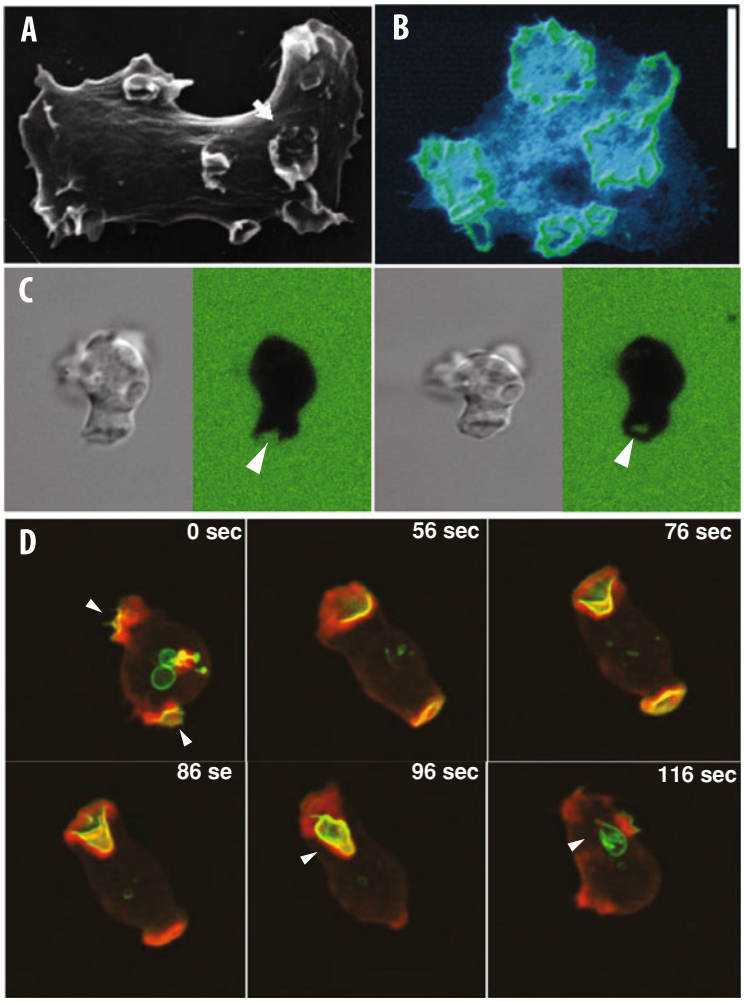

Fig. 3.1 The morphology of macropinocytosis in *Dictyostelium* cells. (**a**) Scanning electron micrograph showing abundant ruffles and macropinocytic cups ("crowns"; one is arrowed) on the surface of a cell grown in liquid medium (from Hacker et al. 1997). (**b**) Immuno-staining of coronin showing four large macropinocytic cups on a growing cell (from Hacker et al. 1997). (**c**) Images of an Ax2 cell growing in liquid medium containing fluorescent dextran, showing a macropinocytic cup closing to form a macropinosome within the cell (arrowed). Paired DIC and fluorescent images made by confocal microscopy. Courtesy of Douwe Veltman. (**d**) Lattice light sheet microscopy of a single cell, expressing reporters for PIP3 (green) and F-actin (red), showing a macropinosome (arrowed at top) growing and closing to release a macropinosome. This first loses its F-actin coat and so appears greener, then loses PIP3 as well and becomes invisible. Macropinosomes made a little earlier in the film sequence are visible in the first panel, but then lose their PIP3 and disappear. The cell attempts to form a second macropinosome (arrowed, bottom in first panel), but this fails and regresses. Unpublished observations of the authors

PIP3 Domains and Macropinocytosis

PIP3 domains are a distinctive feature of macropinocytic cups, discovered in *Dictyostelium* using fluorescent PH-domain reporters that bind PIP3 (Parent et al. 1998; Meili et al. 1999; Dormann et al. 2004). These reporters showed that the plasma membrane of growing cells contains discrete domains of PIP3 a few microns across, around which macropinocytic cups form (Fig. 3.1d). The domains only disappear as cups close, when PIP3 is replaced by PI3,4P2 on the resulting vesicles. Later it was found that active Ras and active Rac also form coincident domains with PIP3, creating a region of intense signalling within macropinocytic cups (Sasaki et al. 2007; Veltman et al. 2016).

PIP3 domains have been studied intensively since their discovery, but often under the assumption that they are involved in chemotaxis (Parent et al. 1998; Funamoto et al. 2002; Iijima and Devreotes 2002). However deletion of all five Ras-activated PI3-kinases in the genome abolishes PIP3 domains but leaves chemotaxis unscathed, showing that they have little to do with chemotaxis (Hoeller and Kay 2007). On the other hand, similar deletions show that PIP3 is essential for macropinocytosis (Buczynski et al. 1997; Hoeller et al. 2013).

PIP3 in Dictyostelium is chemically distinct from that in mammalian cells, having one of its fatty acid chains attached to glycerol by an ether rather than an ester linkage: they are plasmanylinositides, rather than phosphatidylinositides (Clark et al. 2014). This chemical difference results in modified biosynthetic (Kappelt et al. 2020) and breakdown routes for the gylcero moiety, but appears to make no difference to the recognition of the phosphorylated inositol head group by PH-domain proteins.

How Macropinosomes Form

Macropinosomes are readily visible under the light microscope, yet there is no clear consensus on the morphological route for their formation. The standard view from macrophages is that macropinocytic cups form by circularization of linear ruffles, and then close at their lip to form a sealed macropinosome(Swanson 2008; Yoshida et al. 2009). PIP3 builds up within the cup after circularization and is lost from the sealed vesicle. A variant scheme suggests that cups are supported by F-actin "tentpoles," which twist together to close them (Condon et al. 2018); and a less-ordered alternative suggests that ruffles can simply fold back and fuse with the plasma membrane to form a macropinosome (Quinn et al. 2021).

In *Dictyostelium*, scanning electron microscopy, immuno-staining for coronin, and live-cell imaging consistently show cupped structures on the surface of cells, which are rich in F-actin (Fig. 3.1a, b) (Hacker et al. 1997; Lee and Knecht 2002). How these cups form, evolve, and close to form macropinosomes has been less easy to determine by confocal or spinning-disc microscopy, due to their size, dynamism,

and the light-sensitivity of the cells. However, lattice light sheet microscopy (LLSM) allows full cell volumes to be obtained in a few seconds at tolerable light intensities and suggests a variant of the macrophage scheme (Veltman et al. 2016; Chen et al. 2014). A reporter for F-actin shows that macropinocytic cups are irregular and often pit-like, of varied shape and can form de-novo or by splitting of existing ones.

More recent work pairing PIP3 and F-actin reporters shows that the PIP3 domain and F-actin structures develop together almost from their first appearance: there is no obvious linear ruffle/circularization stage (unpublished observations of the authors). En face images show that macropinocytic cups often close at the lip by concerted contraction of the rim. This leads to extinction of the PIP3 domain, but if cups close leaving a remnant PIP3 domain, this can grow to form another cup, and repeated macropinosomes from the same site.

Genetics of Macropinocytosis

Facile homologous recombination and genome-wide screens by REMI insertional mutagenesis (Kuspa and Loomis 1992) have led to the isolation of many macropinocytosis mutants in *Dictyostelium*, often as by-products of other projects. Even severely impaired mutants can be isolated and maintained by growth on bacteria (Paschke et al. 2018). Sifting these gives a set of genes and proteins strongly implicated in macropinocytosis (Table 3.1).

The main criteria for inclusion in this list are a defect in fluid uptake (and usually growth in liquid medium) in the mutant, and localization of the protein to macropinocytic cups, implying a direct function in macropinocytosis (or exclusion from them in the case of PTEN). Genes that are only indirectly involved are excluded where possible. For instance, both clathrin and dynamin mutants have reduced fluid uptake, but neither protein specifically localizes to macropinocytic cups, and the phenotype can be plausibly explained as due to impairment of vesicle trafficking (O'Halloran and Anderson 1992; Damer and O'Halloran 2000; Wienke et al. 1999). Similarly, deletion of geranylgeranyl diphosphate synthase blocks fluid uptake but this may be because it is required for the geranylgeranylation of many small G-proteins involved in macropinocytosis (Jiao et al. 2020). Some important gene families are redundant and require multiple knock-outs to show a strong phenotype: PI3-kinase, Ras, probably Rac and myosin-1, and most likely other cytoskeletal proteins. Actin and the Arp2/3 complex are essential to cell viability and their importance is shown by inhibitor studies.

The genes identified in this way encode proteins that broadly divide into two classes. Cytoskeletal proteins, which are used generically to create a variety of F-actin structures, including pseudopods as well as macropinosomes. And classical signalling proteins of the Ras/PI3-kinase pathway, which create PIP3 domains and are proposed to organize the cytoskeletal proteins for macropinocytosis.

Table 3.1 Proteins critical to macropinocytosis in *Dictyostelium*. The proteins listed have been linked to macropinocytosis by the phenotype of mutants (knock-outs in almost all cases) and by their localization to macropinocytic cups (except for PTEN, which is excluded). Mutants generally have significant defects in fluid uptake compared to their parent and grow poorly in liquid medium, except for mutants of NF1 where uptake is greatly increased compared to its parent. All other mutants are in an axenic background where NF1 is deleted. Gene names can be used as a key into the more extensive literature curated at dictyBase (https://dictycr.org) (Basu et al. 2013)

Protein name or class	Dicty gene	Comment and mutant phenotype	Reference
SCAR/ WAVE	*scrA*	Forms rings	Seastone et al. (2001), Veltman et al. (2016)
WASP	*wasA*	Can replace SCAR	Davidson et al. (2018)
Formin	*forG*	Ras-binding	Junemann et al. (2016)
Myosin-1	*myoB, E, F*	Myo1B: outer zone Myo1E, F: PIP3 zone	Brzeska et al. (2014, 2016)
Profilin	*proA, proB*	Suppressed by deleting LIMP scavenger receptor	Karakesisoglou et al. (1999), Temesvari et al. (2000)
Coronin	*corA*	Forms crowns	Hacker et al. (1997)
Aip1	*Aip1*	Actin binding, WD40 repeat	Konzok et al. (1999)
Carmil	*carmil*		Jung et al. (2001)
Actin cross-linking proteins	*abpA, abpB, abpC*	Alpha actinin, 34 kDa cross linker, ABP120	Rivero et al. (1999)
Ras	*rasG, rasS, rasB*	Many genes; redundancy Active Ras forms domains	Veltman et al. (2016), Junemann et al. (2016), Chubb et al. (2000), Williams et al. (2019)
NF1 (RasGAP)	*axeB*	Deletion: enlarged PIP3 domains; increased macropinocytosis	Bloomfield et al. (2015)
RasGAP/ RhoGEF	*rgbA*	RGBARG. Deletion: enlarged PIP3 domains	Buckley et al. (2020)
IQGAP	*iqgC*		Marinovic et al. (2019)
RasGAP2		Enlarged Ras domains	Li et al. (2018)
Rap	*rapA*	Ras homologue	Seastone et al. (1999)
RapGEF	*gflB*	Implicates Rap in macropinocytosis	Inaba et al. (2017)
PI3-kinase	*pikA, pikB*	Makes PIP3 domains	Buczynski et al. (1997), Hoeller et al. (2013)
PI3-kinase	*pikF*	Closes PIP3 domains?	Hoeller et al. (2013)
PTEN	*ptenA*	Reverts PIP3 to PI4,5P2. Giant PIP3 domains	Veltman et al. (2016)
PI4P5-kinase	*pikI*	Makes PIP2	Fets et al. (2014)
OCRL	*Dd5P4*	PI5-phosphatase; makes PI3,4P2	Loovers et al. (2007)

(continued)

Table 3.1 (continued)

Protein name or class	Dicty gene	Comment and mutant phenotype	Reference
Akt (PKB)	*pkbA*	Redundant with SGK	Williams et al. (2019), Rupper et al. (2001)
SGK (PKBR1)	*pkgB*	Redundant with Akt	Williams et al. (2019)
TORC2	*lst8, piaA, ripA*	Activates Akt & SGK	Williams et al. (2019)
PDK1	*pdkA*	Activates Akt & SGK	Williams et al. (2019)
Rac/Rho	*racC, racE*	Activates actin polymerization	Wang et al. (2013)
RacGEF	*gxcT*		Wang et al. (2013)
RhoGAP	*gacG*	Makes pseudopods instead of MP	Williams et al. (2019)

Cytoskeletal Proteins and the Structure of Macropinocytic Cups

Inhibitors such as latrunculin and cytochalasin show that actin polymerization is essential for macropinocytosis (Hacker et al. 1997; Williams and Kay 2018). A picture of how the actin in macropinocytic cups is organized comes from work on "actin waves" (Bretschneider et al. 2009; Gerisch et al. 2019). We regard these dynamic structures on the basal surface of *Dictyostelium* cells as frustrated macropinocytic cups. They are similarly closed structures with a ring of actin polymerization encircling a central PIP3 domain and have a homologous arrangement of coronin, myosin-1 proteins, and SCAR/WAVE to cups (Veltman et al. 2016) (unpublished observations). They also depend on the same mutation of NF1 for their abundance and size.

Interpreted in this way, the lip of a macropinocytic cup corresponds to the ring of F-actin polymerization in a wave and the body of the cup to the central PIP3 area. Cryo-electron microscopic (cryo-em) tomography of waves shows that the central area largely consist of actin filaments parallel to the membrane, which are denser than outside the wave, and that the wave itself (lip of the cup) contains a dendritic actin network (Jasnin et al. 2019). In the dendritic network, electron densities corresponding to the Arp2/3 complex occur at the junctions between fibers, which are at the characteristic 70° angle to the mother fibers. The daughter fibers point predominantly toward the membrane and would be expected to apply outward force to it as they extend.

In summary, this picture, although in two dimensions, suggests that macropinocytic cups contain a mixture of dendritic actin fibers produced by the Arp2/3 complex and linear fibers produced by formins or other means. The linear fibers dominate in the body of the cup and mainly lie parallel to the membrane;

whereas dendritic actin predominantly forms a ring around the PIP3 domain and is expected to apply outward force to the membrane.

The Arp2/3 complex, which locates to macropinocytic cups and is genetically essential in *Dictyostelium*, initiates dendritic actin; a direct role is indicated by acute inhibition of macropinocytosis by CK666 (Williams and Kay 2018; Insall et al. 2001). The Arp2/3 complex is activated by WASP and the SCAR/WAVE complex, both of which are found in macropinocytic cups (see later). Deletion of SCAR/WAVE already has a substantial effect on macropinocytosis, whereas deletion of WASP has very little; however, deletion of both together abolishes macropinocytosis (Veltman et al. 2016; Seastone et al. 2001; Davidson et al. 2018). This redundancy is also seen in pseudopods, where WASP can replace SCAR/WAVE in genetic deletions (Veltman et al. 2012). Actin polymerization by WASH on closed macropinosomes is also required at both early and late stages in vesicle trafficking; WASH mutants are blocked in the final exocytosis of undigested remnants of medium taken up by macropinocytosis (Carnell et al. 2011; Buckley et al. 2016).

Unbranched actin fibers in macropinocytic cups are initiated by formins, such as ForG (Junemann et al. 2016). This is activated by Ras and locates to cups, where it plays a distinct structural role in maintaining F-actin in the base and is required for efficient fluid uptake.

Myosin-1 proteins are single-headed myosins that link F-actin to membranes. Several locate to macropinocytic cups in two distinct patterns: PIP3-binding Myo1E and Myo1F locate to the inner surface of the cup and are surrounded by a broad zone of Myo1B (Brzeska et al. 2014, 2016). Single mutations of individual myosin-1 genes have minimal effect on macropinocytosis, but multiple deletions seriously impair it, with in the most severe case a multiple knock-out of five myosin genes resulting in a 75% reduction in fluid uptake (Titus 2000) (P. Paschke, unpublished).

The role of myosin-II is less certain: there is no report of a specific localization to macropinocytic cups, but fluid uptake is modestly reduced in null mutants (Shu et al. 2005), while blebbistatin is inhibitory, but only at high concentrations, where its effect is indirect (Shu et al. 2005). Thus, it is doubtful whether myosin-II has a major role in macropinocytosis, such as in forming a contractile ring to close macropinocytic cups.

Profilin binds actin monomers and macropinocytosis is reduced by around 60% in a double mutant of the two major profilin genes (Karakesisoglou et al. 1999). A screen for genetic suppressors yielded LIMP, a homologue of the scavenger receptor LIMP-2, whose deletion restored normal fluid uptake to the profilin mutant (Temesvari et al. 2000). LIMP appears to be recruited to macropinosomes and is also required for fluid uptake. This intriguing genetic interaction between profilin and LIMP is unexplained, but illustrates the power of suppressor genetics for revealing unexpected connections.

Among the F-actin binding proteins, coronin and Aip1 (also known as Wdr1) are believed to cooperate with cofilin in promoting F-actin turnover and remodeling (Tang et al. 2020). Deletion of either causes a significant defect in fluid uptake and both are recruited to macropinocytic cups, with coronin forming distinct crowns

toward the edge of PIP3 domains, as already described (Hacker et al. 1997; de Hostos et al. 1991). Carmil, which can link myosin-1 to the Arp2/3 complex, also localizes to macropinocytic cups and deletion moderately impairs macropinocytosis (Jung et al. 2001).

Dictyostelium possesses a rich set of actin cross-linking proteins, which are somewhat redundant. None have been reported to be individually essential for fluid uptake, whereas double mutants of alpha-actinin and either filamin or the 34 kDa actin-bundling protein are seriously impaired (Rivero et al. 1999). Doubtless further deletions of members of this class of proteins would be further damaging.

The shape and supporting scaffold of macropinocytic cups is provided by the actin cytoskeleton. As expected, a large number of actin-binding proteins are recruited to the cups and, once redundancy is allowed for, are required for macropinocytosis. Some of these proteins, such as coronin and various of the myosin-1 proteins, have specific places in cups, but none so far reported are unique to cups. All can be found in other actin-based structures such as pseudopods. The essential question therefore is how these generic cytoskeletal components are shaped into cupped structures?

Signalling Proteins and the PIP3 Domain

The Ras-PI3-kinase-Akt/SGK axis forms the core of the signalling proteins organizing macropinocytosis (Fig. 3.2a). This axis is traditionally seen as integral to growth factor signal transduction, but *Dictyostelium* has no growth factors and it functions cell-autonomously in macropinocytosis.

Ras lies at the head of the module. *Dictyostelium* has 14 annotated Ras genes, of which genetic knock-outs have implicated RasG, RasS, and RasB in macropinocytosis, with a RasG/RasS double mutant severely impaired in fluid uptake (Veltman et al. 2016; Junemann et al. 2016; Chubb et al. 2000; Williams et al. 2019). Ras is activated by RasGEFs, but those relevant to macropinocytosis have not yet been definitively identified.

The close Ras homologue, Rap, is also implicated in macropinocytosis, but as it is likely essential, null mutants have not been made. Anti-sense knock-down of Rap decreases macropinocytosis (Kang et al. 2002), as does deletion of the activating RapGEF, GeflB, which is recruited to macropinocytic cups (Inaba et al. 2017). The inactivating RapGAP3 is also recruited to cups and deletion causes enlarged domains of activated Rap (Li et al. 2018).

Ras is inactivated by RasGAPs, whose key role in macropinocytosis is increasingly apparent. NF1 is a conserved tumor suppressor and underlying cause of the common genetic disease neurofibromatosis. Deletion of NF1 is the major genetic cause for the growth of axenic strains in liquid medium, causing much-enlarged PIP3 domains and a many-fold increase in fluid uptake (Bloomfield et al. 2015). It is recruited to PIP3 domains and thus able to limit their extent. Nearly all the

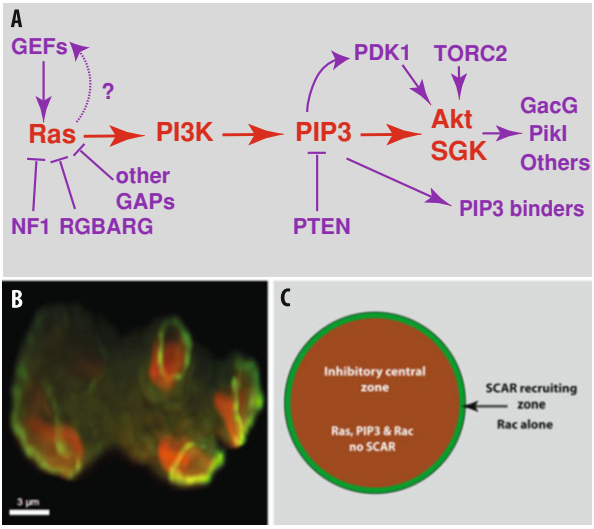

Fig. 3.2 PIP3 domains as templates for macropinocytic cups. (**a**) The Ras-PI3-kinase axis in PIP3 domains. All the named proteins are genetically implicated in macropinocytosis. NF1 and RGBARG are RasGAPs that inactivate Ras; their deletion leads to expanded domains, as does deletion of PTEN which reverts PIP3 to PI4,5P2. Deletion of sufficient PI3-kinases blocks macropinocytosis, as does the double deletion of the Akt and SGK protein kinases or their activators PDK1 and TORC2. Most of the proteins shown are highly conserved and are integral to growth factor signalling in mammalian cells. (**b**) Rings of the SCAR/WAVE complex (green) encircling PIP3 patches (red) in macropinocytic cups. The SCAR/WAVE complex activates dendritic actin polymerization through the Arp2/3 complex and it is proposed that their ringed recruitment to PIP3 domains creates circular actin structures from the plasma membrane. From Veltman et al. (2016). (**c**) Hypothesis for ringed recruitment of SCAR/WAVE to PIP3 domains. PIP3 domains contain active Ras and active Rac as well as PIP3. Rac is an activator of actin polymerization through SCAR/WAVE. The domain of active Rac extends slightly beyond the active domain creating an annulus where SCAR/WAVE is recruited (Buckley et al. 2020; Kay 2020). It is proposed that active Ras, or PIP3, or an actor linked to them, inhibits recruitment to the body of the domain

Dictyostelium mutants discussed here are in the axenic background and so have NF1 deleted.

RGBARG is a complex protein with both RasGAF and RhoGEF domains, and without a clear mammalian counterpart, although the same domains may be distributed among other proteins (Buckley et al. 2020). Deletion causes enlarged PIP3 domains that, combined with NF1 deletion, occupy a large proportion of the cell surface and become inefficient at fluid uptake. RGBARG is recruited to macropinocytic cups, preferentially at the lip. The IQGAP, IqgC, also strongly recruits to macropinocytic cups, but deletion has only a minor effect on fluid uptake in the NF1-background. *Dictyostelium* has another ten or so RasGAPs, of which RG2 at least is relevant to macropinocytosis as it localizes to the base of cups and deletion causes greatly increased Ras domains (Li et al. 2018).

Dictyostelium has five "class 1" PI3-kinases, which are activated by Ras through a Ras-binding domain. Multiple deletions show that collectively these are essential

for macropinocytosis and that two of them—PikA and PikB—produce most of the cellular PIP3 and are required for forming PIP3 domains (Buczynski et al. 1997; Hoeller et al. 2013). In contrast, deletion of PikF impairs fluid uptake without much effect on PIP3 levels or PIP3 domains, suggesting it has a unique function, perhaps in closing macropinocytic cups.

PI4,5P2 is the substrate used to make PIP3, and as the major phosphoinositide in the plasma membrane is important in the interaction of many proteins with the membrane. Not surprisingly, deletion of the major PI4P5-kinase making PI4,5P2 is severely disruptive to macropinocytosis (Fets et al. 2014). PIP3 is reverted to PI3,4P2 by PTEN, a conserved lipid phosphatase and tumor suppressor (Iijima and Devreotes 2002). Deletion of PTEN in the NF1-background leads to enormous PIP3 domains that are very inefficient at fluid uptake (Veltman et al. 2016; Jiao et al. 2020). PIP3 can also be converted to PI3,4P2 by the OCRL phosphatase, which is also likely important for macropinocytosis based on the mutant defect in growth in liquid medium (Loovers et al. 2007).

PIP3 in the inner leaflet of the plasma membrane acts as a recruiting station for proteins carrying PIP3-binding domains, such as the PH domain. The most relevant to macropinocytosis are the PIP3-activated protein kinases Akt and SGK (often called PKB and PKBR1 in *Dictyostelium*) (Meili et al. 1999, 2000). Akt has a PH-domain and is recruited to the plasma membrane by binding to PIP3, whereas the *Dictyostelium* SGK is constitutively bound to the membrane by lipid modification. Both are activated by dual phosphorylation by the TORC2 complex at the hydrophobic motif and through PDK1 at the activation loop (Kamimura et al. 2008; Kamimura and Devreotes 2010). Since PDK1 binds PIP3, both Akt and SGK have PIP3-dependent activation. Deletion of Akt or SGK causes a decrease in fluid uptake, but a double deletion greatly reduces fluid uptake, though PIP3 domains form normally (Williams et al. 2019; Rupper et al. 2001). Consistent with the requirement for Akt and SGK, mutation of their activators PDK1 and the TORC2 complex also inhibits macropinocytosis (Williams et al. 2019).

Phosphoproteomics of Akt and SGK null cells shows that the two protein kinases have a similar specificity and target an overlapping set of proteins, several of which appear to be regulators of the cytoskeleton (Williams et al. 2019). Among those mutated to date, deletion of GacG (a RhoGAP) almost completely suppresses macropinocytosis, with the cells becoming hypermotile and producing pseudopods instead of macropinosomes (Williams et al. 2019).

Additional PIP3 binding proteins relevant to macropinocytosis include myosin 1E and myosin 1F as already discussed, Leep1 (Yang et al. 2021) and possibly others detected biochemically (Zhang et al. 2010) or predicted computationally (Park et al. 2008).

PIP3 domains also harbor active Rac, which is an activator of actin polymerization through SCAR/WAVE and WASP. Fluid uptake is strongly inhibited by the generic Rac inhibitor EHT1864 (Williams and Kay 2018), but of the 20 annotated Rac/Rho family genes, so far only RacC and RacE have been linked to macropinocytosis by the poor growth of deletion mutants in liquid medium (Wang

et al. 2013). Similarly, there are a large number of RacGEFs encoded in the genome, of which only GxcT has been linked to macropinopcytosis so far (Wang et al. 2013).

Shaping Macropinocytic Cups: PIP3 Domains and SCAR/WAVE Rings

Imaging the GFP-tagged HSPC300 or NAP subunits of the SCAR/WAVE complex reveals that it is recruited as an irregular necklace around PIP3 domains and thus at the lip of macropinocytic cups (Veltman et al. 2016). This holds true for all other PIP3 domains examined including both basal waves and phagocytic cups and has been confirmed by LLSM microscopy through the lifetime of macropinocytic cups (unpublished observations of the authors). Similar rings around PIP3 domains are revealed by tagging the Arp2/3 complex (unpublished observations). This suggests a general cytoskeletal "rule" (at least in *Dictyostelium*) that PIP3 domains are encircled by SCAR/WAVE and thus can template rings of actin polymerization under the membrane, creating cupped structures and circular ruffles.

PIP3 domains remain mysterious entities. They contain active Ras and Rac as well as PIP3, plus the proteins that are recruited by binding to these membrane-bound components. Ras, Rac, and PIP3 are expected to be freely diffusible in the membrane, so some mechanism is required to prevent patches from dissipating by diffusion. Several, non-exclusive mechanisms can be envisioned.

Turing type reaction-diffusion process are capable of producing discrete "activated" and "inhibited" domains by, for instance, the interaction of a short-range activator and longer range inhibitor that limits the activation (Meinhardt and Gierer 1974). This has been modeled for PIP3 domains (Saito and Sawai 2021), but many model configurations are possible, and it will be necessary to attach real biochemical entities to the components before they can be distinguished.

Evidence to date suggests that Ras may be the master regulator. This is consistent with its biochemical role of activating PI3-kinase and is suggested genetically. Active Ras domains still form when PI3-kinases are deleted and do not follow PIP3 domains when these are expanded by deletion of PTEN (Veltman et al. 2016). Conversely when Ras domains are expanded by deletion of NF1, the PIP3 domains conform to the new size. These observations argue that Ras may be the activator in a Turing scheme and predict it should be capable of self-activation, for instance via a Ras-activated GEF. Additional activatory or inhibitory loops, for instance between Ras and PIP3 or actin polymerization are not excluded, however.

Two other properties may be involved in PIP3 domain formation. One is a diffusion barrier surrounding the domain, as suggested in macrophages (Welliver et al. 2011). The other, entirely speculative, is that interactions between the cytoplasmic components recruited to the domain may induce a phase separation between the domain and the surrounding membrane.

Whatever the process creating PIP3 domains, they are capable of selective recruitment of SCAR/WAVE to their periphery (Wigbers et al. 2020). Reporters for active Ras and active Rac do not exactly overlap: the Rac domain extends slightly beyond the Ras domain, creating an annulus where active Rac predominates (Fig. 3.2C) (Buckley et al. 2020). If active Ras or PIP3 somehow blocked the effect of active Rac, this could explain the ringed recruitment of SCAR/WAVE. Consistent with this idea, in mutants of RGBARG (which has RasGAP and RhoGEF domains) the annulus is virtually abolished and macropinocytosis is disorganized.

Conclusions and Future Perspectives

Dictyostelium is very convenient for the study of macropinocytosis. Growing cells perform macropinocytosis at a high rate and will do so for hours even in a simple buffer. They are easy to grow and manipulate, and there is an efficient suite of molecular genetic methods, including genetic screens. Although strains that cannot perform macropinocytosis will not grow in liquid medium, they can still be maintained on bacteria.

If one of the essential questions in macropinocytosis is how do macropinocytic cups form and close, then the PIP3 domains, which are so striking in *Dictyostelium*, provide the beginnings of an answer. We hypothesize that dendritic actin polymerization is attracted to the periphery of these domains by recruitment of SCAR/WAVE, and that this provides a general mechanism for forming cups in the plasma membrane, not only in macropinocytosis but also in circular dorsal ruffles and in phagocytosis. The evolution of signalling within domains may then dictate the expansion of the cup and its eventual closure, splitting or shrinkage.

The *Dictyostelium* work also emphasizes the importance of the signalling axis of Ras, Ras-activated PI3-kinase, and the protein kinases Akt and SGK in macropinocytosis, together with their associated regulators NF1, PTEN, TORC2, and PDK1. This axis is highly conserved between amoebae and mammals, not only in the sequence of the proteins, but in many biochemical details. It includes well-known oncogenes and tumor suppressors and is most usually thought of as an arm of growth factor signalling. This however is not its function in *Dictyostelium*, which lacks growth factor receptors (receptor tyrosine kinases) and where the axis functions without known receptor stimulation to organize macropinocytic structures. We propose that this function in organizing the actin cytoskeleton was the original function of the axis in early, single-celled organisms, and that only later, as multicellular organisms evolved was it co-opted into growth factor signalling, as a means of bringing these structures under organismal control. In support of this idea, a limited investigation shows a complete correlation in animals, yeast, and amoebozoa between the presence of the axis and macropinocytosis (King and Kay 2019). For instance, in the evolution of yeast, although Ras is still present, Ras-activated PI3-kinase and Akt have been lost from the genome.

More generally, a fertile interaction between ideas produced in *Dictyostelium* and mammalian cells should stimulate the macropinocytosis field as a whole and lead to a rounded understanding of macropinocytosis, including defining its conserved evolutionary features and how these have been adapted in various organisms and specialized cell type, as well as throwing light on the origins of growth factor signalling.

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Chapter 4
Extracellular ATP and Macropinocytosis: Their Interactive and Mutually Supportive Roles in Cell Growth, Drug Resistance, and EMT in Cancer

Maria Evers, Jingwen Song, and Xiaozhuo Chen

Abstract Macropinocytosis is one of the major mechanisms by which cancer cells uptake extracellular nutrients from tumor microenvironment (TME) and plays very important roles in various steps of tumorigenesis. We previously reported the unexpected finding that intratumoral and extracellular ATP (eATP), as one of the major drastically upregulated extracellular nutrients and messengers in tumors, is taken up by cancer cells through macropinocytosis in large quantities and significantly contributing to cancer cell growth, survival, and increased resistance to chemo and target drugs. Inhibition of macropinocytosis substantially reduced eATP uptake by cancer cells and slowed down tumor growth in vivo. More recently, we have found the eATP also plays a very important role in inducing epithelial-to-mesenchymal transition (EMT), and that macropinocytosis is an essential facilitator in the induction. Thus, macropinocytosis and eATP, working in coordination, appear to play some previously unrecognized but very important roles in EMT and metastasis.

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As a result, they are likely to be interactive and communicative with each other, regulating each other's activity for various needs of host tumor cells. They are also likely to be an integral part of the future new anticancer therapeutic strategies. Moreover, it is undoubted that we have not identified all the important activities coordinated by ATP and macropinocytosis. This review describes our findings in how eATP and macropinocytosis work together to promote cancer cell growth, resistance, and EMT. We also list scientific challenges facing eATP research and propose to target macropinocytosis and eATP to reduce drug resistance and slow down metastasis.

Keywords Cancer metabolism · Metastasis · Epithelial–mesenchymal transition · Endocytosis · ABC transporters · Purinergic receptor signaling · ATP internalization

Introduction

Intratumoral Extracellular ATP

Our studies of extracellular ATP (eATP) started with our anticancer therapeutics study. In one of the earlier studies more than a decade ago, we found that our lead glucose transport inhibitor WZB117 (Zhang et al. 2010a; Liu et al. 2010, 2012) significantly inhibited cancer cell growth by blocking glucose uptake of cancer cells, resulting in eventual reduction of intracellular ATP concentration, while addition of high μM to very low mM eATP significantly reduced the inhibition and increased cancer cell survival (Zhang et al. 2010a; Liu et al. 2010, 2012). After this finding, we discovered that several publications described the surprisingly high intratumoral and extracellular ATP levels in several different tumor models (Pellegatti et al. 2008; Wilhelm et al. 2010; Michaud et al. 2011; Falzoni et al. 2013; Morciano et al. 2017). The ATP-detecting and -measuring technology developed by these cancer researchers was based on a cell line HEK293 that expresses a luciferase reporter gene on the exterior surface of the cells, which can be used as an ATP sensor (HEK293-pmeLUC cells). When the luciferase-expressing cells were injected into nude mice with xenografted human tumors on the flanks of the mice, for some reasons that are still not fully understood, some of these luciferase-expressing cells would migrate to the tumor sites and grow together with tumor cells (Pellegatti et al. 2008). When luciferin, the substrate of luciferase, was injected into mice and the mice were subsequently subjected to fluorescence imaging at various times after tumor cell injections, the sites of the tumors could be shown by the fluorescence imager. At the same time, concentrations of intratumoral extracellular ATP could be measured by detecting fluorescence generated by the luciferase-catalyzed light reaction using intratumoral eATP as the substrate (Lobas et al. 2019; Zhu et al. 2018). Fluorescence microscopy for tumor imaging based on ATP–luciferase reporting technology has been extensively used in cancer research including in metastasis studies (Elshafae et al. 2017; Tweedle et al. 2018; Zarychta-Wiśniewska et al. 2017; Lim et al. 2009). Furthermore, the fluorescent brightness of the tumor is

directly proportional to the ATP concentration in the extracellular space of the tumors, or the tumor microenvironment (TME) (Falzoni et al. 2013; Michaud et al. 2011; Morciano et al. 2017; Pellegatti et al. 2008; Wilhelm et al. 2010). With a luciferase-catalyzed light reaction standard curve, the relative and absolute concentrations of ATP in the TME can be estimated by comparing the light intensity of the tumor with those in the standard ATP curve. Several published studies using the same luciferase system reported relatively consistent observations: the intratumoral eATP found in the xenografted tumors of different types were always in the range of 100–600 μM or more in concentration, which are 10^3 to 10^4 times higher than the eATP concentrations found in normal tissues (Pellegatti et al. 2008; Wilhelm et al. 2010; Michaud et al. 2011; Falzoni et al. 2013; Morciano et al. 2017; Conley et al. 2017; Rajendran et al. 2016; Wang et al. 2013). eATP concentrations found in a given tumor are time dependent and vary as tumors grow. However, factors that control and regulate eATP concentrations in the tumor microenvironment (TME) have not been fully studied and are presently unclear. The information is even scarcer for the intratumoral eATP concentrations in naturally occurring tumors.

Macropinocytosis

Because of the relatively constant and very high intratumoral extracellular ATP (ieATP) concentrations found in several tumor models, we speculated that these would be the ieATP concentrations present in most cell line generated tumors and used these ATP concentrations in our subsequent cancer cell line and tumor studies. After observing that eATP rescued cancer cells from anticancer compound treatment, we hypothesized that eATP is internalized by cancer cells by some mechanisms such as macropinocytosis, partly because macropinocytosis was known to be upregulated in most cancers and partly because macropinocytosis is well known to nonspecifically internalize various molecules present in the extracellular environment (Bloomfield and Kay 2016; Commisso 2019; Commisso et al. 2013; Recouvreux and Commisso 2017; Swanson and King 2019). In these studies, we used the fluorescent macropinocytosis tracer high molecular weight fluorescent dextran (HMWFD) (Commisso et al. 2013, 2014) and non-hydrolyzable fluorescent ATP (NHFATP) to co-incubate with non-small cell lung cancer (NSCLC) A549 and H1299 cells, and nontumorigenic lung NL-20 cells, respectively. We observed the colocalization of green fluorescence NHFATP with red fluorescent HMFD inside the cells after 30–45 min of coincubation, making the combined (superimposed) color of the colocalized molecules orange. The merged orange color indicates that the ATP was internalized by macropinosomes along with the macropinocytosis tracer HMFD (Qian et al. 2014). From this result, we concluded that ATP, in the form of extracellular fluorescent ATP, can be internalized by cancer cells via macropinocytosis (Bloomfield and Kay 2016; Commisso 2019; Commisso et al. 2013; Recouvreux and Commisso 2017; Swanson and King 2019). Since

macropinocytosis can engulf large molecules such as proteins and indiscriminatory to small molecules, we concluded that macropinocytosis would be able to internalize regular ATP as well. Macropinocytosis-mediated NHFATP internalization was also found in non-tumorigenic NL-20 lung cells, but the level of the internalization was much lower compared with A549 cells (Qian et al. 2014). This result supports the notion that macropinocytosis is upregulated in most cancer cells compared with the normal cells from which the cancer cells are derived. When low molecular weight fluorescent dextran (LMFD) and endocytosis inhibitors were used in a similar study, it was found that, in A549 cells, other types of endocytoses such as Clathrin-mediated endocytosis and Caveolae-mediated endocytosis, were also present in A549 cells and contribute to the ATP internalization, although at lower levels compared with macropinocytosis (Qian et al. 2016). We subsequently made the same observation in several other cancer cell lines in other cancer types. We, therefore, made a speculation that most cancer cells use macropinocytosis and/or other endocytoses to internalize eATP, in addition to other extracellular nutrients such as glucose and amino acids. Our experimental results strongly support the notion that eATP and iATP are integral parts of the Warburg effect (Chen et al. 2015; Koppenol et al. 2011) in vivo and a relatively under-explored area of opportunistic uptake of extracellular nutrients by cancer cells, which is a top hallmark of cancer metabolism (Pavlova and Thompson 2016). The above-described phenomenon was originally observed in cultured cancer cells. We were also interested in finding out if the same observation could be made in vivo. To that end, we generated A549 tumors on the flank of nude mice first, and then co-injected NHFATP and HMFD into tumors. Fluorescence microscopy revealed that NHFATP was also internalized by A549 tumor cells, indicating that macropinocytosis was also operational in vivo (Qian et al. 2016). We recently have done more tumor ATP injection studies to confirm that other cancer cell lines of other cancer types also internalize ATP in vivo, with different cell lines showing different levels of macropinocytosis and variable levels of dependence on macropinocytosis (unpublished data). Figure 4.1 summarizes the macropinocytosis-mediated eATP internalization and subsequent release from macropinosome.

Scientific and Technological Challenges in ATP Research

Instability of ATP

Although macropinocytosis-mediated NHFATP internalization has been shown in multiple cancer cell lines and cancer types, NHFATP is not structurally identical to ATP due to the presence of a relatively large fluorescence-generating tag that is absent in regular ATP. Presently, there is no technology that could directly observe and keep track of ATP inside cells, as regular ATP is turned over (hydrolyzed) very rapidly, within minutes. Another technology that can partially solve this problem is by using a radiolabeled nonhydrolyzable ATP, such as regular or radioactive

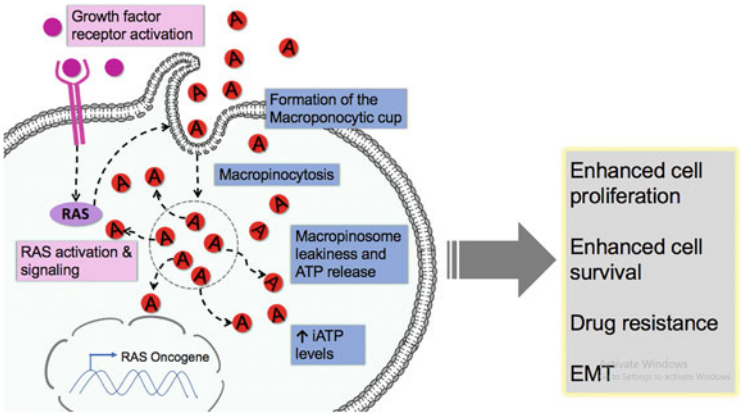

Fig. 4.1 How extracellular ATP is internalized into cancer cells by macropinocytosis. In cancer cells, particularly those that harbor the RAS oncogene, growth factor receptor activation and signaling lead to the downstream activation of RAS, promoting the macropinocytic process. Changes in membrane curvature and invagination form the macropinocytic cup, which indiscriminately engulfs and takes up nutrients, including eATP, into the cancer cells. From here, the ATP is presumably released into the cytosol, perhaps by the leaky nature of macropinosomes, and diffuses through the cell where it functions at multiple levels to promote increased cell proliferation, increased cell survival, drug resistance, and EMT. These are just a few of macropinocytosed-ATP related functions, and this list is likely to continue to expand as our knowledge of ATP in cancer grows

AMP-PNP (Dauter and Dauter 2011; Korkhov et al. 2012; Muftuoglu et al. 2016). The advantages of using radioactive AMP-PNP are that it is much longer-lasting inside cells, it is radioactive so that it can be traced, and it is structurally very similar to ATP so that its intracellular trafficking is likely to be similar to that of ATP. The disadvantage of radiolabeled nonhydrolyzable AMP-PNP, however, is that it can only be quantitatively measured but not visualized like NHFATP.

When we incubate cancer cells with ATP at the concentrations found in tumors, we observed an increase of dose-dependent cell proliferation by either a cell viability assay or a direct cell counting (Qian et al. 2014, 2016). We speculated that internalized eATP increases intracellular ATP (iATP) concentrations inside of cancer cells, and this elevated iATP concentration, in turn, accelerates all biochemical reactions that depend on ATP as either a reaction cofactor or a phosphate donor (as in the case of protein phosphorylation in cell signaling). ATP assays measuring lysate samples of cells treated with or without eATP revealed that the iATP levels increased from 50% to more than 100% within 2–4 h of cell incubation with eATP at the ATP concentrations found in the TME (Qian et al. 2014, 2016). We also observed that the iATP elevation rates were cell line dependent, which is, in turn, macropinocytosis/endocytosis rate dependent. The dependence on macropinocytosis/endocytosis for iATP level increase has been confirmed since the addition of macropinocytosis/endocytosis inhibitors to the ATP containing cell culture media lowers the iATP levels (Qian et al. 2014, 2016).

ATP Release from Macropinosomes

Presently, no study has been done to investigate if and how internalized ATP is released from macropinosomes. However, all the evidence strongly suggests that the internalized eATP is indeed released from macropinosomes. For example, if the internalized ATP is not released and is instead degraded in macropinosomes and their derived endosomes, then how can we explain the phenomenon of eATP promoted cell proliferation and increased drug resistance, let alone the induced EMT? Fortunately, past literature reveals that for reasons still not fully understood, macropinosomes and their derived endosomes appear to be leaky in nature (Khalil et al. 2006; Meier et al. 2002; Norbury et al. 1995; Schwake et al. 2013; Wadia et al. 2004), making it possible that the macropinosome-internalized eATP leaks out and diffuses through the cytosol and even to the nucleus to perform its diverse biological functions. The intrinsic leakiness of macropinosomes makes sense, as macropinocytosis indiscriminately engulfs extracellular nutrients for their immediate usage inside the cell. It does not make biological or energetic sense for macropinosomes to degrade all the engulfed nutrients only to restart biosynthesis from scratch again. It is much more metabolically and energetically favorable for macropinosomes to leak their contents and use them as they are. In principle, ATP release from macropinosomes could be studied using NHFATP and by macropinosome/ trafficking over time with antibodies targeting different macropinosome/endosome protein markers. Alternatively, it can also be studied using low molecular weight dextran coupled with antibodies against macropinosome/endosome markers. Through this study the timing of the release of ATP or its substitutes and from what type of endosomes could be determined. It is both biologically interesting and important to know how and how fast macropinosome-encapsulated ATP is released into the cytosol after it is taken into cells.

Multi-locational and Multi-functional Extracellular ATP in Cancer

ATP as an Energy Provider for Biochemical Reactions

Once the internalized eATP is released from macropinosomes and macropinosome-derived endosomes, the released ATP elevates cytosolic and nuclear ATP concentrations, and participates in and contributes to several biological processes, which drastically affect fates of cancer cells. First, it functions as an energy molecule to speed up all biochemical reactions that use ATP as the energy source. If iATP levels are increased by 100% and if iATP levels are increased relatively uniformly throughout the cell, then the biochemical reaction rates will also be proportionally increased. This is what we observed in the increased drug resistance mediated by the

efflux of anticancer drugs by ATP-Binding-Cassette (ABC) pumps (Komarova and Wodarz 2005; Kovalev et al. 2013; Marquez and Van Bambeke 2011; Wu et al. 2014), in which more ABC pumps are phosphorylated and activated, leading to faster working ABC pumps, increased drug efflux, and augmented drug resistance by reducing intracellular drug concentrations (Wang et al. 2017, 2019). In this situation, due to the increased energy (ATP) supply, ABCs could pump out more anticancer drugs without substantially increasing ABC protein expression or increasing ABC protein concentrations as much as when iATP concentration is not increased. In other words, in this situation, increased ABC efflux of anticancer drugs can be achieved just by iATP increase alone, rather than increasing ABC protein levels. The correlation of iATP levels and cancer cell phenotypes has been known. Cancer cells were found to have higher iATP levels than the cells from which cancer cells are derived (Zhou et al. 2012). Even more dramatic, cancer cells that are resistant to anticancer drugs show even higher iATP levels than those cancer cells from which the resistant cells are selected (Schneider et al. 2013). From these earlier observations, it could be concluded that cancer cells, particularly the drug-resistant cancer cells, have higher iATP levels, either as a consequence of the tumorigenic changes or as a prerequisite for cancer or drug resistance. The correlation between iATP levels and cancer cells and between iATP levels and resistant cancer cells strongly suggest that iATP levels are associated with higher survivability of cancer cells.

ATP as a Phosphate Donor for Protein Phosphorylation and Signal Transduction

iATP can also function as a phosphate donor in those signaling pathways in which protein phosphorylation is used for signal transduction. Therefore, it is also conceivable that, if multiple protein phosphorylation reactions occur in one signaling pathway, then a large enhancement in the signal transduction follows, leading to drastic enhancement of the intensity of the signal transduction. As expected, in our previous studies, we observed that eATP treated cancer cells show increased protein phosphorylation for growth factor receptors such as PDGFR and its downstream signaling proteins such as Akt, ERK, and MAPK, eventually leading to increased cancer cell survival (Cao et al. 2019; Wang et al. 2017; Wang et al. 2019). This effect in combination with accelerated ABC efflux activity further augments drug resistance. It is important to point out that there are different types of ABCs and they efflux different drugs (Linton 2007; ter Beek et al. 2014). ABC's presence in cells and cancer cells are not for anticancer drug efflux alone, but for removing toxic or unwanted molecules from inside of cells. Thus, iATP-resulted increase of ABC efflux is likely to have more and wider fundamental biological functions than just drug efflux.

To confirm macropinocytosis' roles in eATP's activities, we used multiple approaches to inhibit macropinotysis including chemical inhibitors, siRNA knockdown, and CRISPR knockout to downregulate key proteins or genes involved macropinocytosis, resulting in significantly reduced eATP-induced activities in different cancer cell lines. These include reductions in ATP internalization, iATP levels, cell growth and proliferation, drug resistance, and epithelial-to-mesenchymal transition (EMT) (Cao et al. 2019; Qian et al. 2014, 2016; Wang et al. 2017). Depending upon specific activities, the inhibitions of macropinocytosis resulted in up to 50% of reduction of the eATP induced activities, indicating that up to 50% of the eATP-induced activities could be attributed to macropinocytosis-mediated eATP internalization. Looking at the issue from a different angle, up to half of the eATP-induced activities are intracellular in nature. Of note, the contribution of macropinocytosis to eATP internalization is found to be cell type dependent and KRas-status dependent (Qian et al. 2014, 2016).

A hypothetical model for eATP-induced drug resistance in cancer cells is shown in Fig. 4.2.

Beyond Drug Resistance: eATP Induces EMT

ATP and TGF-β

ATP has also been found to play important roles, like TGF-β, in inducing epithelial-to-mesenchymal transition (EMT) (Cao et al. 2019; Kang et al. 2019; Katsuno et al. 2013; Takai et al. 2012; Xu et al. 2009) and cancer stem cells (CSC) (Hao et al. 2019; Ledur et al. 2012; Sciacovelli and Frezza 2017; Yadav et al. 2020; Yang et al. 2019) by different groups. Our new contributions to these fields are: (1) we have generated a stimulatory and regulatory link among eATP/ATP and EMT/CSC, forming a single "unified model" (Fig. 4.3), (2) we have shown that ATP is TGF-β-like and can potentially supplement or even replace TGF-β in inducing EMT (Cao et al. 2019), (3) we have hypothesized that eATP/ATP functions as an emerging master regulator/inducer, similar to TGF-β, of EMT/CSC. Cancer cells in tumors appear to be able to use either ATP or TGF-β for metastasis induction, increasing tumor cells' flexibility in responding to different available inducer molecules, and (4) Identifying ABC (Komarova and Wodarz 2005; Kovalev et al. 2013; Linton 2007; Marquez and Van Bambeke 2011; ter Beek et al. 2014; Wu et al. 2014) and Wnt genes (Zhan et al. 2017; MacDonald et al. 2009; Basu et al. 2018; Martin-Orozco et al. 2019; Zhong and Virshup 2020) as parts of the induction and maintenance system for EMT/CSC/drug resistance, in which their drug resistance function may be a "by-product" of increased survivability and decreased cell death.

Ample experimental evidence strongly supports the notion of eATP being a master regulator and inducer, like TGF-β, for EMT, CSC formation, ABC drug efflux activity, and metastasis. Some of the major evidence is listed below.

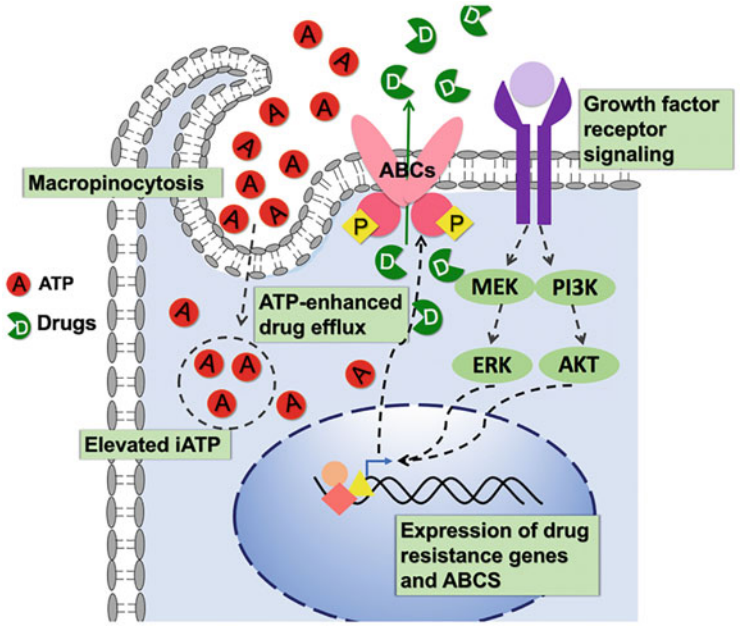

Fig. 4.2 Model for eATP-induced and iATP-augmented drug resistance in cancer cells. ATP in the tumor microenvironment is taken up via macropinocytosis and released inside of the cell to drastically increase intracellular ATP (iATP) levels. ATP participates in a variety of drug resistance-enhancing activities from within the cell. First, ATP functions as a phosphate donor to speed up ABC function and anticancer drug efflux from the cell. At the same time, ATP functions to enhance signaling pathways, including those involving growth factor receptor signaling and downstream signaling pathways including PI3K/AKT and MEK/ERK pathways through phosphorylation. These pathways lead to the transcription of drug resistance-related genes, including ABCs. Thus, ATP functions to promote the expression of ABCs as well as by increasing their drug efflux pumping activities

1. eATP concentrations in the TME (ieATP) are in the range of 100–500+ μM, or 10^3 to 10^4 times higher than its levels in normal tissues (Falzoni et al. 2013; Michaud et al. 2011; Morciano et al. 2017; Pellegatti et al. 2008; Wilhelm et al. 2010), forming a basis for all activities of ATP in tumorigenesis and metastasis. Such high eATP concentrations make it possible that macropinocytosis becomes a self-propelled process that is preferably adopted and almost universally used by cancer cells.
2. High ieATP concentrations have long been recognized as a danger signal for cells ranging from bacteria to humans (Rodrigues et al. 2015; Di Virgilio et al. 2016a, b; Trautmann 2009; Gazzerro et al. 2019; Kouzaki et al. 2011; Feng et al. 2020; Gilbert et al. 2019; Vultaggio-Poma et al. 2020; Ramadan et al. 2017). It is conceivable that ieATP also serves as a danger signal for cancer cells in tumors, warning them of the coming danger triggered by deteriorating hypoxia, poorer nutritional environment, and increased cancer cell death leading to lysis of

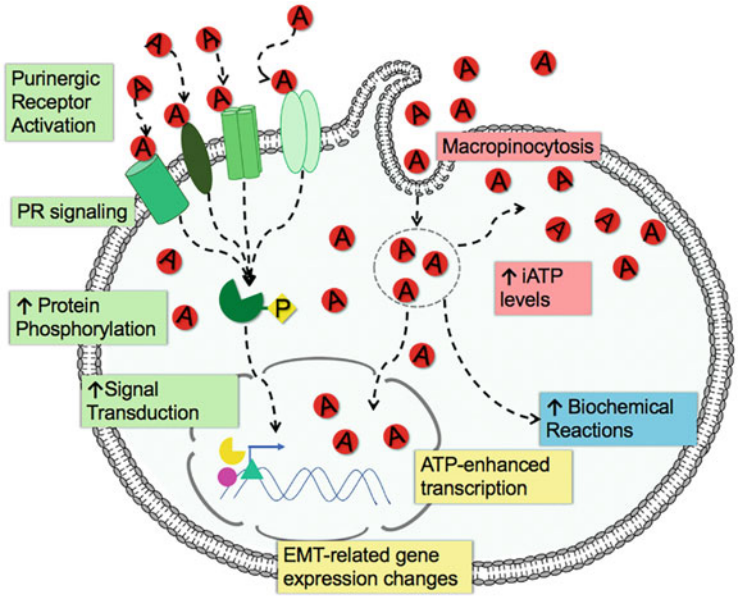

Fig. 4.3 A most current hypothetical model for functions of extracellular ATP and macropinocytosis-mediated eATP internalization. Extracellular ATP functions from inside and outside of the cell. Outside of the cell, ATP at different concentrations in the tumor microenvironment binds and activates various purinergic receptors on the cell surface. Activation of these receptors and subsequent purinergic receptor signaling results in changes in gene expression, including those needed to promote the EMT transcriptional processes and mesenchymal phenotype. Meanwhile, eATP can be taken up by macropinocytosis to function from inside the cell, significantly increasing intracellular ATP levels in an energy gaining and cost-free process. This intracellular ATP then broadly functions from inside of the cell to increase rates of biochemical reactions, increase rates of signaling cascades through enhanced protein phosphorylation (including those signaling reactions involved in purinergic receptor signaling), and in the nucleus where ATP is needed for almost every step of transcription. eATP is indeed an omnipresent and near-omnipotent molecule in cancer

cancer cells and release of intracellular ATP to TME, in addition to stromal cell-released ATP (Aymeric et al. 2010; Ayna et al. 2012; Martins et al. 2014; Zefferino et al. 2021). High levels of ieATP also energetically enable cancer cells to leave their original locations (such as inside of primary tumors) for other safer places (outer surface of primary tumors and at distant sites for forming new colonies as a result of metastasis). This is because directional cell movement is an energy-consuming process. Our ATP-EMT study has shown these effects in human lung cancer cells (Cao et al. 2019).

3. eATP is a messenger/activator for purinergic receptors (PRs), which are located on the plasma membrane of cells, including cancer cells, and play roles in EMT, CSC, and ABC activities (Di Virgilio et al. 2016a, b, 2018; Burnstock and Di Virgilio 2013; Di Virgilio and Adinolfi 2017; Ferrari et al. 2017). It is noteworthy that there are different types (classes) of PRs that have different affinities for ATP,

and these different classes of PRs are activated by different extracellular concentrations of ATP in order to mediate different and specific cellular responses. The ATP concentrations that activate different PRs, in general, are compatible with the eATP concentration range found in the TME.

4. TGF-β is well-known to be an EMT and CSC inducer (Kang et al. 2019; Katsuno et al. 2013; Sciacovelli and Frezza 2017; Takai et al. 2012; Xu et al. 2009). One TGF-β-mediated activity in cancer cells is induced exocytosis of ATP-containing vesicles from the TGF-β activated cells (Sakaki et al. 2013; Takai et al. 2012). The released ATP, now functioning as eATP, activates purinergic receptors (PRs) (Di Virgilio et al. 2016a, b, 2018; Burnstock and Di Virgilio 2013; Di Virgilio and Adinolfi 2017; Ferrari et al. 2017) in a signaling cascade downstream of TGF-β (Kang et al. 2019; Katsuno et al. 2013; Sciacovelli and Frezza 2017; Takai et al. 2012; Xu et al. 2009). Thus, specifically for activating PR signaling, the functions of eATP and TGF-β are the same and redundant but are eATP concentration dependent. This fact supports the notion that eATP can trigger some TGF-β-like activities, replacing TGF-β for inducing certain EMT and metastasis-related activities. We have made observations that 0.5 mM eATP could induce higher activities such as migration and invasion than physiological levels of TGF-β (Cao et al. 2019). One possible reason for this phenomenon is that TGF-β alone is unlikely to trigger the release of ATP at such a high ATP concentration, resulting in lower eATP levels and lower PR-mediated signaling activities.
5. eATP can also be internalized by cancer cells through endocytosis, particularly macropinocytosis, a subtype of endocytosis which is upregulated in most cancer cells (Bloomfield and Kay 2016; Commisso et al. 2013; Recouvreux and Commisso 2017; Commisso 2019; Swanson and King 2019; Canton 2018; Lim and Gleeson 2011; Palm 2019), particularly those with Ras mutations (for example, A549 cells have KRas mutation) (Commisso 2019; Finicle et al. 2018; Hobbs and Der 2020; Jayashankar and Edinger 2020; Lin et al. 2020; Zwartkruis and Burgering 2013). Macropinocytosis, as an important part of opportunistic uptake of extracellular nutrients, has been named a top hallmark of cancer metabolism (Pavlova and Thompson 2016). Macropinocytosis-mediated eATP internalization has been shown by us (Cao et al. 2019; Chen et al. 2015; Qian et al. 2014, 2016 ; Wang et al. 2017, 2019), leading to large (>50–100%) increases in intracellular ATP concentration (Qian et al. 2014, 2016; Wang et al. 2017, 2019). Elevated intracellular ATP (iATP), once diffused into the nucleus, can function as a transcriptional cofactor in gene expression, accelerating transcription process at various steps such as transcription initiation, elongation, termination, and chromatin remodeling (Fishburn et al. 2016; Conaway and Conaway 1988; Kopytek and Peterson 1998; Kim et al. 2000; Porrua and Libri 2015; Kugel and Goodrich 1998; Cho et al. 2013; Wright et al. 2016; Chen et al. 2018) ATP is unique among transcription cofactors in that it can function as an energy provider to trigger structural changes of the transcription complexes necessary for activation of transcription, and it also serves as a substrate for RNA synthesis. In other words, it works on and accelerates its

own polymerization. ATP must be an ancient molecule that actively participated in the RNA world (Cech 2012; Horning and Joyce 2016; Robertson and Joyce 2012), and has been selected to continue to play its vitally important roles in the present "protein world," including the process of transcription.

Macropinocytosis as a Self-Propelled Process in Cancer Cells

On the surface, it appears that macropinocytosis is an energy-consuming and energy-intensive process that may not be used very frequently by animal cells. This is true for normal cells. Only specialized cells such as T lymphocytes, macrophages, dendritic cells, and neurons heavily use macropinocytosis for their specialized functions (Canton 2018; Lim and Gleeson 2011; Mercer and Helenius 2009; Li et al. 2020; Liu and Roche 2015). However, this rule does not apply to cancer cells in tumors. This is because in the TME, ATP concentrations are 10^3 to 10^4 times higher than in normal tissues, making macropinocytosis in tumor cells a self-propelled, "cost-free," or even an "energy gaining" process. Since eATP concentrations are so high in the TME and tumor cells can use macropinocytosis to internalize an unlimited amount of eATP, macropinocytosis can be sustained without requiring tumor cells to synthesize much ATP. The presence of extremely high ATP concentrations in the TME is likely to be a major contributing factor for macropinocytosis to become a key hallmark for cancer metabolism (Pavlova and Thompson 2016). The higher ieATP concentrations (and higher concentrations of other nutrients in the TME) select the tumor cells for their macropinocytotic phenotype, while the macropinocytotic phenotype drastically enhances the numerous tumorigenic effects eATP mediates. Figure 4.3 summarizes the effects of eATP and macropinocytosed eATP.

Table 4.1 also summarizes these effects with references.

Table 4.1 Extracellular ATP's activities promoted by maropinocytosis—an expanding list

Extracellular ATP's activity	Shown in vitro and/or in vivo	References
Increased cell survival	In vitro	Aymeric et al. (2010), Ayna et al. (2012), Basu et al. (2018)
eATP internalization and intracellular ATP concentration increase	In vitro and in vivo	Elshafae et al. (2017), Falzoni et al. (2013), Feng et al. (2020)
Cancer cell growth and proliferation	In vitro and in vivo	Elshafae et al. (2017), Falzoni et al. (2013), Feng et al. (2020)
Increased resistance to both chemo and target anticancer drugs	In vitro	Kang et al. (2019), Katoh (2017)
Induced EMT, increased invasion	In vitro	Khajah et al. (2018)

Targeting Macropinocytosis and eATP in Cancer Therapy

Targeting Macropinocytosis

As macropinocytosis is upregulated in cancer cells and eATP levels in the TME are 10^3 to 10^4 times higher than in normal cells, targeting macropinocytosis and eATP either individually or in combination is likely to be novel and effective strategies in reducing tumor growth, drug resistance, and even metastasis.

In our previous studies, we demonstrated that inhibiting macropinocytosis led to a reduced increase in intracellular ATP levels, slower cancer cell growth and proliferation, and weaker drug resistance (Qian et al. 2014, 2016; Wang et al. 2017, 2019). More recently, using the CRISPR-Cas9 approach, we knocked out sorting nexin (*SNX5*), a key gene involved in macropinosomal trafficking (Cao et al. 2019; Kerr et al. 2006; Lim et al. 2008, 2015; Vanlandingham and Ceresa 2009; Schwake et al. 2013), but not in other cellular functions such as cell movement. The *SNX5*-KO cells grew much slower than the wild-type A549 cells from which the KO cells were derived (Wang et al. 2017, 2019). In addition, the *SNX5*-KO cells-derived tumors grew much slower than the wild-type A549 tumors, indicating the importance of SNX5 and macropinocytosis in tumor growth and implying potential value of targeting macropinocytosis in new cancer therapies. However, careful design of the inhibition must be made to prevent severe side effects since macropinocytosis is also used by some normal cells. Therefore, it may be more advantageous and safer to target the macropinocytosis regulatory process rather than macropinocyotosis effectors which are specific or selective for cancer cells but not normal cells. More studies are needed to identify such target(s).

Targeting Extracellular ATP

Targeting eATP in the TME may be equally or even more effective. First, eATP in the TME is 10^3 to 10^4 times higher than in normal tissues. The very large difference in the ATP concentration itself may make it an attractive target. However, ATP or eATP is used everywhere in the body. A clear distinction between concentrations of TME eATP and eATP in normal tissues may be a prerequisite for effectively targeting cancer cells in the tumors without harming normal cells. One way to distinguish the TME from normal tissues is that the pH of the TME is significantly lower than those of normal tissues (Zhang et al. 2010b; Swietach et al. 2014; Shirmanova et al. 2015; Ward et al. 2020; Thews and Riemann 2019; Hao et al. 2018; White et al. 2017; Boedtkjer and Pedersen 2020). The pH of normal tissues is usually slightly higher than 7, similar to our physiological pH. In contrast, pH in the TME is acidic due to hypoxia and secretion of large amounts of lactate by cancer cells. The pH of the TME ranges from low to high 6 (Zhang et al. 2010b; Swietach et al. 2014; Shirmanova et al. 2015; Ward et al. 2020; Thews and Riemann 2019;

Hao et al. 2018; White et al. 2017; Boedtkjer and Pedersen 2020). If a pH-sensitive ATPase can be developed and delivered, then the ATPase will be inactive at pH above 7, but becomes active at 6.5 or lower, efficiently degrading eATP in the TME without touching eATP in normal tissues. The ATPase approach was tried and shown to be effective but the method used was not pH sensitive (Ledur et al. 2012), providing promising signs for the approach. Although it was effective, it is not applicable in real tumors, as the method used ATPase injections directly into tumors. Therefore, this tool has to be redesigned and redeveloped to produce selectivity for the TME. Inhibitor of V-ATPase was also described as an anticancer method as it is partially responsible for the acidic pH of the TME (Kulshrestha et al. 2019; Pérez-Sayáns et al. 2009). We have demonstrated that eATP's cancer cell proliferation-promoting activity is ATP dose dependent. Its cell proliferating and invasion-inducing activities are minimal when the eATP level is lower than 100 μM, the lower end of the TME ATP concentrations detected (Falzoni et al. 2013; Michaud et al. 2011; Morciano et al. 2017; Pellegatti et al. 2008; Wilhelm et al. 2010). Therefore, an ATPase will show selectivity for eATP in the TME if the chosen ATPase is genetically engineered to have its K_m around 100 μM or above. This way, the ATPase is much less active when the ATP concentration is around or below 100 μM, but much more active when the concentration is above 100 μM. With this double safety feature of pH sensitivity and higher K_m values, the genetically engineered ATPase will be functional primarily in the TME, not in the normal tissues. For an even safer approach, a pro-ATPase prodrug can be designed. Such a pH-sensitive pro-ATPase will not be active until the pro-ATPase is cleaved in an acidic environment, namely the TME. This way, the active ATPase is further restricted to the low pH TME, degrading only intratumoral ATP. However, there are various types of ATPases in animal cells, which are expressed at different locations in the cell or on the plasma membrane of the cell (Clausen et al. 2017; Khajah et al. 2018; Yang et al. 2014). It is vitally important to carefully select an ATPase candidate that will be active extracellularly in an acid pH environment, but also not greatly interfere with the functions of normal cells by near completely depleting extracellular ATP in normal tissues.

Conclusions and Future Research Perspectives

Although some major advancements have been made in eATP research in cancer, eATP research, particularly in drug resistance, EMT, and metastasis, is still in its early stages and a lot more should be and can be done to further enhance our understanding of the roles of eATP and macropinocytosis in these processes. The following is an incomplete list of studies and priorities.

1. eATP internalization in more human cancer cell lines of many cancer types both in vitro and in vivo—Although we have shown that eATP is internalized by macropinocytosis in human NSCLC cell lines and their derived tumors (Qian

et al. 2014, 2016), we still do not know how prevalent this phenomenon is among all cancers. The same methods such as ATP assays, colocalization assays, and macropinocytosis inhibitor studies (Cao et al. 2019; Qian et al. 2014, 2016; Wang et al. 2017) we used for lung cancer cells can be applied to other cancer cell lines and tumors to determine the prevalence of eATP internalization.

2. *ATP release from macropinosomes*—So far, we know that eATP is internalized by macropinocytosomes (Cao et al. 2019; Qian et al. 2014, 2016), but we do not know how the internalized eATP is released from them. Fluorescent microscopy of cancer cells that are incubated with either fluorescent nonhydrolyzable ATP or radioactive nonhydrolyzable ATP for various times can be studied with various fluorescent antibodies against macropinosome marker proteins and endosome marker proteins to trace the ATP trafficking and also detect the leakiness of macropinosomes (Khalil et al. 2006; Meier et al. 2002; Norbury et al. 1995; Schwake et al. 2013; Wadia et al. 2004) and the rates of ATP release from the macropinosomes/endosomes.
3. *Regulation of macropinocytosis rate and iATP levels*. Although the regulation of macropinocytosis has not been studied in my lab, we have indirect evidence that the process of macropinocytosis is likely to be vigorously regulated. In the different cancer cell lines we studied, the intracellular ATP levels reach equilibrium (peak level) 3–4 h after the incubation starts (Cao et al. 2019; Qian et al. 2014, 2016; Wang et al. 2017). After reaching equilibrium, the iATP levels fluctuate in a relatively narrow range. It would be interesting to find out what proteins and pathways control macropinocytosis, which in turn participate in regulating iATP levels.
4. *ATP-induced drug resistance in cancer cells*—Previously, we reported that eATP significantly enhances resistance of multiple cancer cell lines of multiple cancer types to both chemo and target drugs (Wang et al. 2017). The drug resistance mechanisms of eATP include augmented ABC drug efflux activities and enhanced signaling in cell survival pathways due to increased protein phosphorylation (Wang et al. 2017, 2019). However, additional mechanisms are speculated. For example, eATP is found to induce EMT (Cao et al. 2019) and possibly cancer stem cells (CSC), both of which are known to contribute to drug resistance in cancer (Hill and Wang 2020; Huang et al. 2015; Phi et al. 2018; Prieto-Vila et al. 2017; Shibue and Weinberg 2017; Vinogradov and Wei 2012). Thus, eATP-induced EMT and CSC can be studied in the context of drug resistance.
5. *ATP induced EMT*—Recently, we reported that eATP induces EMT as demonstrated by increased cell migration and cell invasion, downregulation of epithelial type proteins and upregulation of mesenchymal type proteins, and corresponding cell morphology changes such as filopodia formations (Cao et al. 2019). However, we still do not know if and how much eATP-induced EMT is different from EMT induced by TGF-β, a well-established EMT inducer (Katsuno et al. 2013; Xu et al. 2009; Takai et al. 2012; Kang et al. 2019). RNA sequencing, proteomics, and metabolomics approaches can be used to characterize and compare EMT induced by eATP and TGF-β at different doses and different times.

6. *ATP-induced cancer stem cell formation*—It has been known that EMT is intimately associated with CSC and that they share some common markers (Ishiwata 2016; Liu and Fan 2015; Wang et al. 2015). As we have observed that eATP induces EMT, we naturally speculate that eATP also induces CSC and we want to demonstrate this new and important feature. To that end, similar approaches, such as RNAseq and metabolomics, can be used to investigate eATP-induced CSC. If this can be confirmed, eATP's seemingly unlimited capabilities in cancer will be further expanded.

In summary, macropinocytosis is upregulated in most cancer cells and plays some very important functions in internalizing extracellular nutrients available in the TME, significantly contributing to and affecting cancer cell behavior changes, including cell growth, drug resistance, EMT, and possibly CSC. One of the most important extracellular nutrients turns out to be ATP, a molecule that is so prevalent and common, yet has been taken for granted and is often ignored. On the other hand, many groups, including ours, have recently identified some previously unidentified novel activities of ATP, such as hydrotropic activity, which have tremendous scientific and medical implications (Patel et al. 2017). eATP cannot and should not be ignored considering its concentrations in tumors are 10^3 to 10^4 times higher than normal, making it possible for cancer cells to upregulate macropinocytosis to take in enough free eATP to propel the macropinocytosis process in a cost-free situation, while accelerating almost all intracellular biochemical reactions and processes. Because of the prevalence and levels of macropinocytosis in cancer cells and high levels of eATP in tumors, cancer cells may be viewed as a system with a partially open membrane system, having much higher material internalization rates with their environment than normal cells. Unfortunately, these research areas are currently severely under-explored. The studies in these areas are bound to significantly increase our understanding of these key processes such as EMT, CSC, and metastasis, changing our views on eATP and generating new strategies for combating cancer and slowing down cancer development and metastasis (Vultaggio-Poma et al. 2020).

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Chapter 5
Macropinocytosis and Cell Migration: *Don't Drink and Drive*. . .

María-Graciela Delgado, Claudia A. Rivera, and Ana-María Lennon-Duménil

Abstract Macropinocytosis is a nonspecific mechanism by which cells compulsively "drink" the surrounding extracellular fluids in order to feed themselves or sample the molecules therein, hence gaining information about their environment. This process is cell-intrinsically incompatible with the migration of many cells, implying that the two functions are antagonistic. The migrating cell uses a molecular switch to stop and explore its surrounding fluid by macropinocytosis, after which it employs the same molecular machinery to start migrating again to examine another location. This cycle of migration/macropinocytosis allows cells to explore tissues, and it is key to a range of physiological processes. Evidence of this evolutionarily conserved antagonism between the two processes can be found in several cell types—immune cells, for example, being particularly adept—and ancient organisms (e.g., the social amoeba *Dictyostelium discoideum*). How macropinocytosis and migration are negatively coupled is the subject of this chapter.

Keywords Macropinocytosis · Migration · Small GTPases · Cytoskeleton · Actin · Arp2/3 complex · Myosin II · Dendritic cell · Barotaxis

Macropinocytosis Versus Cell Migration: Two Antagonistic Processes

Macropinocytosis is a process by which cells internalize particles, fluid, and membranes from the extracellular space into large vacuoles, referred to as macropinosomes. It was first observed by Anton van Leeuwenhoek's in 1677, who described the phenomenon in "animalcules" changing shape as they moved through a drop of water (Fritz-Laylin et al. 2017). Today macropinocytosis has been studied in several organisms in both physiological and pathological contexts.

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Different cell types are capable of macropinocytosis under the right circumstances. Certain cellular house-keeping processes rely on their macropinocytic capacity: for instance, primary innate immune cells are particularly adept at this task. Immature dendritic cells (DCs) (Sallusto et al. 1995) and macrophages (Redka et al. 2018) can explore their environment and take up antigens via macropinocytosis, which are either subsequently presented to T cells (in the case of DCs) or degraded (in the case of macrophages). Moreover, macropinocytosis enables modulation of synaptic signaling in neurons, by adjusting cell surface receptor availability (Clayton and Cousin 2009). Importantly, macropinocytosis contributes to the development of several diseases, including: (1) neurodegenerative diseases, promoting cell-to-cell transmission of prions (Zeineddine and Yerbury 2015); (2) cancer—an example being pancreatic duct adenocarcinoma cells (Commisso et al. 2013; White 2013)—in which Ras-activated tumor cells use macropinocytosis to obtain nutrients from the environment; and (3) pathogenesis of atherosclerotic disease (Michael et al. 2013).

During macropinocytosis, membrane ruffles form a cup-like structure that closes up at its distal tips to form a relatively large vacuole (greater than 250 nm), known as the macropinosome. Once in the cytoplasm, a macropinosome follows the endosomal pathway, participating in membrane fusion–fission interactions with components of the endocytic compartment. This leads to the formation of a mature, acidic, and often tubular, structure—a process known as macropinolysosome formation. This series of events has mainly been described in non-myeloid cell types that perform macropinocytosis induced by growth factors (reviewed nicely in Bloomfield and Kay 2016).

Today, there is a consensus that macropinocytosis proceeds in one of two modes: induced or constitutive. The “induced” form is a transient endocytic specialization, which is growth factor-induced and actin-dependent (Canton 2018). Induced macropinocytosis involves the generation of dynamic protrusions of the whole plasma membrane of the cell, and it is triggered by the activation of the cytoskeleton connected to the plasma membrane. An alternative, “constitutive”, form of macropinocytosis has been observed both in vitro and in vivo; however, it is restricted to primary immune cells (von Delwig et al. 2006). Although its mechanism has remained understudied (Canton 2018), it was shown that it requires the presence of extracellular calcium. Furthermore, constitutive macropinocytosis is less sensitive to perturbations in intracellular/cytosolic pH, and it leads to morphologically distinct macropinosomes (Canton et al. 2016).

Constitutive macropinocytosis is the less understood type, mainly because of the notorious difficulty in culturing and genetically manipulating primary immune cells. Several questions remain unanswered: for example, little is known about the cellular energy cost of macropinocytosis. It is conceivable that it would be very sizeable, considering that macrophages, for example, internalize their entire cell surface every 33 min (Steinman et al. 1976). How cells deal with this “compulsive” tendency to internalize extracellular material and satisfy the energy demand associated with this dynamic whole-cell rearrangement remains a fascinating open question.

Cells in many eukaryotic phyla create a variety of dynamic protrusions that project forward in the direction of their migration (see Box 5.1). Cells performing constitutive macropinocytosis can be highly motile. Striking examples are dendritic cells (DCs), which navigate complex environments without forming specific molecular adhesions, and are responsible for capturing antigens in peripheral tissues, transporting them to lymph nodes and present them to T lymphocytes on major histocompatibility complex (MHC) molecules (RicciardiCastagnoli et al. 1996). A crucial aspect of this phenomenon is competition for the limited cytoplasmic resources required for cell motility on the one hand, and the establishment of macropinosomes on the other. How does a cell deal with these intrinsic limitations? A fascinating dilemma.

One of the first pieces of evidence for this antagonism came from *Dictyostelium discoideum*, a social amoeba that constitutes a valuable model for studying cell migration and endocytic processes. In the wild, *Dictyostelium* cells feed themselves by phagocytosing bacteria. However, most lab work has been done in axenic strains (i.e., those able to grow in the absence of living prey). These strains are suitable for studies of fast constitutive macropinocytosis, because they have been selected by growth in medium lacking bacteria. In this organism, chemoattractants such as cyclic AMP and folic acid stimulate both migration and macropinocytosis (Jowhar et al. 2010).

Box 5.1 Generation of tridimensional pseudopods allows cells to migrate

Many studies targeting free-living amoebae and immune cells have shown that both make highly dynamic tridimensional pseudopods at the cell leading-edge. These 3D pseudopods are filled with branched actin networks nucleated and organized by the actin-related protein ARP2/3 complex (Buenemann et al. 2010; Butler et al. 2010). Amoebae and immune cells are also highly motile, their migrating velocities typically being in the range of tens of μm/min (100–1000 times faster than fibroblast motility). Interestingly, during this navigation mode, there is an absence of specific, high-affinity adhesions to the extracellular environment—a locomotory mode known as α-motility (Fritz-Laylin et al. 2017). Some organisms using α-motility may employ additional methods for generating forward movement, such as contractility, retrograde flow, and/or blebbing (Mierke 2015). α-motility appears to be restricted to the animal lineage, where it relies on the presence of specific ligands in the extracellular environment. Nevertheless, the evolutionary conservation of both cell migration and constitutive macropinocytosis confirm their functional significance and interrelation.

Using mouse fibroblasts, it was shown that macropinocytosis could also have a beneficial effect on growth factor-stimulated cell migration. Engulfed surface integrins proceed to transit inside the cells in endosomal compartments, and are

subsequently recycled to the plasma membrane to replenish new focal adhesions (Gu et al. 2011). Interestingly, live imaging showed that this process seems to be uncoupled in time, and hence, cells reduce their motility while cycling integrins via macropinocytosis.

Other relevant evidence supporting the antagonism and its conservation among the animal kingdom has recently been found in an emerging eukaryotic model: the amoeba *Naegleria* (Velle and Fritz-Laylin 2020). In this work, involvement of the Arp2/3 complex in migration and phagocytosis processes was described. Importantly, *Naegleria* almost completely lacks microtubules, expressing a divergent type of tubulin that is only present during mitosis (Chung et al. 2002). It is furthermore intriguing to note that *Naegleria* belongs to a completely distinct lineage of eukaryotes, the Discoba, which are evolutionary far from the Opisthokonts, which include *Mus* and *Homo* among many other genera, and also different from the Amoebozoa group, which includes the genus *Dictyostelium*. Interestingly, analysis of actin-related genes in *Naegleria gluberi* and *Naegleria fowleri*, also known as the brain-eating amoeba, revealed high expression of actin regulators. When the Arp2/3 complex was inhibited, a decrease in actin-rich structures such as lamellar ruffles was observed, together with a decrease in migration speed. The phagocytic capacity of the cells was also impaired by disruption of the Arp2/3 complex, suggesting that even in evolutionarily distant organisms these two processes may be related (Dey et al. 2020).

In the next sections, we will analyze the molecular mechanisms supporting antagonism between macropinocytosis and cell migration, and its biological impact.

Antagonism Between Macropinocytosis and Cell Migration: Molecular Mechanisms

How are macropinocytosis and cell migration integrated in space and time to optimize cell function, for example in a phagocyte that is patrolling its environment in search of food? How are cytoskeleton regulators selectively targeted to allow a cell to control such as an enormous rate of membrane remodeling? Both questions can be answered by reference to a confirmed observation: macropinosome formation inhibits oriented movement, and the activities are mutually exclusive (Chabaud et al. 2015; Veltman et al. 2016).

Macropinocytosis is initiated by the actin-dependent extension of plasma membrane into ruffles, which implies an active modulation of the actin cytoskeleton. A striking observation is that the cytoskeletal activities required for creating macropinosomes are also needed for oriented cell migration (see Fig. 5.1 and Box 5.2). While epithelial cells move as collective sheets, there are many cell lineages that naturally migrate as individuals or small groups of cells (Martin et al. 2020). Individual cell motility is a fundamental cell behavior that is crucial for a wide range of biological processes, including embryonic development, immune surveillance,

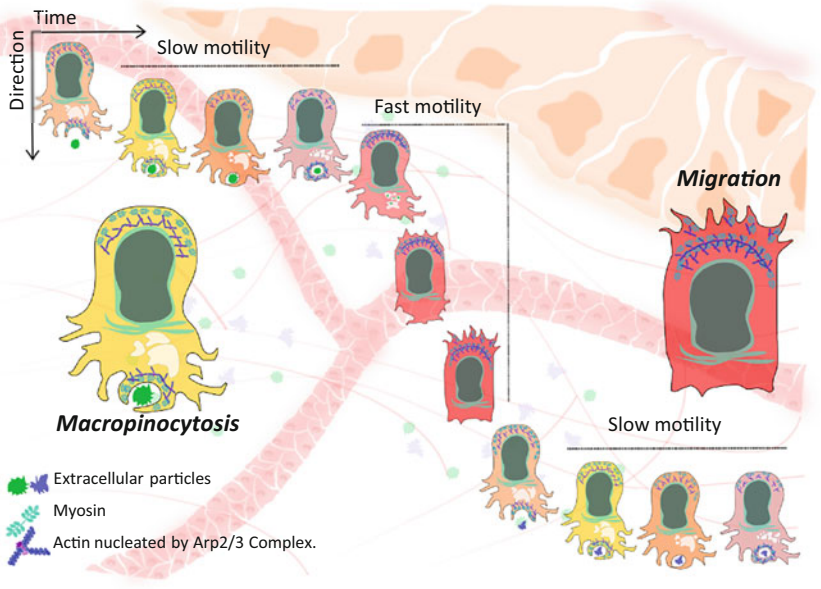

Fig. 5.1 The cycle of migration/macropinocytosis allows cells to explore tissues, and it is key to a range of processes both physiological and pathological. Cells alternate phases of slow motility where they mainly perform macropinocytosis and phases of fast motility when migrating

and wound healing; in contrast, deregulated migration is a key prerequisite for cancer cell dissemination.

Box 5.2 Asymmetrically localized components lead to the antagonism between macropinocytosis and migration

At the *leading edge*, the following molecules and events are detected: the activation of several Ras and Rac family GTPases, activation of mTORC2 and its substrates of the Akt/protein kinase B (PKB) family kinases, accumulation of phosphatidylinositol 3-kinases (class I PI_3 kinases), which catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-trisphosphate (PIP_3), and recruitment of a number of cytoskeletal regulators, such as the Scar/WASP-family verprolin-homologous protein (WAVE) and Arp2/3 complexes responsible for actin polymerization and pseudopod projection (Cai et al. 2010; Pino et al. 2015; Smith et al. 2020). At the macropinocytic and phagocytic cups, PIP_3, activated Ras or Rac, the Arp2/3 actin nucleator complex and its modulators Scar/WAVE proteins take up their positions (Veltman 2014; Buckley and King 2017); whereas *at the back of the cell* it is possible to identify molecules such as phosphatase and

(continued)

Box 5.2 (continued)
tensin homolog deleted on chromosome 10 (PTEN), which are implicated in the sensitivity of cancer tumors to insulin and IGF1, and in calorie restriction. The generation of formin and myosin II–dependent actin cortex is necessary for back retraction (Ramalingam et al. 2015; Litschko et al. 2017; Damiano-Guercio et al. 2020). These asymmetrically localized components (both leading and tailing edge molecules) exhibit signature behaviors in cells stimulated with chemoattractants, by transiently relocalizing with respect to the cell periphery. For example, upon the addition of uniform cAMP, many of the leading-edge molecules translocate transiently to the cell periphery within ~10 s, and redistribute to the cytosol by 30 s. With the same kinetics, the trailing edge molecules transiently descend from the cell periphery into the cytosol, before returning to the cell membrane or cortex (Swaney et al. 2010; Sobczyk et al. 2014).

Recently, a leucine-rich repeat (LRR) domain–containing protein named "leading edge enriched protein 1" (Leep1) was described: Leep1 is a novel leading edge–localized polarity regulator, which interacts with PIP_3 and the Scar/WAVE complex to modulate protrusion dynamics (Yang et al. 2021). PIP_3 is the principal mediator of membrane association of Leep1, which in turn modulates leading edge activities by negatively regulating the Scar complex. Leep1 is localized to the macropinocytic cups and pseudopods, its concentrations at these locations being fine-tuned for optimal leading-edge function. Deletion or overexpression of Leep1 impairs macropinocytosis and alters actin dynamics, even when Leep1 association specifically to the Scar complex is weak. The phenotypic overlap between overexpression of Leep1 and disruption of the Scar complex implies that Leep1 is a negative regulator of the complex (Yang et al. 2021).

The Role of Small GTPases

Motility and endocytic mechanisms are probably mutually exclusive because they both rely on the actin-remodeling machinery. The RasGEFB/RasS signaling pathway, which controls cytoskeleton remodeling, emerges as an obvious candidate for the modulator of this tight balance. In general, the Rho family of GTPases has been shown to regulate cell migration as they exert effects on actin assembling, actomyosin contractility, and microtubules organization (Crespo et al. 2011). While the Ras family of GTPases has mostly been linked with processes occurring during cellular proliferation, both families (Rho and Ras GTPases) share regulatory mechanisms (Crespo et al. 2011). Migration is tightly regulated by *Rho family GTPases*: tridimensional movements, such as those in confined spaces (i.e. in tissues), can be performed as (1) *Cell elongation*, which is important for tumor cells and for

activation of Rac1; and (2) *Rounded movement*, which requires specific Cdc42 and Rho signaling pathways, and is necessary in cells that perform amoeboid migration or α-motility. Regulators such as RasGRF2, a Ras activator, can suppress rounded movement by inhibiting Cdc42 activation, independently of the activation of Ras. Additionally, functional loss of the Ras GTPase regulator RasGEF-B results in impaired macropinocytosis and enhanced speed of migration (Crespo et al. 2011).

Dictyostelium macropinosomes are large structures (up to 5 μm in diameter) that efficiently take up liquid and soluble nutrients (Williams and Kay 2018). Interestingly, the Ras family member RasS in *Dictyostelium* is implicated in the regulation of both migration and macropinocytosis. Loss of RasS activity leads to defective fluid-uptake capacity and slower phagocytosis; however, it increases cell polarity and F-actin-rich structures, namely pseudopodia, which elongate roughly three times faster in comparison with wild-type organisms (Wilkins et al. 2000; Ghoshal et al. 2019). Events downstream of Ras GTPases are also involved in regulating multiple aspects of cell migration and have been shown to be key in the invasion of tumor cells (Crespo et al. 2011).

The Role of Phosphoinositides

The small GTPase Ras directly activates class I PI_3 kinases leading to production of PIP_3. Deregulation of this pathway leads to massive macropinocytosis, a key event for cancer metastasis (Commisso et al. 2013). In both mammalian and *Dictyostelium* cells, macropinocytic cups (or circular ruffles) form around patches of high PIP_3 concentration within the plasma membrane. Here, active Ras and Rac are found forming a domain measuring a few microns in diameter (Welliver and Swanson 2012; Veltman et al. 2016). PIP_3 patches remain detectable in the membrane even after the cup has closed, detached itself from the membrane and is underway through the cytoplasm as an intracellular vesicle (Dormann et al. 2004). These PIP_3 patches are observed in macrophages, and they form concurrently with circular ruffles (the origins of macropinosomes) in the plasma membrane (Yoshida et al. 2009).

PIP_3 is dispensable for *Dictyostelium discoideum* migration toward folate, but actively serves as an inhibitor of chemotaxis. Growing cells need PIP_3 to form macropinosomes but not for pseudopod formation (Veltman et al. 2014). Interestingly, PIP_3 and Ras-signaling help to spatially organize the actin cytoskeleton inside the macropinocytic cups. It has been shown that Ras/PIP_3 signaling triggers not only macropinosome formation, but is also a spatial organizer of macropinocytic cups leading to a signal amplification within the cup (Kay et al. 2018). Therefore, the orchestration of both macropinocytosis and cell migration is achieved via compartmentalized distribution of cytoskeletal macromolecules together with a tight spatiotemporal regulation. During macropinocytosis, many downstream signaling and cytoskeletal components are localized or activated specifically at either the leading or trailing edge of cells. These domains of plasma membrane create functionally distinct opposing ends that promote cell migration and downregulate

macropinocytosis in parallel. Moreover, the observation of PIP_3 at the cell surface witnesses the underlying compartmentalization of actin-related machinery.

Compartmentalization of the Actomyosin Cytoskeleton

The molecular machinery required for macropinocytosis and migration is clearly shared between the two processes and involves membrane availability, which is constant, and active cytoskeleton remodeling. If perceived as a logic circuit diagram, regulation of the actin cytoskeleton is the point at which molecular macropinocytosis and migration meet. We will therefore focus on the active component—cytoskeleton remodeling. These rearrangements of the actin cytoskeleton occur by de novo formation of filaments through actin nucleation mechanisms. Arp2/3 has been extensively studied because it specifically assembles branched actin filament arrays by binding to the side of an existing filament and initiating branch formation (Goley and Welch 2006; Pollard 2007; Firat-Karalar and Welch 2011). The resulting two new filaments can then each be split again, creating a natural feed-forward mechanism, limited only by the supply of monomers (Veltman 2014). A striking observation is that Arp2/3 complex, which organizes the actin cytoskeleton during cell motility, is also used for fluid uptake during macropinocytosis (Insall et al. 2001).

How nucleation activity is precisely regulated remains a largely open question. Key players in the signaling for actin polymerization are the small GTPases of the Rho family (Caron and Hall 1998). It has been reported that Cdc42 activation in the nascent phagocytic cup activates effectors such as N-WASP, an actin nucleation-promoting factor that acts on the Arp2/3 complex (see Box 5.3). Furthermore, Rac1 is essential for F-actin polymerization during the extension and closure of the cup: accomplishes this via activation of another NPF, the WAVE complex (Hoppe and Swanson 2004; Swanson 2008; Niedergang and Grinstein 2018).

Box 5.3 Regulation of Arp2/3 activity by NPFs

Regulation of this nucleation capacity is achieved by several means:

(1) *Nucleation-promoting factor (NPF)*: strong nucleation requires its association with a stimulatory co-factor or nucleation-promoting factor (NPF), the best known being Wiskott Aldrich syndrome protein (WASP) and WASP family VErproline-homologous (WASP / WAVE) family proteins (Higgs and Pollard 2001).

(2) *Inhibitors*: nucleation can be inhibited by some binding partners, including Coronin (Humphries et al. 2002; Cai et al. 2008; Liu et al. 2011), glia maturation factor (GMF) (Gandhi et al. 2010; Luan and Nolen 2013; Ydenberg et al. 2013), Gadkin (Maritzen et al. 2012), and Arpin (Dang et al. 2013).

The actin filament nucleation-promoting factor SCAR/WAVE has been described as having a prominent role in the switch between pseudopod and macropinosome formation. It is localized in pseudopods, specifically at the extreme leading edge (Veltman et al. 2012). In cells growing in liquid medium, SCAR/WAVE is relocalized to the macropinocytic cup's boundaries. How SCAR/WAVE acquires this distribution is currently not understood. Interestingly, it has been shown that the SCAR/WAVE complex accumulates at the periphery of PIP_3 patches. These regions are present in the cup of the macropinosome (Veltman et al. 2014).

Proteins that directly inhibit Arp2/3 are Gadkin, PICK1, and Arpin (Rocca et al. 2008; Maritzen et al. 2012; Dang et al. 2013), for which no role in macropinocytosis has been reported yet. The major functions of Arpin described to date are the inhibition of cell migration (Gorelik and Gautreau 2015) and the control of cell steering (Dang et al. 2013), also is a negative regulator of Arp2/3 activity that fine-tunes actin nucleation activity at the leading edge of the lamellipodium to steer the cell (Dang et al. 2013). Arpin exposes its COOH terminal acidic tail to inhibit the Arp2/3 complex (Fetics et al. 2016; Sokolova et al. 2017). In the cell types studied so far, Arpin localizes at lamellipodial edges along with the WAVE complex. The ability of Arpin to interact with Arp2/3 was found to depend on Rac1 signaling. In response to Rac1 signaling, Arpin inhibits Arp2/3 at lamellipodial tips, where Rac1 also stimulates actin polymerization through WAVE. This places Arpin downstream of Rac1 in a cascade where Rac1 induces and inhibits actin polymerization (Dang et al. 2013). Arpin now joins a short list of mammalian proteins with true carboxy-terminal acidic Arp2/3-binding motifs that counteract the activator signals provided by the WAVE/Rac1 complex. Recently, Arpin has been shown to be critical for phagocytosis in macrophages, and it is targeted by human rhinovirus 16, allowing the virus to perturb bacterial internalization and phagocytosis in macrophages (Jubrail et al. 2020).

Similar to Arpin, coronin7 proteins have a highly conserved carboxy-terminal acidic motif, but their exact function has not yet been established (Chan et al. 2011). In mammalian cells coronin7 is recruited to the Golgi, where it helps maintain proper Golgi morphology, whereas the coronin7 homologue of the social amoeba *Dictyostelium* is recruited to crown-like structures associated with fluid-phase endocytosis. Disruption of coronin7 leads to an increase in phagocytosis (Shina et al. 2010).

The relevance of the antagonism between macropinocytosis and cell migration has also been explored in the mammalian immune system, demonstrating an essential role of the actin-based molecular motor, Myosin II, which is responsible for actomyosin contractility. Analysis of Myosin II dynamics in migrating DCs has revealed that their migratory behavior is often adapted to their immune function (Chabaud et al. 2015). These differences result from distinct localizations of Myosin II due to the differential use of actin nucleation machineries and intracellular calcium dynamics (Solanes et al. 2015; Bretou et al. 2017; Sáez et al. 2018). Moreover, a novel concept has emerged from this work: DCs are endowed with the capacity to adapt their locomotion mode to their functional requirements and optimize their

chances of finding rare targets, providing a putative explanation for their efficiency as immune sentinels. This will be discussed in the next section of this chapter.

Overall, actin-based molecular motors and their regulators play a key role in the establishment and regulation of the antagonism between macropinocytosis and motility. The dynamics of the actin cytoskeleton allow a cell to switch between "drinking" and "moving."

Physiological Impact of the Antagonism Between Macropinocytosis and Cell Migration

Environment Exploration

DCs are one of the best examples of how the antagonism between macropinocytosis and cell migration, as well as its tight regulation, serves a key physiological function: the environment patrolling capacity of immune cells (see Box 5.4).

Box 5.4 Dendritic cells, the sentinels of the immune system
DCs constitute a complex cell population, the main function of which is to link innate and adaptive immune responses, thereby playing a critical role in both the establishment of tolerance and immunity (Joffre et al. 2009). By switching between actin-nucleating machineries, DCs adapt their migration mode to their distinct functional requirements: tissue patrolling/antigen uptake for immature DCs, and fast migration to lymph nodes for mature DCs (Vargas et al. 2016). Whereas fast DC migration results from the enrichment of the Myosin II actin-based molecular motor at the rear of immature DCs, slow migration phases are due to the diversion of this motor protein from the rear to the front of the cells by Ii (Faure-André et al. 2008).

In peripheral tissues, immature DCs continuously sample their environment by internalizing and processing extracellular material. Immature DCs capture antigens mainly by phagocytosis and macropinocytosis (Sallusto et al. 1995). This actin-dependent mode of internalization allows the nonspecific uptake of large amounts of extracellular fluid and, in DCs, relies on the small GTPases Cdc42 and Rac1 (Garrett et al. 2000; West et al. 2000). Endocytosed antigens are delivered to endolysosomes, where they are degraded into peptides to be loaded onto MHC class II molecules (Norbury 2006). Interestingly, physical modeling shows that such intermittent modes of migration are efficient for searching for antigens present at low concentrations in large spaces (nicely review in Heuzé et al. 2013). Alternation of fast and slow migration orchestrated by distinct actin subcellular pools is observed in many immune cells.

Interestingly, in immature DCs, this intermittent migration mode might facilitate their ability to detect scattered antigens, as suggested by a model based on optimization of intermittent search strategies (Chabaud et al. 2015; Moreau et al. 2018). DCs lack focal adhesions and migrate in a myosin II-dependent manner, displaying an amoeboid phenotype (Paluch et al. 2016). Such adaptation is particularly evident when analyzing the migration modes of immature and mature DCs.

Immature DCs, whose main function is tissue patrolling, alternate phases of slow and fast migration (Faure-André et al. 2008). Antigen capture and migration in three-dimensional (3D) environments both require myosin IIA (Lämmermann et al. 2008; Solanes et al. 2015). Efficient antigen uptake by macropinocytosis is associated with periodic enrichments of Myosin IIA at the DC front (Chabaud et al. 2015). These enrichments were shown to disrupt the asymmetry in myosin IIA dynamics responsible for fast locomotion (the cell rear characterized by actin bundles, and the leading edge by rapid turnover of polymerization and adhesion); the result was a reduction in cell migration speed. Migration of immature DCs depends on two main actin pools: a RhoA–mDia1 [mDiaphanous1 (mDia1) is the main formin involved in nucleation of the bulk actin cortex (Bovellan et al. 2014)]-dependent actin pool located at their rear, which facilitates forward locomotion; and a Cdc42–Arp2/3-dependent actin pool present at their front, which limits migration but promotes antigen capture (Vargas et al. 2016). These results provide an additional demonstration of the cell-intrinsic antagonism between fast cell migration and antigen uptake by macropinocytosis.

This antagonism was further shown to be dependent on the regulation of myosin IIA localization by Ii (CD74), which is required for the recruitment of the motor protein at the front of DCs (Chabaud et al. 2015). Ii-dependent Myosin II enrichment at the DC front has two consequences: (1) it disrupts back-to-front Myosin II polarity and thereby forces immature DCs to slow down; and (2) it exerts local mechanical forces on macropinosomes, allowing their intracellular retrograde transport and the retrieval of antigens into endolysosomes for loading onto MHC class II molecules (Chabaud et al. 2015).

Hence, *mature DCs*, which transport antigens to lymph nodes to initiate the immune response, migrate at a relatively constant high speed, and with greater persistence (the ability of a cell to maintain its direction of motion). In parallel, mature DC, following TLR4–MyD88-induced maturation, Arp2/3-dependent actin enrichment at the cell front is markedly reduced. Consequently, mature DCs switch to a faster and more persistent mDia1-dependent locomotion mode that facilitates chemotactic migration to lymphatic vessels and lymph nodes (Vargas et al. 2016). Thus, the differential use of actin-nucleating machineries optimizes the migration of immature and mature DCs according to their specific function, leading to downregulate macropinocytosis and coordinately increase rates of migration (Vargas et al. 2016). Additionally, back-to-front Myosin II polarity depends on intracellular calcium release through IP_3 receptors (Solanes et al. 2015).

In vivo evidence of this phenomenon has been found via intravital two-photon microscopy in fluorescent reporter mice. The systems under study in this work were the dynamic response of $CD103^+$ DCs to *Salmonella* challenge and the cellular

behavior that underlies sampling of bacteria and soluble antigens. It was found that bacterial challenge recruits $CD103^+$ DCs from the lamina propria (LP) into the epithelium, a process in which DCs crawl laterally while sending dendrites into the intestinal lumen. Luminal bacteria are captured by these dendrites and their antigens are subsequently presented in the mesenteric lymph nodes (MLNs) of mice harboring $CD103^+$ DCs (Farache et al. 2013).

Furthermore, considering the low number of tissue-resident DCs and the relatively large space to be scanned, optimized antigen searching behaviors are likely to be required for efficient patrolling of the environment. How immature DCs take up antigens in vivo while patrolling their environment has recently started to be documented. Two-photon imaging experiments suggest that in certain tissues, such as the mouse ear and gut, DCs randomly migrate to scan the environment (Lai et al. 2008; Farache et al. 2013). In contrast, in the mouse foot-pad and lung, DCs were shown, to remain sessile and take up luminal antigens through membrane projections that cross the epithelia (Rescigno et al. 2001; Tal et al. 2011; Lelouard et al. 2012; Thornton et al. 2012).

Cell Guidance by Extracellular Cues

As we mentioned earlier, cells polarize their molecular components in order to perform two distinct and mutually exclusive functions: sampling their extracellular media by macropinocytosis, and migrating toward chemical and physical cues. During migration, cells encounter complex tridimensional environments and must carefully balance environmental stimuli, e.g. chemical cues over the cell surface, and shifting gradients sensed by adhesion receptors. This process is collectively known as "durotaxis" (Lo et al. 2000). Additionally, while moving through a confined space (interstitial tissue) a cell will push water ahead of itself, generating hydraulic pressure. The ability to detect and respond to local differences in hydraulic resistance (HR) as a physical input driving migration has been referred to as "Barotaxis." It was first studied in neutrophils derived from HL60 cells (Human promyelocytic leukemia) (Prentice-Mott et al. 2013), but it has also been investigated in chemotactic cells, such as DCs, neutrophils, and Dictyostelium (Parent 2004; Insall 2010; Swaney et al. 2010; Moreau et al. 2019). Barotaxis depends on geometrical parameters and fluid viscosity, and these constitute an intrinsic property of the tissue. HR can oppose cell migration because it restricts the movement of fluid that cells must displace as they move. Interestingly, it has been observed that although cells migrate along the path of least hydraulic resistance, chemotaxis always overrides barotaxis (Prentice-Mott et al. 2013, 2016).

Strikingly, it was shown that immature DCs do not respond to HR (Moreau et al. 2019). This is explained by the elevated macropinocytic activity of immature DCs that sample their environment by continuously ingesting extracellular fluid. This implies that macropinocytosis at the leading edge of immature DC allows them to move against HR gradients when they enter blind-ended capillaries, thereby

permitting immature DC to explore tissues more effectively than non-macropinocytic cells. In contrast, when these cells encounter a microbial signal and become mature DCs, they downregulate macropinocytosis and, accordingly, they now become sensitive to hydraulic resistance. As a consequence of this, HR can bias the migration of mature DCs, thereby allowing their guidance to lymph nodes by defining the shortest migration path. Therefore, although HR can represent a physical obstacle for cell migration, DCs can overcome or use this physical constraint to exert their immune-surveillance function (Moreau et al. 2019).

Recently, it has been shown that *Dictyostelium* cells also respond differentially to hydraulic resistance/chemical gradients when studied in an asymmetric bifurcating microchannel (Belotti et al. 2020). Cells confronted to a microchannel bifurcation are often observed to partly split their leading edge and start moving into both channels. Moreover, cells moving faster in steeper cAMP gradients split more readily. The decision to retract the pseudopod moving away from the cAMP source is made when the average velocity of the pseudopod moving up the cAMP gradient is 20% higher than the average velocity of the pseudopod moving down the gradient. Surprisingly, this decision threshold is independent of the steepness of the cAMP gradient and speed of movement. This finding indicates that a critical force imbalance threshold underlies the repolarization decision. *Dictyostelium* cells always migrate up the chemical gradient despite the hundred times higher hydraulic resistance, thus, as in neutrophils, chemotaxis overrides barotaxis in *Dictyostelium*. When analyzing the splitting dynamics of cell leading edge, it was found that when cells face the bifurcation inside channels, there is a response threshold that is independent of the cAMP concentration gradient but dependent on the tension gradient between the competing pseudopods in *Dictyostelium* (Belotti et al. 2020). Whether macropinocytosis plays a role in this process is unclear.

Conclusion

We here describe the molecular mechanisms involved in the antagonism between macropinocytosis and cell migration and further discuss the impact of such process in cell behavior and function. Based on multiple evidence obtained in amoeba and immune cells, we highlight that this antagonism results from the use of molecular machineries that control the dynamics of the actomyosin cytoskeleton and are common to both biological processes. Consistently, an antagonism between cell migration and phagocytosis, which relies on a similar machinery than macropinocytosis, has also been reported in hemocytes, the *Drosophila melanogaster* macrophage-like cells. Excessive phagocytosis of apoptotic corpses by these cells impairs their migration and thereby compromises their immune function (Evans et al. 2013). Whether the use of common cytoskeleton-related molecular machineries might represent a more general strategy for cells to coordinate different functions remains an opened question.

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Chapter 6
Macropinocytosis in Phagocyte Function and Immunity

Johnathan Canton

Abstract Phagocytes play critical roles in the maintenance of organismal homeostasis and immunity. Central to their role is their ability to take up and process exogenous material via the related processes of phagocytosis and macropinocytosis. The mechanisms and functions underlying macropinocytosis have remained severely understudied relative to phagocytosis. In recent years, however, there has been a renaissance in macropinocytosis research. Phagocytes can engage in various forms of macropinocytosis including an "induced" form and a "constitutive" form. This chapter, however, will focus on constitutive macropinocytosis and its role in the maintenance of immunity. Functions previously attributed to macropinocytosis, including antigen presentation and immune surveillance, will be revisited in light of recent revelations and emerging concepts will be highlighted.

Keywords Macropinocytosis · Endocytosis · Pinocytosis · Macrophage · Dendritic cell · Phagocyte · Antigen presentation · Cross-presentation · Pattern recognition receptor (PRR) · Microbe-associated molecular pattern (MAMP) · Innate immunity

Introduction

It was Warren H. Lewis who, in a series of articles published in the early 1930s, first described pinocytosis or "cell drinking." Lewis defined pinocytosis as a relatively common process whereby certain cells internalize "globules" of culture medium by the projection of "wavy ruffle pseudopodia" from their dorsal surface (Lewis 1937). Although not able to observe such processes in tissues given the technical limitations of the time, he also suggested that "instead of sitting around and doing nothing" macrophages are "always actively engaged in drinking tissue juices" thereby predicting the constitutive nature of pinocytosis (Lewis 1937). Over the years, pinocytosis has been divided and subdivided into mechanistically distinct endocytic

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processes including the well-studied caveolae-mediated uptake and clathrin-mediated endocytosis (Doherty and McMahon 2009). However, many pinocytic pathways remain poorly understood and new forms continue to be discovered. One pathway that has been receiving increasing attention in recent years is macropinocytosis (Swanson and King 2019).

In macropinocytosis, actin-driven protrusions are extended from the plasma membrane and, by an entirely unknown mechanism, seal at their distal tips to form relatively large (>250 nm) vesicles referred to as macropinosomes (Donaldson 2019). Although primarily studied in phagocytes and malignant cells, macropinocytosis is now being described in an increasing variety of cells including T cells, B cells, and neurons (Clayton and Cousin 2009; Rosales-Reyes et al. 2012; Charpentier et al. 2020). However, under non-transformed, homeostatic conditions, phagocytes are uniquely endowed with the capacity for continuous or constitutive macropinocytosis. Indeed, estimates for the rate of membrane turnover in resting macrophages are believed to exceed the equivalent of the entire plasma membrane surface area every half hour (Steinman et al. 1976; Freeman et al. 2020). Figures for the energy requirements of this constitutive membrane turnover do not exist but it is likely to represent a substantial portion of the cells' energy equivalents. The evolutionary conservation of this process, then, speaks to its importance in phagocyte function.

This chapter will focus on the role that macropinocytosis plays in the maintenance of immunity by phagocytes. An emphasis will be placed on the mechanistic differences between the constitutive macropinocytosis of phagocytes and the more ubiquitous growth factor-induced macropinocytosis. Then, its role in antigen acquisition, processing, and presentation as well as immune surveillance will be revisited in light of recent discoveries. Finally, emerging concepts in the function of macropinocytosis in phagocytes will be explored.

Constitutive Macropinocytosis: Turning Ruffles in Vesicles

For almost a century, the basic series of events resulting in the formation of a macropinosome have been known. The first step is the formation of dynamic, branched-actin networks that propel the plasma membrane outward from the cortex of the cell. These structures can vary depending on the inducing stimulus but generally resemble wave-like structures and are referred to as membrane ruffles. Next, in the most cryptic step in macropinosome formation, some membrane ruffles form cup-like structures that then seal at their distal tips to generate large, fluid-filled vesicles, or macropinosomes. The mechanics of this process has been mostly studied by treating cells with a macropinocytosis-inducing stimulus, most often a growth factor (Yoshida et al. 2018; Doodnauth et al. 2019; Freeman et al. 2020). The treatment of macrophages with high concentrations of macrophage-colony stimulating factor (M-CSF), for example, induces a tremendous burst of membrane ruffling followed by the formation of numerous macropinosomes. The details of growth

factor-induced macropinocytosis will be covered in a separate chapter. Here, I will highlight the mechanisms by which phagocytes engage in a separate and distinct form of macropinocytosis—constitutive macropinocytosis.

Live-cell microscopy of phagocytes, including macrophages and dendritic cells, reveals that they continuously extend membrane ruffles from their dorsal surfaces. This process occurs even in minimal medium containing no source of growth factors (Canton et al. 2016). These constitutive membrane ruffles resemble growth factor-induced ruffles but are noticeably smaller. Similarly, constitutive ruffling results in the continuous generation of macropinosomes (West et al. 2004; von Delwig et al. 2006; Redka et al. 2018). For many years, it was assumed that the mechanistic basis of constitutive macropinocytosis was identical to that of growth factor-induced macropinocytosis. However, recent studies have revealed key differences in the molecular mechanism driving constitutive macropinocytosis.

Unlike growth factor-induced macropinocytosis, constitutive macropinocytosis in phagocytes is uniquely driven by the presence of extracellular calcium (Canton et al. 2016). G protein-coupled receptors that bind extracellular calcium (calcium-sensing receptors; CaSRs) are expressed by phagocytes, including macrophages and dendritic cells, and sense extracellular calcium ions (Ca^{2+O}) in the range of roughly 0.5–1.5 mM (Olszak et al. 2000; Lee et al. 2012; Conigrave 2016; Canton et al. 2016; Redka et al. 2018). Typical serum concentrations of Ca^{2+O} range from 1.1 to 1.2 mM (Conigrave 2016). Common cell culture media for phagocytes also contain $Ca^{2+}{}_{O}$ within that range. Importantly, removal of extracellular calcium from the medium or direct inhibition of CaSRs results in the complete cessation of constitutive membrane ruffling and macropinocytosis while having little to no effect of growth factor-induced macropinocytosis (Canton et al. 2016).

Mechanistically, ligation of CaSRs by $Ca^{2+}{}_{O}$ results in the dissociation of the Gα and Gβγ subunits of the heterotrimeric G protein. The GTP-bound Gα subunit activates its effector phospholipase C gamma (PLCγ) to convert plasmalemmal phosphatidylinositol 4,5-*bis*phosphate [PtdIns(4,5)P_2] into the second messengers diacylglycerol (DAG) and inositol 1,4,5-*tris*phosphate (IP_3). DAG is subsequently converted into phosphatidic acid (PtdOH) by diacylglycerol kinases (DGKs). Simultaneously, the Gβγ subunit activates phosphatidylinositol 3-kinases (PI3K) to convert PtdIns(4,5)P_2 into phosphatidylinositol 3,4,5-*tris*phosphate [PtdIns(3,4,5)P_2]. This accumulation of negatively charged lipid species on the cytosolic leaflet of the plasma membrane facilitates the recruitment of polybasic domain-containing Rho family guanine nucleotide exchange factors (RhoGEFs), which in turn activate small GTPases such as Rac1/2. PtdOH further facilitates this pathway by promoting the dissociation of the small GTPase Rac1/2 from its Rho-specific guanine nucleotide dissociation factor (RhoGDI). Ultimately, Rac1/2 promotes the activation of the actin nucleation machinery that drives constitutive membrane ruffling and macropinocytosis (Canton et al. 2016; Schlam and Canton 2016). These pathways are further summarized in Fig. 6.1. To date, CaSRs are the only receptors known to drive the constitutive macropinocytosis of phagocytes. Whether other receptors can similarly drive this process remains to be explored.

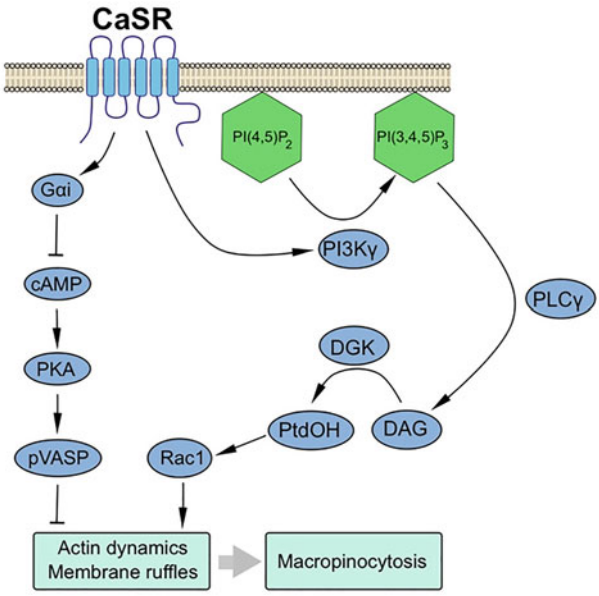

Fig. 6.1 The mechanics of constitutive macropinocytosis. Phagocytes, such as macrophages and dendritic cells, express G protein-coupled receptors that sense extracellular calcium ions (CaSRs). Upon ligation, CaSRs signal through distinct pathways. Inhibitory G protein ($G\alpha_i$)-dependent pathways limit the elevation of cyclic AMP (cAMP) and therefore prevent the phosphorylation and activation of vasodilator-stimulated phosphoproteins (VASP) via protein kinase A (PKA). VASP is a regulator of actin dynamics and has been implicated in macropinocytosis, although its role in constitutive macropinocytosis remains unclear. CaSR-dependent signals simultaneously activate PI3K resulting in the generation of PtdIns(3,4,5)P_3 on the cytosolic aspect of the plasma membrane. PtdIns(3,4,5)P_3 accumulation fosters the recruitment of PLC via its PH domain. PLC catalyzes the conversion of PtdIns(4,5)P_2 into IP_3 and DAG. DAG is further converted into PtdOH by diacylglycerol kinases (DGKs). These events result in the accumulation of active Rac1 on the plasma membrane and the generation of the actin-driven membrane ruffles that drive constitutive macropinocytosis

Constitutive Macropinocytosis in Pathogen and Damage Sensing

Phagocytes are sentinel cells and survey the environment for potentially harmful encounters. They do so by expressing on their surface an impressive array of sensor proteins referred to as pattern recognition receptors (PRRs). PRRs are capable of recognizing molecular signatures referred to as microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) in the extracellular milieu (Brubaker et al. 2015). Ligation of PRRs by their respective ligands induces a series of acute antimicrobial responses as well as more long-term gene transcription/translation responses that result in metastable changes to the phenotype of the sensor cell and the production of soluble factors that can alert and induce

changes in neighboring cells (Zindel and Kubes 2020). Constitutive macropinocytosis is emerging as a critical regulator of the ability of phagocytes to sense danger and damage via PRRs.

The first mechanism by which macropinocytosis facilitates PRR sensing in phagocytes is through the delivery of ligand to intracellular PRRs. Although many PRRs are located at the cell surface and contain ligand-binding domains on the exofacial aspect of the plasma membrane, some PRRs are located in endosomal compartments and others in the cytosol (Brubaker et al. 2015). Engagement of these receptors requires internalization and delivery of ligands to the appropriate compartments. A number of pathways have been implicated in the delivery of ligand to intracellular PRRs including phagocytosis and clathrin-dependent endocytosis. However, under certain circumstances neither of these pathways can efficiently deliver ligand to intracellular compartments. A notable example is the case of outer membrane vesicles (OMVs) shed by microbes during the establishment of a replicative niche in host organisms. OMVs are known to activate intracellular PRR pathways (Bielig et al. 2011; Guidi et al. 2013; Vanaja et al. 2016; Cañas et al. 2018). Importantly, OMVs range in size, but are typically larger than 300 nm in diameter (Roier et al. 2016). This places them out of the range of particle sizes that can be internalized by clathrin-mediated endocytosis (<150 nm in diameter) (Doherty and McMahon 2009). Similarly, phagocytosis efficiency decreases dramatically for particles below 1 μm in diameter (Champion et al. 2008). Macropinosomes on the other hand range in size from 250 nm to 5 μm in diameter and can conceivably be an efficient route for the delivery of material to intracellular compartments. In line with this, OMVs contain ligands for cytosolic PRRs such as the nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1/2) (Bielig et al. 2011; Cañas et al. 2018). Indeed, the blockade of constitutive macropinocytosis dramatically dampens the ability of both macrophages and dendritic cells to sense NOD1/2 ligands (Canton et al. 2016). Interestingly, macropinosomes facilitate this process by two distinct mechanisms. First, upon formation, macropinosomes immediately undergo a dramatic shrinkage in size (Freeman et al. 2020). This is the result of the opening of lipid-gated ion channels that allow the flux of monovalent ions across the macropinosomal membrane. Osmotically obliged water follows resulting in crenation of the membrane and a reduction of the luminal volume. The reduction in size increases the effective concentration of ligand and therefore lowers the threshold of detection in the lumen relative to the extracellular space. Second, as macropinosomes mature they accumulate vacuolar-type H^+-ATPase (V-ATPase) proton pumps (Racoosin and Swanson 1993). The action of V-ATPases creates a steep gradient of protons across the macropinosomal membrane. The acidification of the lumen simultaneously activates degradative enzymes that enhance the extraction of ligand and activates transmembrane transporters that pump the ligand down the proton gradient, out of the macropinosome and into the cytosol (Lee et al. 2009; Marques et al. 2017; Canton 2018). Once in the cytosol, the ligand is free to engage cytosolic PRRs (Fig. 6.2) (Lee et al. 2009; Nakamura et al. 2014; Canton et al. 2016). The same principles apply to transmembrane PRRs located in endosomal compartments such as Toll-like receptor 3 (TLR3) which preferentially binds its ligand under

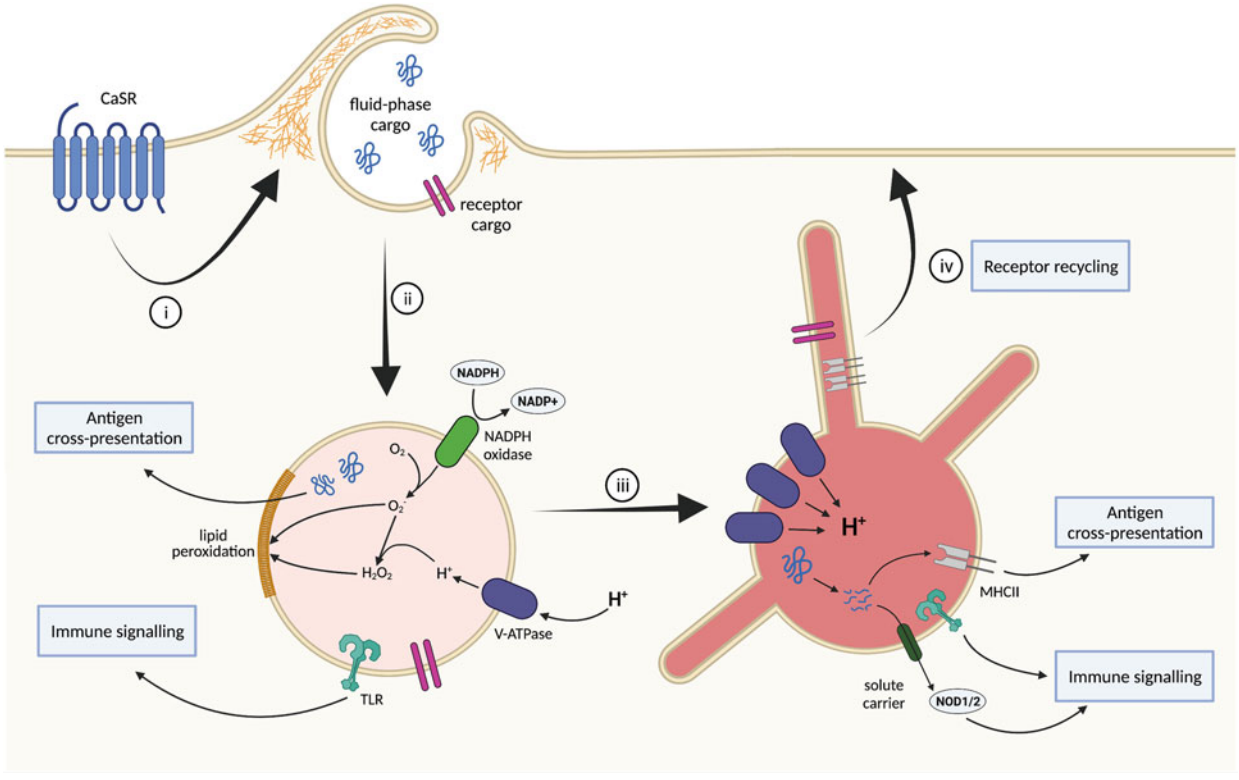

Fig. 6.2 The contribution of constitutive macropinocytosis to antigen presentation and immune signalling. (i) CaSRs signal constitutive macropinocytosis in phagocytes such as macrophages and dendritic cells. (ii) Both fluid-phase and transmembrane cargo are internalized into macropinosomes. In the early macropinosome, reactive oxygen species such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) result in damage to the limiting membrane of the macropinosome via lipid peroxidation. This results in the escape of minimally processed material into the cytosol. This exogenous material can be processed by the proteasome for subsequent presentation on MHC-I molecules in a process referred to as antigen cross-presentation. Material in the lumen of the

Fig. 6.2 phagosome can also be sensed by endosomal TLRs such as TLR7 and TLR9. (iii) As macropinosomes mature, they continue to accumulate V-ATPases resulting in the precipitous acidification of the lumen. This activates degradative enzymes with acidic pH optima. Digested cargo can be transported out of the lumen and into the cytosol through transmembrane solute carriers. Cytosolic PRRs, such as NOD1/2 can then sense those ligands. Peptides from the digested cargo may also be loaded onto MHC-II molecules in the lumen of the macropinosome for subsequent antigen presentation. (iv) The opening of lipid-gated ion channels results in the movement of monovalent ions across the macropinosomal membrane. This results in the simultaneous movement of osmotically obliged H_2O out of the macropinosome resulting in macropinosome shrinkage. Shrinkage is accompanied by membrane crenation and the binding of curvature-sensing proteins such as the BAR-domain containing a family of proteins. This ultimately results in membrane tubulation and the recycling of cargo including transmembrane receptors and peptide-loaded MHC-II to the plasma membrane

acidic conditions and TLR7 and TLR9 which are predominantly found in endosomal compartments (Leonard et al. 2008; Chaturvedi and Pierce 2009).

A second way that macropinocytosis contributes to the tissue surveillance function of phagocytes is in receptor recycling (Fig. 6.2). This is best demonstrated by the response of tissue-resident macrophages to local damage. Upon acute injury, tissue-resident macrophages sense the damage via PRRs and extend dynamic actin-driven processes to "cloak" the damage site from neutrophils (Uderhardt et al. 2019). This "cloaking" mechanism prevents the excessive recruitment of neutrophils, in a process called "swarming," to the damage site. This has the effect of limiting the collateral damage generated by neutrophil activity. Crucially, the rapid internalization and redistribution of cell surface receptors, such as integrins, involved in the "cloaking" of the damage site require bulk membrane turnover via macropinocytosis. Under conditions where the recycling of receptors from macropinosomes to the plasma membrane is blocked, tissue-resident macrophages can no longer shield the damage site from neutrophils and a "swarm" ensues resulting in excessive damage at sites of local tissue injury (Freeman et al. 2020). This demonstrates the importance of macropinocytosis in the responsiveness of tissue-resident macrophages and in the maintenance of tissue homeostasis.

Phagocytes can also control the rate at which they perform constitutive macropinocytosis depending on the particular environmental conditions to which they are exposed. The tuneable nature of this process has important implications in the ability of phagocytes to sense via their PRRs. For example, anti-inflammatory and homeostatic macrophages perform constitutive macropinocytosis, whereas inflammatory macrophages do not (Redka et al. 2018). This shifts the balance in the capacity for sensing via PRRs at the cell surface versus in intracellular compartments. It is as yet unclear how this is related to the respective function of polarized macrophages but is likely to reveal distinct outcomes depending on the site of PRR engagement. Furthermore, dendritic cells demonstrate very high rates of constitutive macropinocytosis in their immature state, but upon maturation completely turn off macropinocytosis (West et al. 2000; Garrett et al. 2000; Calmette et al. 2016). This is likely to facilitate the retention of peptide-loaded MHC on the surface of the cell for presentation to T cells. In particular, cycling of peptide-loaded MHC-I through an acidic compartment, as would be the case during constitutive macropinocytosis, would likely result in the dissociation of peptide-MHC-I complexes in the acidic lumen of the macropinosome (Chefalo et al. 2003). In summary, macropinocytosis has emerged as a critical and tuneable mechanism that facilitates the responsiveness to both pathogens and sterile damage by phagocytes.

Constitutive Macropinocytosis and Antigen Presentation

As discussed above, phagocytes, mainly macrophages and dendritic cells, are uniquely capable of performing constitutive macropinocytosis. Macrophages and dendritic cells also form a bridge between the innate and adaptive arms of the

immune system. They are capable of internalizing and processing exogenous material for presentation to T cells in their function as antigen-presenting cells (APCs). Given this critical role in the initiation of immunity, a role in the acquisition of soluble antigen has been proposed for constitutive macropinocytosis (Fig. 6.2) (Sallusto et al. 1995; Norbury et al. 1997; Hackstein et al. 2002, 2007; Sarkar et al. 2005; von Delwig et al. 2006; Lim et al. 2012; Singla et al. 2018). However, dissecting the contribution of macropinocytosis to the acquisition of soluble antigen has proven challenging.

Much of the evidence describing a role for constitutive macropinocytosis in antigen presentation comes from a model in which APCs are pulsed with soluble ovalbumin (OVA) and subsequently used to activate model T cells that recognize OVA peptides. Presumably, soluble OVA is internalized via macropinocytosis and processed for presentation in the maturing macropinosomes. However, studies have rarely distinguished between macropinocytosis and other modes of endocytosis. It cannot be ruled out, for example, that OVA does not engage a cell surface receptor that elicits a receptor-dependent mode of endocytosis. Indeed, OVA is known to bind the mannose receptor (MR) on the surface of APCs. MR mediates internalization via clathrin-dependent endocytosis and not macropinocytosis (East and Isacke 2002; Sorvillo et al. 2012). Furthermore, under conditions where the MR is genetically deleted, soluble OVA is not internalized efficiently enough to be processed and presented to T cells (Burgdorf et al. 2006). Findings such as this make it particularly difficult to understand the contribution of constitutive macropinocytosis to the presentation of peptides derived from OVA.

The major difficulty in formally linking macropinocytosis to antigen presentation lies in the inability to specifically inhibit it. Many of the drugs traditionally used to inhibit macropinocytosis simultaneously inhibit a broad range of cellular pathways. PI3K inhibitors, such as LY294002 and wortmannin, for example, are often used to inhibit macropinocytosis despite PI3K having roles in other modes of endocytosis such as phagocytosis (Schlam et al. 2015). Likewise, another commonly used macropinocytosis inhibitor the amiloride derivative 5-(N-ethyl-N-isopropyl) amiloride (EIPA) is extraordinarily nonselective and in fact inhibits Na^+/Ca^{2+} exchangers, Na^+ channels, and Na^+/H^+ exchangers (de la Rosa et al. 2000; Masereel et al. 2003; Koivusalo et al. 2010; Orlowski and Grinstein 2011). The inhibitory effect of amiloride derivatives is a result of dysregulated submembranous pH at sites of macropinocytosis due to impaired Na^+/H^+ exchange (Koivusalo et al. 2010). The inhibition of macropinocytosis by amiloride derivatives then is indirect at best and importantly not unique to macropinocytosis. In fact, amiloride derivatives have been shown to inhibit a broad range of cellular activities in immune cells including cell proliferation, differentiation, migration, and even apoptosis (De Vito 2006). Yet, these nonspecific approaches have been used to probe the contribution of macropinocytosis both in vitro and in vivo (von Delwig et al. 2006). Clearly, more specific means of interrogating the role of macropinocytosis in antigen acquisition and presentation are required. The discovery that CaSRs uniquely drive constitutive macropinocytosis in phagocytes will likely represent a valuable tool in dissecting the role of macropinocytosis in antigen presentation.

Emerging Roles for Macropinocytosis in Phagocyte Function

The recent advent of techniques in both the culture and the genetic manipulation of the historically intractable phagocytes has resulted in renewed interest in their biology. Not surprisingly, previously unappreciated roles for macropinocytosis are being recognized. One such role is in the capacity of immature DCs to migrate through complex environments. As phagocytes survey peripheral tissues they encounter external cues that guide their movement. In general, they follow the path of least resistance and their forward motion can be inhibited by a build-up of hydraulic resistance. This is the case for phagocytes such as neutrophils (Prentice-Mott et al. 2013). Immature DCs, however, are less sensitive to hydraulic resistance as they are able to mitigate it by transferring liquid from the front to the back of the migrating cell via macropinocytosis (Moreau et al. 2019). This mechanism allows immature DCs to more broadly survey tissues. Interestingly, as DCs mature they downregulate their capacity for macropinocytosis, which favors their migration along the path of least hydraulic resistance to draining lymph nodes (Moreau et al. 2019). The details of how macropinocytosis contribute to cell migration will be covered in further detail in a separate chapter.

Constitutive macropinocytosis is also implicated in the removal of pro-inflammatory debris from circulation. This is best demonstrated by the clearance of oxidized low-density lipoprotein (oxLDL) particles by macrophages (Doodnauth et al. 2019). LDL particles can become oxidized by exposure to oxidative stress in circulation. The resultant oxLDL particles are well-established contributors to the establishment of atherosclerotic plaques (Tabas Ira et al. 2007). Macrophages express several scavenger receptors, such as CD36, that harbor cationic patches on their extracellular domains that can capture the highly negatively charged oxLDL particles (Canton et al. 2013). A unifying feature of scavenger receptors, however, is that remarkably few harbor signalling motifs or domains on their cytosolic tails. This has resulted in a great deal of confusion as to how the scavenger receptor family mediates the internalization of their ligands. Remarkably, the oxLDL receptor CD36 overcomes this apparent inability to signal by "hitching a ride" on macropinosomes. This phenomenon has been referred to as receptor-assisted macropinocytosis and will likely emerge as a critical pathway involved in the clearance of pro-inflammatory and homeostatic debris by phagocytes (Doodnauth et al. 2019). In line with this, phagocytes uniquely express a strikingly impressive array of scavenger receptors (Areschoug and Gordon 2009; Canton et al. 2013).

Conclusion

Phagocytes are simultaneously key players in the maintenance of organismal homeostasis and at the front lines of the host response to infection. They are uniquely endowed with an array of PRRs that allow them to detect potentially harmful signals

and to respond appropriately. As these pathways continue to be defined, it is increasingly recognized that the subcellular compartmentalization of PRRs results in nuanced responses to discrete stimuli. Constitutive macropinocytosis has emerged as an important route for the delivery of signals to intracellular PRRs. Phagocytes also are responsible for internalizing, processing, and presenting material from their surroundings to cells of the adaptive immune system. It is likely that constitutive macropinocytosis contributes to the delivery of exogenous material to intracellular compartments for loading onto the antigen presentation machinery. Nevertheless, a more careful approach to manipulating macropinocytosis is required to better dissect its role in antigen presentation. Lastly, better tools for genetically manipulating primary phagocytes continue to be developed and have resulted in a renaissance in macropinocytosis research. New roles in diverse phenomena are being ascribed to macropinocytosis and it is therefore likely to emerge as an exciting new field in immunobiology and in the design of future immunotherapeutic strategies.

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Conflict of Interest The author declares that the manuscript was prepared in the absence of any commercial or financial relationships that may be construed as a potential conflict of interest.

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Part II
Signaling Mechanisms Driving Macropinocytosis

Chapter 7
Roles for 3' Phosphoinositides in Macropinocytosis

Joel A. Swanson and Nobukazu Araki

Abstract The distinct movements of macropinosome formation and maturation have corresponding biochemical activities which occur in a defined sequence of stages and transitions between those stages. Each stage in the process is regulated by variously phosphorylated derivatives of phosphatidylinositol (PtdIns) which reside in the cytoplasmic face of the membrane lipid bilayer. PtdIns derivatives phosphorylated at the 3' position of the inositol moiety, called 3' phosphoinositides (3'PIs), regulate different stages of the sequence. 3'PIs are synthesized by numerous phosphoinositide 3'-kinases (PI3K) and other lipid kinases and phosphatases, which are themselves regulated by small GTPases of the Ras superfamily. The combined actions of these enzymes localize four principal species of 3'PI to distinct domains of the plasma membrane or to discrete organelles, with distinct biochemical activities confined to those domains. Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) and phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P_2) regulate the early stages of macropinosome formation, which include cell surface ruffling and constrictions of circular ruffles which close into macropinosomes. Phosphatidylinositol 3-phosphate (PtdIns3P) regulates macropinosome fusion with other macropinosomes and early endocytic organelles. Phosphatidylinositol (3,5)-bisphosphate (PtdIns(3,5)P_2) mediates macropinosome maturation and shrinkage, through loss of ions and water, and subsequent traffic to lysosomes. The different characteristic rates of macropinocytosis in different cell types indicate levels of regulation which may be governed by the cell's capacity to generate 3'PIs.

Keywords Phosphatidylinositol 3-kinase · Macrophage · Macropinosome closure · Ruffling

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Introduction

Increased appreciation for the importance of macropinocytosis in health and disease has highlighted limits to our understanding of the mechanisms of its regulation. The formation of macropinosomes requires several distinct movements of cytoplasm which must be coordinated spatially and temporally. The movements include transient extension of curved protrusions from the plasma membrane followed by retraction and contraction of those protrusions that close them into membrane-bounded macropinosomes derived from plasma membrane. Subsequent intracellular movements of macropinosomes are accompanied by their fusion with endosomes and lysosomes (collectively referred to as the endolysosomal network (Huotari and Helenius 2011)), or by reversal of the process through fusion with plasma membrane. These activities are mediated by the assembly, contraction, and disassembly of the actin filament network that underlies plasma membrane protrusions, by localized fusion and fission between the macropinosome and endolysosomes, and by regulated flux of water, ions, and solutes across the bounding membrane of the macropinosome. The movements are analogous to the movements that phagocytic cells use to ingest particles. However, unlike phagocytosis, the movements of macropinocytosis occur without a particle surface to guide the process. The organization of cytoplasm and signaling molecules during macropinosome morphogenesis varies between cell types and different kinds of stimulation. In all cases, macropinosomes form through a self-organized series of distinct chemical activities which require mechanisms to coordinate the timing of their activation and inhibition.

3' phosphoinositides (3'PIs) are essential to many of these activities and their coordination. Although 3'PIs provide no mechanical or structural support for macropinosome morphogenesis, they do serve to organize the component activities in space and time. This chapter summarizes the known roles for 3'PIs in the component activities and overall organization of macropinocytosis.

Phosphoinositides and the Enzymes that Affect their Abundance

Phosphatidylinositol (PtdIns) is a minor species of phospholipid in cellular membranes, localizing primarily in the leaflet of the membrane lipid bilayer that faces the cytosolic space (inner leaflet). The hydroxyl groups of the inositol sugar moiety of PtdIns (Fig. 7.1a) are readily modified by phosphorylation. Thus, PtdIns is substrate for enzymes that generate phosphatidylinositol 3-phosphate (PtdIns3P), PtdIns4P, and PtdIns5P, which are substrates for lipid kinases that generate phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2), PtdIns(3,4)P_2, and PtdIns (3,5)P_2. Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) is generated by phosphorylation of PtdIns(4,5)P_2 (Fig. 7.1b). These phosphoinositides may be dephosphorylated by lipid phosphatases; for example, the phosphatase PTEN

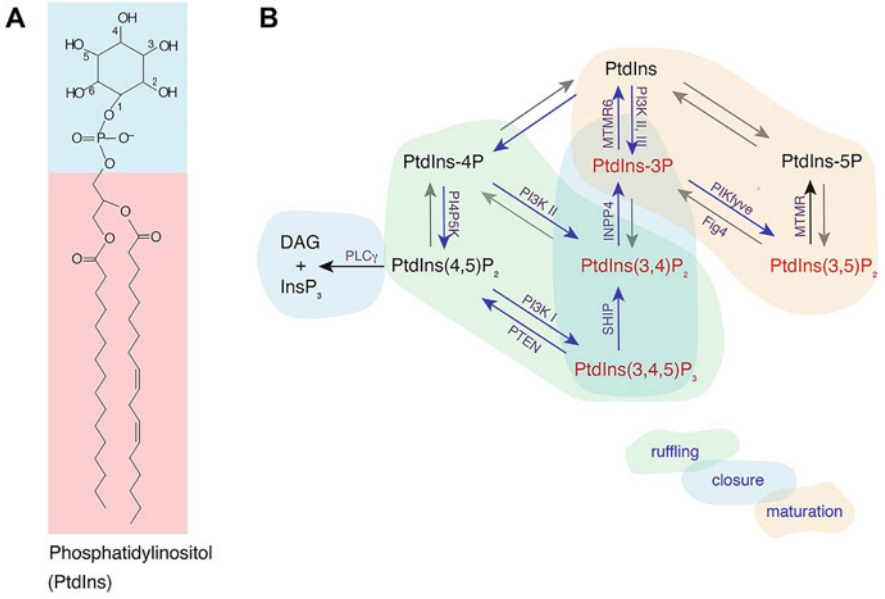

Fig. 7.1 (**a**) The chemical structure of phosphatidylinositol (PtdIns). The blue box highlights the hydrophilic inositol moiety, the phosphodiester linkage to glycerol, and the positions of the hydroxyls which may be variously phosphorylated. The pink box highlights the hydrophobic diacylglycerol moiety which resides in the membrane lipid bilayer. (**b**) Essential pathways of 3'PI metabolism. 3'PIs most relevant to macropinocytosis are indicated in red, the principal pathways of their synthesis and degradation for macropinocytosis are indicated with blue arrows, and the essential enzymes are indicated in purple font. Overlays indicate the biochemical activities associated with ruffling (green), macropinosome formation (blue), and macropinosome maturation (orange). PI3K I: class I PI3K; PI3K II: class II PI3K; PI3K III: Vps34 or class III PI3K. Other labels are indicated in the text

(phosphatase and tensin-homolog) synthesizes PtdIns(4,5)P_2 from PtdIns(3,4,5)P_3 (Di Paolo and De Camilli 2006).

The 3'PIs in metazoan cells are PtdIns3P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3, which are synthesized and degraded by localized activities of phosphoinositide kinases and phosphatases. For example, PtdIns3P may be synthesized by the phosphorylation of PdtIns by the class III phosphoinositide 3-kinase (PI3K) VPS34, by the dephosphorylation of PtdIns(3,4)P_2 by INPP4, or by dephosphorylation of PtdIns(3,5)P_2 by Fig. 4 (Di Paolo and De Camilli 2006). The different 3'PIs distribute into different and characteristic membrane compartments. The reversibility of the phosphorylation reactions allows for the abundance and location of different phosphoinositide species to be regulated rapidly. Also, phosphoinositides diffuse laterally in the plane of the membrane leaflet, conferring on them the ability to integrate laterally the membrane-associated chemical activities within an organelle or membrane domain. Thus, the cytosolic surface of a

macropinosome or phagosome can be rapidly enriched in, or rapidly depleted of, a single species of 3'PI (Henry et al. 2004).

Phosphoinositides can also be hydrolyzed by reactions which are less readily reversed. Phospholipase C (PLC) hydrolyzes PtdIns(4,5)P_2 on the glycerol side of the phosphodiester bond, yielding diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate ($InsP_3$). Other important phospholipases, D, A1 and A2 (PLCD, PLCA1, PLCA2), primarily hydrolyze phospholipid substrates other than phosphoinositides. The reaction products of phospholipases have potent biological activities. Although 3'PIs are not hydrolyzed by phospholipases, the activities of PLCγ1 and PLCγ2 are regulated in part by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Falasca et al. 1998), which may organize PLC-mediated signaling spatially inside cells through their localization and concentrations.

Enzymes that Synthesize or Degrade 3'PIs

Metazoan cells have three classes of PI3K (Jean and Kiger 2014; Vanhaesebroeck et al. 2012). Class I PI3Ks, which synthesize PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 (Toker and Cantley 1997; Vanhaesebroeck et al. 2012), are comprised of two subsets. PI3K class IA includes the catalytic proteins p110α (protein symbol: PIK3CA), p110β (PIK3CB), and p110δ (PIK3CD), and the regulatory proteins p85α, p55α and p50α (PIK3R1), p85β (PIK3R2), p55γ (PIK3R3), and p150 (PIK3R4). They can be activated by tyrosine kinase receptor signaling, and dimers containing p110β can also be activated by heterotrimeric G proteins. Class IB PI3K is comprised of the catalytic protein p110γ (PIK3CG), and the regulatory protein p101 (PIK3R5), which are activated by heterotrimeric G proteins. Class I PI3K catalytic proteins have domains that bind to the small GTPase Ras (p110α, p110δ, and p110γ) or to the GTPases Rac and Cdc42 (p110β), which increase PI3K catalytic activity (Fruman et al. 2017). Class II PI3Ks (PIK3C2A, PIK3C2B, PIK3C2G) synthesize PtdIns3P from PtdIns, and PtdIns(3,4)P_2 from PtdIns4P. Class III PI3K (PIK3C3), also called VPS34, synthesizes PtdIns3P from PtdIns.

Some 3'PIs are synthesized by phosphatases or lipid kinases other than PI3K. SHIP1 (INPP5D) and SHIP2 (INPPL1) are 5'PI phosphatases that hydrolyze PtdIns (3,4,5)P_3 to produce PtdIns(3,4)P_2. Inositol polyphosphate-4-phosphatase (INPP4) synthesizes PtdIns3P from PtdIns(3,4)P_2 (Maekawa et al. 2014). PIKfyve (PIP5K3) is a 5'PI kinase that synthesizes PtdIns(3,5)P_2 from PtdIns3P (Shisheva 2012). 3'PI phosphatases relevant to macropinocytosis include myotubularin-related protein 6 (MTMR6: PtdIns3P to PtdIns (Maekawa et al. 2014)), INPP4 (PtdIns(3,4)P_2 to PtdIns3P; (Maekawa et al. 2014)), Fig. 4 (PtdIns(3,5)P_2 to PtdIns3P; (McCartney et al. 2014)), and PTEN (PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 and PtdIns(3,4)P_3 to PtdIns-4P) (Goulden et al. 2019; Jiao et al. 2020; S. M. Kim et al. 2018). PTEN is an important inhibitor of class I PI3K functions (Maehama et al. 2001). PTEN deletion occurs in many cancers, highlighting the significance of PtdIns(3,4,5)P_3 in supporting cell growth (S. M. Kim et al. 2018).

The strong association of PI3K metabolism with cancer and other diseases has led to the development of many inhibitors of PI3K and related enzymes. Broad specificity PI3K inhibitors include wortmannin and LY294002, both of which inhibit macropinocytosis (Araki et al. 1996). Numerous inhibitors of class I PI3Ks are in clinical use or various stages of evaluation. Class III (VPS34) PI3K inhibitors, including 3-methyladenine, VPS34-IN1, and SAR405 (Araki et al. 2006; Bago et al. 2014; Miller et al. 2010; Ronan et al. 2014), have potential for therapeutic treatments in cancer. Class II PI3K inhibitors have not been described. The PIKfyve inhibitor apilimod has been used to treat autoimmune disease and cancer (de Campos et al. 2020; Ikonomov et al. 2019).

How Phosphoinositides Organize Cytoplasm

A membrane lipid bilayer containing phosphoinositides presents a surface decorated with variously phosphorylated inositide sugars anchored to the membrane by their diacylglycerol moieties. Cytoplasmic proteins with phosphoinositide-binding domains concentrate at these membrane surfaces by diffusion and binding, where they are activated allosterically or by their increased proximity to membrane-localized binding proteins. Many kinases, phosphatases, and hydrolases bind 3'PIs, as do proteins that regulate small GTPases of the Ras superfamily which can modulate other effectors allosterically. Thus, an organelle membrane enriched in a particular 3'PI recruits and activates a distinct combination of enzyme activities that stabilize the identity of that domain, execute a defined set of effector activities, and guide its transition to a specific different identity. The identities of some membranous compartments in cells, especially those that comprise the endolysosomal system, are transient and vectorial, meaning that the molecular profile of the organelle membranes remains stable for a limited period before changing to another specific profile as the organelle ages. During the two to five minutes it takes to form a macropinosome, the biochemical profile of the membrane changes transiently and sequentially from that of the plasma membrane to that of early endosomes. A newly formed macropinosome is enriched in the GTPase Rab5a. Active Rab5a activates the class III PI3K VPS34, which synthesizes PtdIns3P, thereby increasing concentrations of PtdIns3P in the macropinosome membrane. The GTPase-activating protein (GAP) that inactivates Rab5a is itself activated by PtdIns3P (Law et al. 2017). Thus, increasing concentrations of PtdIns3P on the macropinosome membrane activate feedback inhibition that leads to the loss of Rab5a and the arrival of Rab7 (Langemeyer et al. 2020). Consequently, the Rab5a-positive, PtdIns3P-rich membrane of the nascent macropinosome transitions after several minutes to another profile depleted of those molecules and enriched in Rab7 and PtdIns(3,5)P_2.

These chemistries underlie the progression of different stages during macropinosome maturation. The characteristic profiles organize the local effector activities: actin polymerization, actomyosin contractility, membrane fusion, and the

transitions to different stages of the maturation sequence. These transitions sometimes define decision branch-points between two maturation routes. In phagocytosis, 3'PI concentrations must reach thresholds for commitment to particle ingestion (Zhang et al. 2010). In this way, 3'PIs can integrate and direct the activities of cytoplasm.

Localization and Mapping of 3'PIs and Associated Chemistries

Much of what is known about the organization of 3'PIs in macropinocytosis has been discovered through fluorescence microscopy (Maekawa and Fairn 2014). When expressed in cells, fluorescent protein (FP) chimeras of PI-binding domains can concentrate near membranes enriched for the target PI. The net synthesis and degradation of the target PIs can be monitored by confocal microscopy or by ratiometric widefield fluorescence microscopy of the FP chimeras (Araki et al. 2007; Hoppe and Swanson 2004; Vieira et al. 2001). For example, a yellow fluorescent protein (YFP) chimera with the PtdIns(3,4,5)P_3-binding PH domain of the enzyme Bruton's tyrosine kinase (YFP-BtkPH), when expressed inside a cell, distributes uniformly through the cytoplasm of an unstimulated cell. When stimulation increases class I PI3K activity, the YFP-BtkPH concentrates on membranes enriched in PtdIns(3,4,5)P_3. This has allowed study of the distributions and dynamics of PtdIns(3,4,5)P_3 in living cells during macropinosome formation (Araki et al. 2007; Yoshida et al. 2009). A drawback of this method is that high levels of expression of 3'PI-binding FP chimeras can interfere with the 3'PI-dependent reactions they are meant to reveal (Wills et al. 2018; Wills et al. 2021). Control experiments are needed to ensure such artifacts do not alter the essential 3'PI dynamics significantly.

The Cellular Activities Essential for Macropinocytosis

Some cancer cells exhibit macropinocytosis constitutively. In many non-transformed cells, however, macropinocytosis occurs in response to stimulation of cell surface receptors, most notably growth factor receptors, which initiate cell movements that lead to macropinosome formation. The morphologies of these movements vary widely among cell types and even within a single cell. The protrusions which close into macropinosomes are called ruffles, which are curved folds of plasma membrane with underlying meshworks of actin filaments. The actin filaments inside ruffles are polymerized into roughly planar arrays of parallel filaments or cross-linked networks of filaments. Actin polymerization occurs either at the distal margins of ruffles, which are enriched in the growing ends of actin

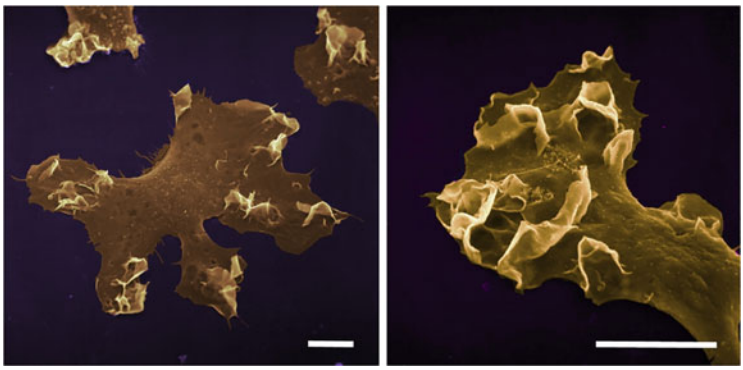

Fig. 7.2 Scanning electron micrographs of bone marrow-derived macrophages stimulated with CSF-1, showing dorsal surface ruffling and macropinocytic cup formation. Scale bars: 5 μm

filaments, or at branch-points along the sides of actin filaments. Actin cross-linking proteins may reinforce the meshwork structure (Sasaki et al. 2001). The organization of ruffles is regulated by cytoplasmic microtubules (Rosania and Swanson 1996; Waterman-Storer et al. 1999). In actively macropinocytic cells observed on coverslips, ruffles extend as protrusions from the dorsal surface (i.e., the surface facing away from the coverslip; Fig. 7.2). Most ruffles form as curved sheet-like extensions, which either continue growing into fully circular, crater-shaped extensions of the cell surface or close back against the cell as a cresting wave, trapping extracellular fluid into plasma membrane-derived vesicles. In a cell which is spread out on a coverslip, single ruffles that cover large areas of the surface, called circular dorsal ruffles, often appear after acute stimulation with growth factors. They mature by constricting centripetally and forming macropinosomes near the ruffling regions. More commonly, the ruffles on a cell surface are smaller and short-lived, lasting only one to five minutes. They resolve by receding back into the cell or by closing into macropinosomes. Closure was thought to occur at the distal margins of ruffles (J. A. Swanson 2008). This occurs sometimes but the more common processes involve either an asymmetric wavelike closing against the cell (Quinn et al. 2021) or a circumferential constriction of the macropinocytic cup near the base of the circular ruffles, creating macropinosomes which are small relative to the ruffles that precede them (Fig. 7.3). The basal constriction that closes into a macropinosome may involve a twisting movement of tent-pole like actin bundles within the ruffle (Condon et al. 2018), or other kinds of constrictions away from the distal margin of the ruffle (Quinn et al. 2021).

Once a macropinosome has separated from plasma membrane as an intracellular organelle, which we refer to here as macropinosome closure, it takes either of two routes. It may reverse course and return to the plasma membrane (Feliciano et al. 2011) or begin a series of changes that ultimately lead the macropinosome to merge with endolysosomes. The regurgitation may simply be a redistribution of plasma membrane following incomplete closure of the macropinosome. Macropinosomes

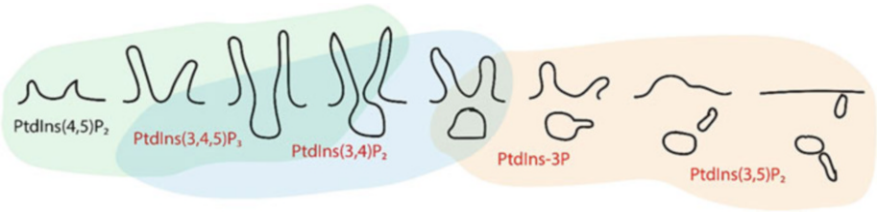

Fig. 7.3 Summary of the stages of macropinocytosis and the corresponding 3'PIs. The stages of macropinosome formation and maturation are indicated as side view sections progressing from left to right. The predominate phosphoinositides at each stage are indicated. Overlays indicate the membrane movements associated with ruffling (green), macropinosome formation (blue), and macropinosome maturation (orange). Small ruffles on a quiescent surface are activated by Arf6 and PtdIns-4P to generate early ruffles, enriched in PtdIns(4,5)P_2 and active Cdc42. As ruffles enlarge, concentrations of PtdIns(3,4,5)P_3 increase, as well as the activities of Ras, Rac, and Rab35. Ras and Rab35 promote the feedback amplification of class I PI3K and Rac activities. Closing macropinosomes are enriched in PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. During or just after closure, Rac is deactivated and the activities of Rab5 and Vps34 increase. Nascent macropinosomes are stabilized by elevated concentrations of PtdIns3P and by increased activities of Rab5, Rab35, Rab20, Rab21, and Arf6. Maturing macropinosomes fuse with other macropinosomes and with endolysosomes. Increasing concentrations of PtdIns(3,5)P_2 on macropinosomes increase the activities of TPC1, TPC2, and Septins

that do not recycle immediately to the plasma membrane swell initially, transforming from irregular shapes into more rounded shapes, then shrink by the export of the ingested sodium and chloride and the osmotically obliged water (Freeman et al. 2020). The ions introduced into cytoplasm by export from macropinosomes are expelled from the cell by plasma membrane ion transporters such as the Na/K ATPase, thereby allowing equilibration of cell volume (Freeman et al. 2020). Macropinosome shrinkage allows the formation of small vesicles or narrow membranous tubules which break away from the macropinosome and return membrane and plasma membrane proteins to the cell surface (Freeman et al. 2020; Kerr et al. 2006). Meanwhile, newly formed macropinosomes migrate from the cell periphery to perinuclear positions, first fusing with others of their kind or with early endosomes, then merging with endolysosomes (Racoosin and Swanson 1993). This later stage of maturation sometimes occurs by transient and reversible connections between the macropinosome and the endolysosomes, which has been called pyranhalysis or kiss-and-run (Willingham and Yamada 1978; Yoshida et al. 2015b). Eventually the macropinosome merges completely into the endolysosomal network, where the internalized macromolecular solutes are degraded by acid hydrolases. Thus, in a cell which is continuously forming macropinosomes, internalized water and ions move across macropinosome membranes into cytoplasm and out of the cell, internalized membrane is recycled to plasma membrane via recycling tubules, and extracellular macromolecules are scavenged efficiently for hydrolytic degradation to smaller molecules that support cell metabolism.

3'PI-Dependent Activities of Macropinocytosis

Macropinocytosis requires the localized synthesis of 3'PIs at different stages of the process (Fig. 7.3). PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are concentrated in circular ruffles and closing macropinosomes (Yoshida et al. 2009). PtdIns3P is concentrated in newly formed macropinosomes (Araki et al. 2006). Quantitative fluorescence microscopy of 3'PI-binding FP chimeras expressed in macrophages showed that macropinocytic cups formed in response to stimulation with Colony-stimulating Factor-1 (CSF-1) exhibited a sequence of associated phosphoinositides during their formation and closure; PtdIns(4,5)P_2 increased first in circular ruffles, followed by transient, sequential peaks of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdIns3P as macropinosomes closed into the cell (Welliver and Swanson 2012). Imaging of other cell types indicated similar patterns of PtdIns(4,5)P_2 and 3'PIs during macropinocytosis (Araki et al. 2007; Porat-Shliom et al. 2008). Genetic analysis of macropinocytosis in *Caenorhabditis elegans* embryos demonstrated an essential sequence of 3'PI-modifying enzymes necessary for macropinosome formation and maturation that was consistent with the fluorescence microscopic studies (Maekawa et al. 2014). The implied sequence of 3'PIs in the *C. elegans* study was PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, PtdIns3P, PtdIns. PtdIns(3,5)P_2 is not readily visualized in living cells, but experimental manipulation of PIKfyve activity indicates the importance of PtdIns(3,5)P_2 for late stages of macropinosome maturation (Krishna et al. 2016). With this sequence of phosphoinositides in the various stages of macropinocytosis, we next review the roles for the principal 3'PI species in the underlying biochemical activities.

Class I PI3K Is Necessary for Some But not all Ruffling

The four class I PI3K catalytic proteins have been implicated in macropinocytosis to varying degrees depending on their levels of expression and the receptor pathways that initiate the process. They can be activated by growth factor receptors, Toll-like receptors (TLR), chemokine receptors, and G-protein-coupled receptors (GPCR). p110β is regulated by inputs from both GPCR and tyrosine kinase receptors, and functions as a coincidence detector or integrator of signaling inputs (Bresnick and Backer 2019). Receptors bind to PI3K directly or to adapter proteins that recruit and activate p85 regulatory proteins (Fruman et al. 2017). PtdIns(3,4,5)P_3 synthesis increases by allosteric activation of PI3K catalytic proteins and by the increased proximity of the enzymes to their substrates. The actin cytoskeleton, organized by cytoplasmic microtubules (Rosania and Swanson 1996) or some other structural feature of the cups themselves, facilitates amplification of PI3K activities in plasma membrane domains circumscribed by ruffles (Erami et al. 2017; Pacitto et al. 2017; Yoshida et al. 2018). FP chimeras of PH domains show increased concentrations of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in the membranes associated with ruffles (Araki

et al. 2007; Yoshida et al. 2018). The phosphatase PTEN lowers levels of PtdIns (3,4,5)P_3 in cells and provides a brake on stimulation of PI3K leading to macropinocytosis. PTEN deletion or inhibition often stimulates macropinocytosis (S. M. Kim et al. 2018). For some cells, class I PI3K is required for ruffling, a necessary prerequisite for macropinosome formation. In murine embryonic fibroblasts (MEF) and PTEN-deficient cancer cells, p110β is required for ruffling and macropinocytosis in response to PDGF (Salloum et al. 2019). In contrast, PI3K inhibition in macrophages inhibits macropinosome closure but not ruffling in response to CSF-1 (Araki et al. 1996). In summary, class I PI3Ks are required for both ruffling and closure in some cells, but only for closure in others.

How do class I PI3Ks organize ruffling? The localized and oriented polymerization of actin filaments beneath ruffles requires the small GTPase Rac, an essential regulator of ruffling and macropinosome formation (Buckley et al. 2020; Fujii et al. 2013; Grimmer et al. 2002). The Rac effectors p21-activated kinase-1 (Pak1) and WAVE are important for macropinocytosis (Dharmawardhane et al. 2000; Veltman et al. 2016). Phosphorylated Pak1 binds to and activates LIM kinase, which phosphorylates proteins that regulate actin filament dynamics, including filament-uncapping proteins and the filament-severing protein cofilin (Delorme et al. 2007). Pak1 also activates Ctb1/BARS, a protein essential to macropinosome closure which works through activation of phospholipase D (Haga et al. 2009). Rac also activates WAVE, which stimulates ruffle extension by activating Arp2/3, which mediates the formation of actin filament branches on other actin filaments (Eden et al. 2002). PtdIns(3,4,5)P_3-dependent guanine nucleotide exchange factors (GEFs) which activate Rac include Tiam-1, Vav, and TRIO (Bai et al. 2015). For cells that require PI3K for ruffling, these Rac GEFs may initiate the ruffles leading to macropinocytosis. However, as inhibition of class I PI3K does not inhibit ruffling in all circumstances, it is not certain that the PtdIns(3,4,5)P_3-dependent Rac GEFs are necessary for macropinocytosis.

The mechanism by which ruffles become circular is still unclear. The signaling pathway and machinery of large circular dorsal ruffle formation are distinct from those of relatively small circular ruffles or macropinocytic cups formed by the curling of peripheral ruffles (Itoh and Hasegawa 2013). The mechanism of large circular dorsal ruffle formation is well characterized in PDGF-stimulated fibroblasts. The formation of circular ruffles from peripheral ruffles is not perturbed by PI3K inhibitors in macrophages or EGF-stimulated A431 cells (Araki et al. 2007; Araki et al. 1996). However, the formation of large circular dorsal ruffles ($>$ 20 μm in diameter) observed in some types of culture cells, such as PDGF-stimulated fibroblasts, is dependent on class I PI3K activity (Salloum et al. 2019; Wymann and Arcaro 1994). In cells that form circular dorsal ruffles, increased PtdIns(3,4,5)P_3 concentrations persist for several minutes within the domain of plasma membrane circumscribed by the actin-rich ruffles (Yoshida et al. 2018). These domains may facilitate PI3K amplification (Pacitto et al. 2017). Macropinosomes form at the base of the contracting circular dorsal ruffles. Lanzetti et al. showed that Rab5 organizes circular dorsal ruffle formation through coordinated activities of PI3K, Ras, and Rac (Lanzetti et al. 2004). SH3YL1 (SH3 domain containing Ysc84-like 1), which binds

to PtdIns(3,4,5)P_3, is an important regulator of dorsal ruffle formation (Hasegawa et al. 2011). PtdIns(3,4)P_2 synthesis from PtdIns(3,4,5)P_3 by the 5'PI phosphatase SHIP2, which also binds to SH3YL1, is correlated with formation of the circular ruffles. ARAP1 (Arf GAP with Rho GAP domain, ankyrin repeat, and PH domain 1), which is an Arf GAP with multiple PH domains that bind to PtdIns(3,4,5)P_3, localizes to the membrane inside the circular ruffles after PDGF-stimulation. ARAP1 and its substrate Arf1/5 are involved in the ring size control of circular ruffles (Hasegawa et al. 2012). The actin cytoskeleton machineries N-WASP, WAVE, and Arp2/3, which are effectors of Rac1, are also localized to circular ruffles (Krueger et al. 2003; Legg et al. 2007; Suetsugu et al. 2003). Also, the F-actin-bundling protein actinin-4 localizes in circular ruffles of macrophages (Araki et al. 2000) and PDGF-stimulated fibroblasts (Lanzetti et al. 2004). PtdIns(4,5)P_2 and PdtIns(3,4,5)P_3 differentially regulate actinin flexibility and actin-bundling function through their binding to the calponin homology domain 2 of α-actinin (Corgan et al. 2004; Fraley et al. 2003). The Rab5 GAP RN-tre interacts with both F-actin and actinin-4 and is also necessary for circular ruffle formation (Lanzetti et al. 2004).

As mentioned above, Rac1 is indispensable for membrane ruffling. However, strong overexpression of constitutively active Rac1 produces long straight linear ruffles in RAW264 cells, indicating that a Rac1 effector promotes ruffle formation but not circularization of the ruffles (Ikeda et al. 2017). Lanzetti et al. (2004) showed the same result in PDGF-stimulated MEFs. Local and temporal modulation of Rac1 activity within a small cell surface area may be required for circular ruffle formation.

Oncogenic Ras stimulates macropinocytosis in many cells (Bar-Sagi and Feramisco 1986). Stimulation may occur through the binding of GTP-Ras to the Ras-binding domains of PI3K catalytic subunits p110α, p110δ, or p110γ, which leads to local generation of PtdIns(3,4,5)P_3. H-Ras-dependent macropinocytosis in HeLa cells leads to formation of PtdIns(3,4,5)P_3-rich macropinosomes (Porat-Shliom et al. 2008). Alternatively, stimulation of macropinocytosis may be due to the effects of oncogenic Ras on the redistribution of cholesterol to plasma membrane which consequently increases Rac localization to plasma membrane (Ramirez et al. 2019). The requirement for wild-type Ras in macropinocytosis is uncertain, however, as deletion of H-, K-, and N-Ras in MEFs did not inhibit macropinocytosis (Palm et al. 2017).

Ruffling and macropinocytosis also require other Class I PI3K-dependent GTPases, including Arf6, Cdc42, RhoG, and Abi1. Arf6 is required for macropinocytosis in H-Ras-transformed HeLa and HT1080 cells (Porat-Shliom et al. 2008; Williamson and Donaldson 2019). The Arf6 GEF cytohesin 2 is activated by PI3K (Davies et al. 2014). Arf6 effectors are PI4P5K, WAVE, and JIP3/4, which together increase actin polymerization and recycling of internalized membrane to the plasma membrane. The RhoG GEF SGEF was shown to stimulate macropinocytosis (Ellerbroek et al. 2004). P-Rex1 is a PtdIns(3,4,5)P_3-dependent GEF for RhoG (Damoulakis et al. 2014). RhoG activates Rac by binding to ELMO in complex with the Rac GEF DOCK180. Thus, 3'PIs may promote ruffling through RhoG, upstream of Rac. Abi1, in complex with Abl and PI3K p85, promotes macropinocytosis (Dubielecka et al. 2010; N. Kim et al. 2019; Kotula 2012).

Rab-family GTPases can also activate class I PI3Ks for macropinocytosis. Rab35 immunoprecipitates with PI3K p85α and stimulates actin dynamics (Marat et al. 2012) and the formation of phagosomes (Egami et al. 2011), circular dorsal ruffles, and macropinosomes (Corallino et al. 2018). Rab35 inhibits Arf6 (Egami et al. 2015). The Rab35 effector ACAP2 is an Arf6 GAP which may be relevant to macropinocytosis (Kobayashi and Fukuda 2012). Rab8a is activated by TLRs and activates p110γ (Wall et al. 2019; Wall et al. 2017). Rab10 is recruited to PtdIns(3,4,5)P_3-positive macropinosomes in macrophages and regulates the formation of recycling vesicles (Liu et al. 2020). However, Rab10 recruitment is PtdIns(3,4,5)P_3-independent (Kawai et al. 2021).

Class I PI3K and Macropinosome Closure

Although class I PI3Ks are not always necessary for cell ruffling, they are nearly always required for macropinosome closure. The constriction that closes ruffles and cups into macropinosomes requires a PI3K-dependent contractile activity mediated by nonmuscle myosins (Araki et al. 1996; J.A. Swanson et al. 1999). High local concentrations of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at the base of the ruffles may activate PLCγ1 or PLCγ2, generating DAG from PtdIns(4,5)P_2. The only known exception to this requirement for class I PI3K in macropinosome closure is in phorbol myristate acetate (PMA)-stimulated macropinocytosis, which does not require class I PI3K for ruffling or closure (Yoshida et al., 2015a). Moreover, PMA-elicited macropinosomes do not generate significant levels of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 in murine bone marrow-derived macrophages (Yoshida et al. 2015b). This indicates that PMA bypasses class I PI3K-dependent activities necessary for closure; likely through mimicry of DAG, the product of PLCγ1. However, studies of other cells indicate roles for type I PI3K in PMA-stimulated macropinocytosis, so further studies will be needed to explain the different results.

Protein kinase C isoforms (PKCs) essential to macropinosome formation are activated by DAG and possibly also by calcium released by InsP_3-binding channels in endoplasmic reticulum. PKC (Yoshida et al. 2015a) or another DAG-dependent activity (Ard et al. 2015) then stimulates the contractile activities of myosin that constrict the cup. Myosin 1B, myosin 1E, and myosin IF are also implicated in macropinosome formation in *Dictyostelium discoideum* (Brzeska et al. 2016; Chen et al. 2012). Because Myosin 1E and myosin 1F, which have a PtdIns(3,4,5)P_3-binding tail homology region 1 (TH1) domain, are recruited to the membrane through interaction with PtdIns(3,4,5)P_3 during chemotaxis and phagocytosis in neutrophils (Chen and Iijima 2012) and RAW macrophages (Ikeda et al. 2017), these isoforms of myosin may contribute to macropinosome closure in mammalian cells as well as *Dictyostelium cells*. Macropinocytosis is inhibited by the myosin II inhibitor blebbistatin (Jiang et al. 2010; Lou et al. 2014; Williamson and Donaldson 2019; Yoshida et al. 2015b) and by the myosin light chain kinase (MLCK) inhibitor ML-7 (Araki et al. 2003). Unlike the myosin I isoforms, the recruitment and contractile activity of myosin II is independent of PtdIns(3,4,5)P_3 (Araki et al. 2003).

Macropinosome closure requires the inactivation of Rac. Fluorescence microscopy of YFP-BtkPH in macrophages showed a transient spike (ca. 90 sec) of PtdIns(3,4,5)P_3 in cup membranes associated with closure of macropinosomes. Fluorescence resonance energy transfer (FRET)-based imaging showed a coincident spike of Rac activity, which suggested that Rac activation and deactivation are both necessary for macropinosome closure. Consistent with this idea, Fujii et al. (Fujii et al. 2013) showed that although Rac activity is necessary for the ruffling that creates macropinosomes, Rac must be inactivated to allow closure of the macropinosome. Experimentally forcing Rac to remain in its active, GTP-bound conformation inhibited macropinosomes from fully closing into the cell. This suggests that inactivation of Rac by a GTPase-activating protein (GAP) is necessary for closure. PtdIns(3,4,5)P_3-binding GAPs for Rac and Cdc42 were shown to be necessary for phagocytosis of large particles (Schlam et al. 2015). Similar PtdIns(3,4,5)P_3-dependent Rac GAP activities may be required for macropinosome closure.

PtdIns(3,4)P_2 may have distinct functions in macropinocytosis. Most of the class I PI3K activities that increase PtdIns(3,4,5)P_3 also increase PtdIns(3,4)P_2, and both species can activate many class I PI3K-dependent activities. Specific roles for the dephosphorylation of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by SHIP-1 or SHIP-2 in ruffles have not been demonstrated; however, there are suggestions that PtdIns(3,4)P_2 facilitates scission of macropinosomes from plasma membrane into the cytoplasm (Hawkins and Stephens 2016).

PtdIns3P Facilitates Macropinosome Formation and Macropinosome Maturation

All macropinocytosis requires synthesis of PtdIns3P. In metazoan cells, PtdIns3P accumulates on membranes of cups and nascent macropinosomes. In A431 cells, the class III PI3K inhibitor 3-methyladenine did not inhibit macropinosome formation but prevented accumulation of PtdIns-3P and EEA1 on macropinosomes, as well as homotypic fusion of macropinosomes and subsequent macropinosome maturation (Araki et al. 2006). PtdIns3P synthesis on macropinosomes may occur by a sequential cascade in cup membranes, in which PtdIns4P is phosphorylated to PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, then dephosphorylated to PtdIns(3,4)P_2 then PtdIns3P (Welliver and Swanson 2012). Alternatively, macropinosomal PtdIns3P may be synthesized simply by VPS34-mediated phosphorylation of PtdIns. Which of these pathways to PtdIns3P predominates, and how these pathways are selected, remains unknown.

Rab5a is also required for macropinosome closure. Experimentally limiting Rab5a activation leads to the formation of unstable macropinosomes which either fail to close into the cell or fuse back with the plasma membrane without maturing (Feliciano et al. 2011). Rab5a may stabilize macropinosomes by recruiting and activating Vps34, thus promoting the synthesis of PtdIns3P (Christoforidis et al.

1999). PtdIns3P stabilizes macropinosomes for fusion with other endosomes and for continued maturation. The PtdIns3P-binding sorting nexins (e.g., SNX5) and other PtdIns3P-binding proteins associate with the tubular extensions of macropinosomes that mediate membrane recycling following shrinkage. Rab5a markedly accumulates on nascent macropinosomes after the PtdIns(3,4,5)P_3 spike, coincident with the rise in PtdIns3P levels (Welliver and Swanson 2012). PtdIns3P-rich membrane may prevent regurgitation by promoting activation of CORVET-HOPS complexes, which regulate PtdIns3P-dependent homotypic fusion of endosomes (CORVET) and the Rab5 to Rab7 transition (HOPS) (Solinger and Spang 2013). Nascent macropinosomes lacking PtdIns3P often fail to mature, and in some cells macropinosomes recycle without fusing to endolysosomes (Hewlett et al. 1994). In EGF-stimulated macropinocytosis by A431 cells, PtdIns3P and EEA1 persisted on membrane of macropinosomes as long as the macropinosomes were present in the cells. Macropinosomes decreased in size and number with time but did not mature into late endosome/lysosomes (Araki et al. 2006; Hamasaki et al. 2004). Their content was not delivered to endolysosomes but instead recycled to extracellular space. It remains unclear why this cell behaves differently than most cells.

Synthesis of PtdIns(3,5)P_2 by PIKfyve Mediates Macropinosome Shrinkage

PIKfyve activity is required for the shrinkage of macropinosomes that accompanies maturation (Krishna et al. 2016). Macropinosome shrinkage is mediated by the lysosomal cation channel TRPML1/MCOLN1 and by the two-pore channels TPC1 and TPC2, which mediate the PtdIns(3,5)P_2-dependent export of sodium and calcium from macropinosomes (Freeman et al. 2020; Krishna et al. 2016). Depletion of PtdIns(3,5)P_2 by PIKfyve inhibition prevents ingested sodium and chloride of internalized fluids from being transported out of macropinosomes via TPC1; consequently, water accumulates and distends the vacuolar compartments and thereby inhibits the return of membrane to the cell surface via recycling tubules (Freeman et al. 2020). PtdIns(3,5)P_2 also regulates macropinosome fusion through recruitment of septin GTPases (Dolat and Spiliotis 2016).

Roles for PI3K in Macropinocytosis by *Dictyostelium discoideum*

The free-living amoeba *Dictyostelium discoideum* feeds by phagocytosis of smaller microbes and, to a limited extent, by macropinocytosis of extracellular fluids. Laboratory strains of *Dictyostelium* selected for axenic growth in liquid medium exhibit increased macropinocytosis, which allows sufficient ingestion of soluble

nutrients to support their metabolism (Hacker et al. 1997). Macropinosomes of axenic strains form similarly to those of metazoan cells and wild-type *Dictyostelium*: protrusions of plasma membrane organize into cup-shaped cell extensions which constrict and close into macropinosomes. The macropinosomes of axenic strains are larger than those of wild-type strains because of spontaneous mutations in the gene for the Ras GAP NF-1. The deficiency of NF-1 increases the activity of *Dictyostelium* Ras proteins and associated PI3K activity in the domains of plasma membrane that form macropinocytic cups, which allows the formation of larger cups and macropinosomes. The versatility of *Dictyostelium* for genetics and fluorescence microscopy has allowed insightful and revealing analyses of the roles for 3'PIs in macropinosome formation.

PI3K is essential for macropinocytosis in *Dictyostelium*, but it does not synthesize 3' phosphoinositides. Rather, the variously phosphorylated inositol headgroups are anchored to the membrane by an ether linkage between a fatty acid chain and the glycerol backbone, rather than an ester linkage, and the substrates for *Dictyostelium* PI3K isoforms and PTEN are plasmanylinositols (Clark et al. 2014). Thus, macropinosome formation in *Dictyostelium* is regulated by a molecule with the same inositol headgroup as PtdIns(3,4,5)P_3, which we refer to here as PIP_3, but with a different lipid backbone. PIP_3 is concentrated within the borders of the circular cup or patch of membrane, with a distinct boundary between the PIP_3-rich interior and the PIP_3-poor membrane outside the cup. In contrast to metazoan cells, PI3P does not accumulate in forming cups or nascent macropinosomes. PTEN localizes to plasma membrane outside of the cup, which suggests that its exclusion helps define the PIP_3 patch. *Dictyostelium* PI3K1 and PI3K2 support ruffling and cup formation; PI3K4 supports closure (Hoeller et al. 2013). They are activated allosterically by GTP-RasG or GTP-RasS and their Ras-binding domains are required for macropinocytosis (Hoeller et al. 2013). PIP_3 and active Ras coincide in the cup membranes, which suggests a positive feedback amplification mechanism involving Ras and PI3K (Veltman et al. 2016). Such feedback interactions have been identified in other motility systems (Thevathasan et al. 2013).

Macropinosome formation in *Dictyostelium* also requires the GTPase Rac and its effector SCAR/WAVE, which activates Arp2/3-based actin polymerization at the outer rim of the cup. Coronin and formins are also essential for macropinocytosis (Junemann et al. 2016; Kelsey et al. 2012). Thus, despite the different mechanisms between metazoans and *Dictyostelium* of inositol anchorage to membranes, the conserved requirement for Ras-regulated PI3K in macropinocytosis indicates the importance of anchored inositol phosphates for organizing actin into cups and macropinosomes.

Feedback Regulation of Macropinocytosis by 3'PIs

The cell's ability to generate PtdIns(3,4,5)P_3 and macropinosomes may be limited by metabolism or cellular dimensions. Macropinocytosis is a source of nutrients for some cancer cells, which suggests that ingestion may be regulated by nutrient supply

or other metabolic needs. AMP kinase, whose activity increases when ATP levels are low, is required for macropinocytosis in starved PTEN-deficient cancer cells (S. M. Kim et al. 2018). Activation of Akt by PtdIns(3,4,5)P_3 increases activity of the metabolic regulatory complex mTORC1 (Laplante and Sabatini 2012). mTORC1 can limit protein scavenging by macropinocytosis (Palm et al. 2017). mTORC1 is negatively regulated by TSC1/2, which is itself negatively regulated by Akt1. However, TSC2-deficiency, which increases mTORC1 activity, upregulates VPS34-dependent macropinocytosis (Filippakis et al. 2018), and this discrepancy is not simply explained.

The ability to synthesize 3'PIs necessary for macropinocytosis may be regulated by larger-scale feedback related to the actin and microtubule cytoskeleton, or to the dimensions of the vacuolar compartment or of the cell itself. PI3K is required for phagocytosis of large but not small particles (Cox et al. 1999), which suggests a role for 3'PIs in regulating the size of permissible gulps. Phagocytosis requires concentrations of PtdIns(3,4,5)P_3 to exceed a threshold concentration for particle ingestion (Zhang et al. 2010). The cell's ability to attain such concentrations of PtdIns(3,4,5)P_3 in phagosomal or macropinocytic cups may be regulated by the cell's capacity for enlargement. That is, 3'PIs may serve as permissive gates for invagination or for progression through the stages of macropinosome maturation.

Remaining Questions

Most of the essential components of macropinocytosis have been identified, yet we remain largely ignorant about how their activities are regulated overall. What factors regulate the characteristic rates or capacity of macropinocytosis in different cell types? If macropinocytosis occurs by self-organized chemistries, then what controls the magnitude of those reactions? Do 3'PIs regulate cup size, frequency of macropinosome formation, or macropinosome stability? If so, how? Why is only some ruffling PI3K-dependent? How does PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 organize cup closure? What regulates the curvature of ruffles? How does Ras regulate macropinocytosis? Answers to these questions will likely reveal how macropinocytosis contributes to health and disease.

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Chapter 8
Signaling Pathways that Regulate Macropinocytosis in Mammalian Cells

Wilhelm Palm

Abstract Macropinocytosis is an evolutionarily conserved endocytic pathway that mediates non-selective uptake of extracellular fluid in bulk. Macropinocytosis is initiated by localized polymerization of the actin cytoskeleton, which generates plasma membrane protrusions that enclose part of the environment into large endocytic vesicles. From amoebae to mammalian cells, the actin dynamics that drive macropinosome formation are regulated by a conserved set of intracellular signaling proteins including Ras superfamily GTPases and PI3-kinases. In mammalian cells, multiple upstream signaling pathways control activity of these core regulators in response to cell-extrinsic and cell-intrinsic stimuli. Growth factor signaling pathways play a central role in macropinocytosis induction. In addition, an increasing number of functionally diverse processes has been identified as macropinocytosis regulators, including several nutrient-sensing and developmental signaling pathways. Many of these signaling pathways have proto-oncogenic properties, and their dysregulation drives the high macropinocytic activity that is commonly observed in cancer cells. These regulatory principles illustrate how macropinocytosis is controlled by complex upstream inputs to exert diverse cellular functions in physiological and pathological contexts.

Keywords Macropinocytosis · growth factor signaling · nutrient-sensing · developmental signaling · oncogenic signaling · Ras GTPase · PI3-kinase · AMPK · mTORC1

Introduction

Macropinocytosis, or "large-scale cell drinking", is a non-selective endocytic pathway that internalizes extracellular fluid and therein contained solutes into large vesicles referred to as macropinosomes (Mercer and Helenius 2009; Swanson

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2008; Bloomfield and Kay 2016). Macropinocytosis emerged early in the evolution of eukaryotic cells and is conserved from unicellular amoebae to multicellular animals (King and Kay 2019). In mammals, macropinocytosis has been documented in many cell types, including endothelial and epithelial cells, fibroblasts, monocytes, neurons, and T-cells. High macropinocytic activity is also commonly observed in cancer cells (Lewis 1937; Commisso 2019). The molecular mechanisms of macropinocytosis and its physiological functions have been studied primarily in mammalian cells in culture and in the social amoeba *Dictyostelium discoideum*. In *Dictyostelium*, macropinocytosis serves as a nutrient uptake pathway that non-selectively internalizes macromolecules from the environment, which are subsequently broken down into their building blocks by lysosomal hydrolases (Bloomfield and Kay 2016). Over the past decade, a similar function has been characterized in mammalian cancer cells: various malignant cells exploit macropinocytosis to feed on macromolecular nutrients, thereby gaining the ability to survive and grow in nutrient-poor tumor microenvironments (Commisso et al. 2013; Kim et al. 2018; Palm et al. 2015; Kamphorst et al. 2015). While cancer cells subvert macropinocytosis, evidence suggests that macropinocytosis could similarly function in macromolecular nutrient uptake in physiological contexts where vascular supply is compromised, for example in wound tissue. Conceivably, the uptake of macromolecular nutrients represents the ancestral function of this endocytic pathway (King and Kay 2019; Palm 2019).

Beyond nutrient acquisition, mammalian cells utilize macropinocytosis in diverse processes that repurpose its unique characteristics in novel and sometimes unexpected ways. For example, it has been known for a long time that macropinocytosis plays an important role in the immune system, where macrophages and dendritic cells exploit macropinocytosis to survey their environment. The non-selective nature of macropinocytosis allows monocytes to internalize any foreign macromolecule, which then can be processed for antigen presentation to activate the adaptive immune response (Norbury et al. 1995, 1997; Doodnauth et al. 2019). In epithelial cells, macropinocytic internalization of large membrane patches mediates rapid remodeling of the plasma membrane proteome. Thereby, macropinocytosis facilitates integrin redistribution during cell migration and alters cell surface components of signal transduction pathways (Chiasson-MacKenzie et al. 2018; Donaldson et al. 2009; Gu et al. 2011). Migrating dendritic cells use macropinocytosis to transport large quantities of fluid through the cell from front to back. Thereby, dendritic cells decrease their hydraulic resistance, which might facilitate movement through the confined space of blood capillaries (Moreau et al. 2019).

Besides its versatile functions in diverse cellular processes, another distinguishing feature of macropinocytosis is its intricate regulation by upstream signaling processes. Some endocytic events occur spontaneously, as is the case for constitutive pinocytosis. Other endocytic pathways are acutely triggered by binding of cargo to cell surface receptors, as is the case for receptor-mediated endocytosis and phagocytosis (Thottacherry et al. 2019). By contrast, the molecular processes that shape a macropinosome display exquisite inducibility but occur independently of endocytic cargo; rather, they are orchestrated by complex signaling cascades. The

core components of the intracellular signaling processes that induce macropinosome formation are evolutionarily conserved from unicellular eukaryotes to multicellular animals (King and Kay 2019). In amoebae, these processes function cell-autonomously to activate macropinocytosis constitutively. By contrast, mammalian cells do not display basal macropinocytic activity with the exception of macrophages and dendritic cells, which engage in constitutive macropinocytosis (Doodnauth et al. 2019). Rather, most mammalian cell types acutely induce macropinocytosis in response to extrinsic stimuli. Already in the 1970s, macropinocytosis was identified as an immediate cellular responses to growth factor stimulation (Brunk et al. 1976; Haigler et al. 1979). Since then, an increasing number of signaling pathways has been associated with the acute or long-term regulation of this endocytic pathway. These findings reveal an astonishing degree to which the induction of macropinocytosis is hardwired to signaling networks that regulate diverse biological processes, ranging from autonomous control of cellular functions to coordination of cellular behavior within the organism.

In this chapter, I discuss the signaling pathways that induce macropinocytosis in mammalian cells (Fig. 8.1). I first review evolutionarily conserved cell-intrinsic signaling pathways that regulate the molecular events which orchestrate the formation of macropinosomes, and the growth factor signaling pathways that are at the center of macropinocytosis induction in mammalian cells. I then review recent findings concerning the emerging roles of metabolic inputs and developmental regulators in macropinocytosis induction, and exemplify how dysregulated signaling processes allow cancer cells to co-opt macropinocytosis. By doing so, I hope to shed light on the intriguing questions of how macropinocytosis responds to complex upstream inputs to exert manifold functions in diverse mammalian cell types.

Cell-Intrinsic Signaling Events that Regulate Macropinosome Formation

The Process of Macropinocytosis

Macropinocytosis is a non-selective endocytic pathway that internalizes large quantities of extracellular fluid and any molecules contained therein into vesicles of 0.2–10 μM diameter, referred to as macropinosomes. The formation of macropinosomes is preceded by vigorous, local movement of the plasma membrane as protrusions and ruffles (Swanson 2008; Buckley and King 2017). These membrane deformations arise in response to localized, directed actin polymerization, which pushes the plasma membrane outward to generate sheet-like extensions. Circular ruffles give rise to macropinocytic cups, which enclose portions of extracellular fluid; subsequent cup closure and pinching off from the plasma membrane into the cytoplasm generates macropinosomes. Similarly, linear plasma membrane ruffles can fold back, thereby generating macropinosomes.

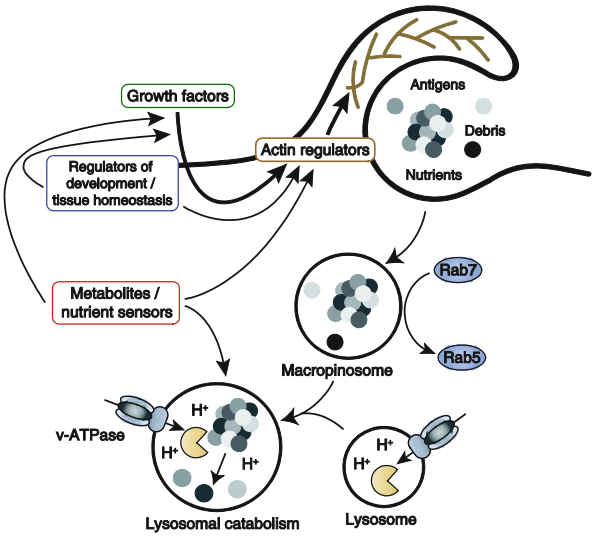

Fig. 8.1 Regulatory principles of macropinosome formation. Macropinosomes form through actin-driven protrusions of the plasma membrane, which enclose portions of extracellular fluid and any macromolecules contained therein into large endocytic vesicles. Subsequently, macropinosomes can mature in a process that is regulated by the small GTPases Rab5 and Rab7 and eventually fuse with lysosomes. Lysosomal v-ATPase and hydrolytic enzymes then mediate degradation of the macromolecular cargo of macropinosomes. Growth factor signaling plays a central role in the induction of macropinocytosis, activating the regulators of actin polymerization that drive plasma membra the macromolecular cargo of macropinosomes. Growth factor signaling plays a central role in the induction of macropinocytosis, activating the regulators of actin polymerization that drive plasma membrane ruffling and macropinosome formation. Several other processes, including signaling pathways that sense nutrients or regulate development and tissue homeostasis, potentiate growth factor signaling, or converge with growth factor signaling on the downstream actin regulators. These processes also regulate the intracellular fate of macropinosome cargo at the step of lysosomal degradation. *Rab5/7* Ras-related protein rab-5/7 *v-ATPase* vesicular ATPase

While macropinocytosis is unique among the different pinocytic pathways, it shares mechanistic aspects with phagocytosis or "cell eating" (Swanson 2008). Both macropinocytosis and phagocytosis commence with actin-driven protrusions of the plasma membrane that lead to the formation of large endocytic vesicles (Mylvaganam et al. 2021). While macropinocytosis and phagocytosis share the molecular machinery that mediates actin-driven membrane protrusions, they differ in their mode of induction. Phagosomes form through interactions of plasma membrane receptors with the surface of solid particles or bacteria, which act as a template around which the nascent phagosome membrane closes. By contrast, macropinosomes form spontaneously in the absence of extracellular cargo in a self-assembly process that is coordinated by intracellular signaling cascades (Swanson 2008).

Ras and PI3-Kinase: The Central Regulators of Macropinocytosis

Across different eukaryotic organisms and mammalian cell types, an evolutionarily conserved set of intracellular signaling proteins orchestrates the actin dynamics that lead to membrane ruffling and macropinosome formation (Fig. 8.2). At the center of this signaling cascade are Ras GTPases and phosphoinositide 3-kinases (PI3-kinase) (Mercer and Helenius 2009; King and Kay 2019; Swanson 2008). Ras GTPases localize to the plasma membrane, where they function as binary molecular switches that cycle between GTP-bound, active and GDP-bound, inactive states (Bar-Sagi and Hall 2000; Simanshu et al. 2017). Mammals express three major Ras proteins, H-Ras, K-Ras, and N-Ras. Like other members of the Ras superfamily of small GTPases, Ras proteins have low intrinsic rates of GTP hydrolysis and nucleotide exchange. This allows control of the nucleotide loading state of Ras GTPases by regulatory proteins: guanine nucleotide exchange factors (GEFs), which activate Ras by inducing exchange of GDP with GTP, and GTPase activating proteins (GAPs), which terminate Ras signaling by increasing the rate of GTP hydrolysis. Multiple upstream signaling pathways regulate the activities of Ras GEFs and Ras GAPs and hence the activity of Ras. In response to these inputs, Ras activates several

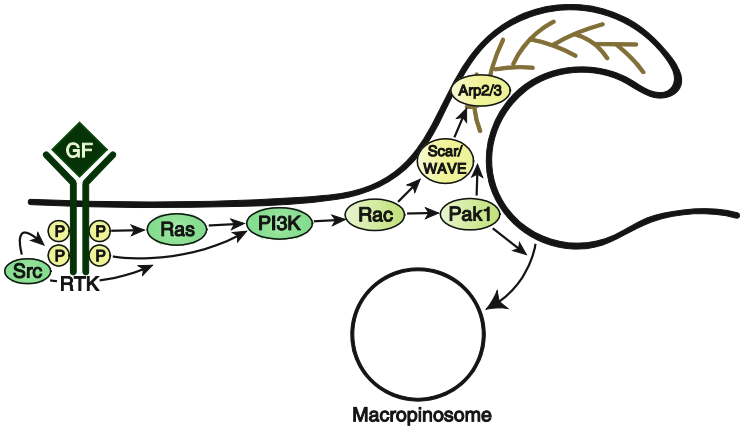

Fig. 8.2 Regulation of macropinocytosis by growth factor signaling. Binding of growth factors to their cognate receptor tyrosine kinase activates the small GTPase Ras and the lipid kinase PI3-kinase. Receptor tyrosine kinase signaling can further be enhanced by the non-receptor tyrosine kinase Src. Downstream in the signaling pathway, the small GTPase Rac and the serine/threonine kinase Pak1 orchestrate activities of several actin regulators, including the Scar/WAVE complex and its effector Arp2/3. The resulting localized polymerization of actin drives plasma membrane ruffling and macropinosome formation. Note that growth factors induce macropinocytosis across different cell types, with individual growth factors acting on specific cell types depending on expression of the respective receptors. *Arp2/3* actin-related protein 2/3, *GF* growth factor, *P* phosphorylated tyrosine residue, *Pak1* p21-activated kinase, *PI3-kinase* phosphoinositide 3-kinase, *RTK* receptor tyrosine kinase, *Src* proto-oncogene tyrosine-protein kinase Src

downstream effectors, including the signaling pathways that regulate actin dynamics and macropinosome formation.

PI3-kinases are lipid kinases that phosphorylate the inositol ring of phosphoinositides at the 3' hydroxyl group, thereby converting phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) to $PI(3,4,5)P_3$ (Cantley 2002). Mammalian cells express several PI3-kinases, which differ in signaling function and subcellular localization (Engelman et al. 2006). Macropinocytosis is regulated by class I PI3-kinase, which consists of a catalytic p110 subunit and a regulatory p85 subunit (for simplicity, hereafter referred to as PI3-kinase) (Mercer and Helenius 2009; Bloomfield and Kay 2016). Through localized production of $PI(3,4,5)P_3$, PI3-kinase generates docking platforms at the cytosolic leaflet of the plasma membrane, which recruit and activate effectors of downstream signaling pathways. The activity of PI3-kinase is antagonized by phosphatase and tensin homolog (PTEN), an enzyme that dephosphorylates the 3' hydroxyl group of $PI(3,4,5)P_3$. The activation of PI3-kinase by upstream signaling events involves its recruitment to the plasma membrane where the enzyme comes into contact with its substrate phosphoinositide lipids. The catalytic p110α subunit of class I_A PI3-kinase, which is encoded by the PIK3CA gene, harbors a Ras-binding domain; therefore, Ras GTPases contribute to membrane recruitment and activation of class I_A PI3-kinase (Gupta et al. 2007; Kodaki et al. 1994).

Activation of Ras signaling potently stimulates the formation of macropinosomes. In fact, Ras was the first intracellular signaling pathway that was shown to activate macropinocytosis. Mutant forms of Ras that have lower intrinsic GTPase activity or decreased interaction with GAPs are constitutively active and represent convenient tools to study Ras signaling. Cellular microinjection or genetic expression of constitutively active variants of H-Ras or K-Ras potently enhances membrane ruffling and macropinosomes formation (Porat-Shliom et al. 2008; Bar-Sagi and Feramisco 1986; Amyere et al. 2000). Similarly, an upregulation of PI3-kinase signaling, for example by expression of constitutively active mutants of PIK3CA or deletion of PTEN, enhances macropinocytosis (Palm et al. 2017; Kim et al. 2018). Ras and PI3-kinase play central roles in macropinocytosis across eukaryota. In *Dictyostelium*, genetic experiments indicate that local activation of Ras constitutes the initial signaling event during the formation of macropinosomes (Williams et al. 2019). Like mammalian p110α, several *Dictyostelium* PI3-kinase isoforms have Ras-binding domains, which mediate their Ras-dependent recruitment to the plasma membrane (Funamoto et al. 2002). Indeed, activated Ras and high levels of $PI(3,4,5)P_3$ have been detected in plasma membrane signaling patches of *Dictyostelium* and macrophages at sites where macropinosomes form (Veltman et al. 2016; Welliver and Swanson 2012).

Downstream Effectors of Ras and PI3-Kinase

Downstream of Ras and PI3-kinase, several effectors have been identified to participate in regulating macropinocytosis (Fig. 8.2). During this process, multiple small GTPases and kinases work together to locally recruit and activate regulators of the actin polymerization events that drive plasma membrane ruffling and macropinosomes formation (Mylvaganam et al. 2021; Buckley and King 2017; Swanson 2008). The Rho-family small GTPase Rac is a key downstream effector of Ras and PI3-kinase that orchestrates the rearrangements of the actin cytoskeleton mediating macropinosomes formation (West et al. 2000; Ridley et al. 1992). Cdc42, another member of the Rho GTPase family, also localizes to forming macropinosomes and contributes to this endocytic process (Garrett et al. 2000; Tkachenko et al. 2004). In concerted action with the phosphoinositides generated at sites of macropinosome formation, Rac activates the SCAR/WAVE complex. In turn, SCAR/WAVE activates the actin-related protein 2/3 (Arp2/3) complex, which nucleates branched actin filaments, thereby pushing the plasma membrane out to shape the nascent macropinosome (Miki et al. 1998; Veltman et al. 2016). The serine/threonine kinase p21-activated kinase 1 (Pak1) is another important downstream effector of Rac that regulates macropinocytosis at several steps. Pak1 localizes to membrane ruffles and phosphorylates various proteins that regulate actin dynamics and macropinosome formation (Dharmawardhane et al. 1997; Even-Faitelson et al. 2005; Dharmawardhane et al. 2000). Pak1 also promotes the late stages of macropinosome formation—closure and membrane fission of the macropinocytic cup (Liberali et al. 2008). While signaling proteins that regulate actin dynamics during macropinosome formation have been identified, how their activity is regulated with temporal and spatial precision to orchestrate the associated membrane deformations remains incompletely understood.

Intracellular Fate of Macropinosomes

Once formed, macropinosomes can either be recycled back to the cell surface or mature through a series of trafficking events that eventually lead to their fusion with lysosomes (Mercer and Helenius 2009) (Fig. 8.1). The signaling events that regulate the intracellular fate of macropinosomes are only partially understood. However, it is clear that macropinosomes share a common intracellular trafficking machinery with other endocytic pathways. Macropinosomes rapidly become decorated with the small GTPase Rab5 (Schnatwinkel et al. 2004). Subsequent exchange of Rab5 for Rab7 destines maturing macropinosomes to the lysosomal compartment (Racoosin and Swanson 1993; Kerr et al. 2006). Fusion with lysosomes containing the vacuolar ATPase (v-ATPase) and acid hydrolases—proteases, lipases, glycosidases, and nucleases, among others—causes acidification of the macropinosome lumen and subsequent degradation of its macromolecular cargo. While this chapter focuses on

the initial signaling events during the formation of macropinosomes, recent insights into the regulation of subsequent steps are briefly discussed where appropriate.

Regulation of Macropinocytosis by Growth Factor Signaling

Cell-Extrinsic Regulation of Macropinocytosis by Growth Factors

In order to respond to changes in their environment, cells constantly receive signals from the exterior. All cells are able to detect chemical signals and initiate adaptive responses. For example, cells can respond to a change in extracellular nutrient levels by initiating movement along nutrient concentration gradients or by adjusting their metabolic activities to the altered environment. Another layer of complexity in cell signaling has evolved in multicellular animals, which coordinate the behavior of individual cells within tissues and throughout the organism. To this end, animal cells constantly communicate with each other through secreted signaling molecules that move through the extracellular space and bind to specific receptors on the surface of receiving cells. Growth factors are a diverse group of secreted signaling proteins that regulate cellular processes associated with metabolism, growth, and proliferation. Intriguingly, plasma membrane ruffling and macropinosome formation are an immediate cellular response to growth factor stimulation (Swanson 2008; Mercer and Helenius 2009). This represents a major difference in the regulation of macropinocytosis between unicellular amoebae and mammalian cells. In *Dictyostelium*, cell-autonomous signaling events continuously trigger the formation of macropinosomes (Bloomfield and Kay 2016). By contrast, most mammalian cells have lost the ability to control the induction of macropinocytosis cell-autonomously, but rather depend on extrinsic stimulation with growth factors (Palm and Thompson 2017). Even constitutively macropinocytic cell types, macrophages and dendritic cells, respond to stimulation with growth factors or chemokines with further induction of macropinocytosis (Doodnauth et al. 2019). Despite differences in the initial signaling events, growth factor-induced macropinocytosis and constitutive macropinocytosis converge on the Ras and PI3-kinase pathways to regulate the localized actin dynamics that orchestrate macropinosome formation (Fig. 8.2).

Transduction of Growth Factor Signals by Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) comprise a large group of cell surface receptors that bind to growth factors and several other extracellular signaling proteins (Hubbard and Till 2000; Lemmon and Schlessinger 2010) (Fig. 8.2). RTKs consist of an

extracellular ligand-binding domain, a single transmembrane helix, and a cytoplasmic part, which contains the tyrosine kinase domain as well as additional regulatory sequences. Ligand binding to the extracellular domain leads to dimerization or in some instances structural changes of preassembled dimers, resulting in transphosphorylation of tyrosine residues in the cytoplasmic domains. Tyrosine autophosphorylation increases the kinase activity of RTKs and generates protein-binding motifs. Thereby, phosphorylated tyrosine residues on RTKs or RTK-bound adaptor proteins generate docking platforms for intracellular effectors that regulate various downstream processes. Some extracellular signals are transduced by non-receptor tyrosine kinases, which are recruited to transmembrane receptors lacking intrinsic kinase activity. However, aside from the lack of a covalent linkage of receptor and tyrosine kinase, activation and intracellular signal transduction resembles that of RTKs (Hubbard and Till 2000).

Mammals possess a large variety of receptor tyrosine kinases and corresponding extracellular ligands (Lemmon and Schlessinger 2010; Hubbard and Till 2000). Cell-type specific expression of secreted ligands and their receptors generates a complex vocabulary of cell–cell signaling to coordinate distinct responses in individual cell types. Consequently, different cell types induce macropinocytosis in response to different growth factors. For example, epithelial cells express high levels of the receptor for epidermal growth factor (EGF) and immediately respond to EGF stimulation with induction of macropinocytosis (Haigler et al. 1979; Brunk et al. 1976). Similarly, fibroblasts express the receptor for platelet-derived growth factor (PDGF), and macrophages the receptor for macrophage colony-stimulating factor (M-CSF), and these cell types initiate macropinocytosis upon stimulation with the respective ligands (Mellström et al. 1988; Racoosin and Swanson 1989). The non-receptor tyrosine kinase Src (short for sarcoma) also participates in macropinocytosis induction. Src is recruited by and in turn phosphorylates activated RTKs. Thereby, Src functions both as effector and upstream activator of RTKs and contributes to strength and persistence of RTK signaling (Bromann et al. 2004). Consistently, over-expression of Src enhances the induction of macropinocytosis in response to growth factor stimulation (Kasahara et al. 2007).

Receptor Tyrosine Kinase Signaling Through the Ras and PI3-Kinase Pathways

Although mammalian cells express a large variety of RTKs, the different RTKs signal through common downstream effectors to induce membrane ruffling and macropinocytosis—Ras and PI3-kinase. Several other cell surface receptors including chemokine receptors and Toll-like receptors also converge on activation of Ras and PI3-kinase to induce macropinocytosis (Mercer and Helenius 2009; Swanson 2008). Ras is activated by RTKs through stimulated exchange of bound GDP for GTP (Simanshu et al. 2017; Bar-Sagi and Hall 2000). A complex of the adaptor

protein growth factor receptor bound protein 2 (Grb2) and the Ras GEF Son of Sevenless (Sos) binds to phosphotyrosine residues on activated RTKs. Translocation to the plasma membrane brings Sos in close contact with Ras, leading to conversion of Ras to its GTP-bound, active state. PI3-kinase is activated by RTKs through binding of the regulatory p85 subunit to phosphotyrosine residues on RTKs, which induces a conformational change that relieves autoinhibition of the p110 catalytic subunit (Cantley 2002). In addition, recruitment of PI3-kinase to RTKs brings the enzyme in close proximity with its substrate lipid $PI(4,5)P_2$ in the cytosolic leaflet of the plasma membrane. Translocation of PI3-kinase to the plasma membrane is further supported by interaction of its Ras-binding domain with activated Ras (Gupta et al. 2007; Kodaki et al. 1994). Hence, RTKs recruit PI3-kinase to the plasma membrane through binding directly to the regulatory p85 subunit, and indirectly through activation of Ras.

Increasing evidence suggests that activation of PI3-kinase is necessary and sufficient to initiate macropinocytosis in mammalian cells. Pharmacological inhibition of PI3-kinase completely blocks the induction of macropinocytosis in response to different upstream stimuli, including growth factor stimulation and expression of hyperactivated Ras mutants (Nobes et al. 1995; Rodriguez-Viciana et al. 1997; Amyere et al. 2000). The atypical $K\text{-}Ras^{G12R}$ mutant, which is constitutively active but deficient in binding to PI3-kinase, fails to upregulate macropinocytosis (Hobbs et al. 2020). This suggests that PI3-kinase activation is the key effector through which Ras regulates macropinocytosis. By contrast, Ras potently induces macropinocytosis but appears to be dispensable in this regard: Fibroblasts deficient for all major Ras isoforms, H-Ras, K-Ras and N-Ras, do not display any defect in macropinocytosis induction in response to growth factor stimulation (Palm et al. 2017). Similarly, activated T-cells that lack Ras guanyl releasing protein 1 (RasGRP1), a Ras GEF that is required for its activation in T-cells, do not show defects in macropinocytosis (Charpentier et al. 2020). This presents an interesting difference to *Dictyostelium*, where Ras is essential for macropinocytosis (Williams et al. 2019). Conceivably, the reason for these discrepancies lies in the mechanisms through which PI3-kinase is recruited to the plasma membrane in mammalian cells and in *Dictyostelium*. Mammalian PI3-kinase is recruited to the plasma membrane through interaction with RTKs and thus can be activated by growth factor stimuli independently of Ras. By contrast, *Dictyostelium* like other unicellular eukaryotes lacks RTKs, and Ras depends on PI3-kinase for recruitment to the plasma membrane in this organism (King and Kay 2019).

Metabolic Regulation of Macropinocytosis

Coordination of Cellular Functions with Metabolic Environment Through Nutrient Sensors

Across all kingdoms of life, cells employ nutrient-responsive signaling pathways to monitor the abundance of various metabolites and adjust cellular processes accordingly (Chantranupong et al. 2015; Yuan et al. 2013). By surveying the levels of energy and select metabolites, nutrient sensors play a key role in cellular homeostasis. In mammalian cells, extensive crosstalk between nutrient-sensing and growth factor-regulated signaling pathways ensures the integration of extrinsic signals and metabolic state to coordinate cellular functions. While the regulation of macropinocytosis by growth factor signaling has been characterized in detail, recent studies have begun to define a role for nutrient sensors and metabolic environment in regulating the induction of macropinocytosis (Palm 2019).

Regulation of Macropinocytosis by AMPK

The serine/threonine kinase AMP-activated protein kinase (AMPK) is the central sensor of intracellular energy availability (Garcia and Shaw 2017; Lin and Hardie 2018). AMPK binds to AMP, which activates its kinase activity through an allosteric process that is opposed by ATP. Through this mechanism, AMPK is able to sense cellular energy status: a decline in energy levels directly translates to an increased AMP/ATP ratio, which subsequently activates AMPK. The activity of AMPK is further regulated by two upstream kinases, liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2). LKB1 is the primary activator of AMPK in response to low energy conditions, which arise, for example, upon glucose deprivation or perturbed mitochondrial respiration. CaMKK2 activates AMPK upon an increase of intracellular calcium levels. Evidence suggests that CaMKK2 also activates AMPK in response to various stressors including amino acid deprivation (Ghislat et al. 2012). Binding of AMP to AMPK enhances its phosphorylation by LKB1 and CaMKK2, thereby further contributing to AMPK activation. Once active, AMPK promotes energy-generating processes and suppresses energy-consuming processes, thereby restoring cellular energy balance.

Recent findings have identified a role for AMPK in stimulating macropinocytosis in response to nutrient starvation (Fig. 8.3). The first evidence that AMPK regulates actin-driven endocytic processes came from the study of phagocytosis in macrophages and neutrophils. AMPK can be activated pharmacologically with AICAR, an analogue of AMP, or with metformin, which elevates AMP levels by inhibiting mitochondrial respiration. AMPK activation by either treatment increases the activity of Rac and its downstream effector Pak1, which then promote cytoskeletal dynamics and enhance the phagocytic capacity of immune cells (Bae et al. 2011).

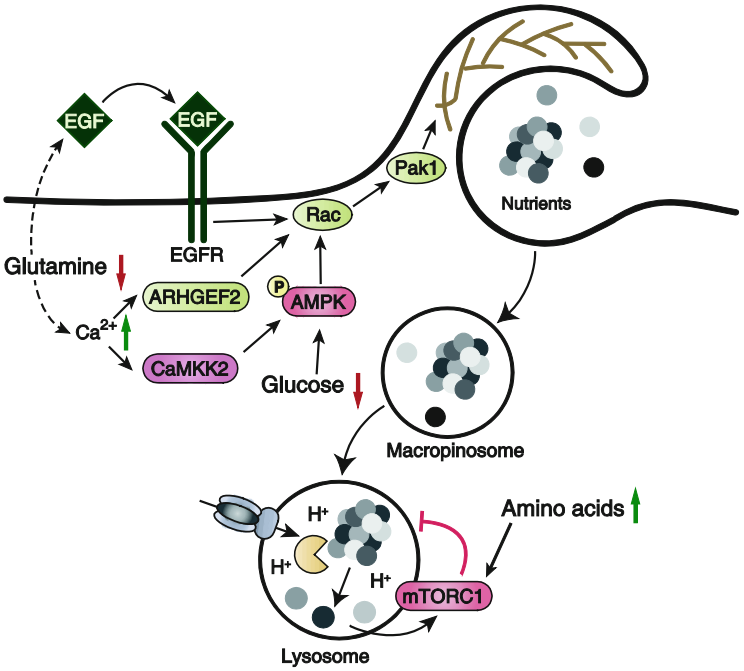

Fig. 8.3 Metabolic regulation of macropinocytosis. Several forms of nutrient starvation enhance macropinocytosis. Deprivation of glutamine enhances secretion of EGF, which leads to autocrine activation of EGFR and thus increased macropinocytosis. Glutamine deprivation also increases intracellular calcium levels, which leads to CaMKK2-mediated activation of the serine/threonine kinase AMPK and increased transcription of the Rac GEF ARHGEF2. Concerted activities of AMPK and ARHGEF2 increase the activity of Rac, which initiates macropinosome formation. Activation of AMPK upon glucose starvation also promotes macropinocytosis through enhanced activation of Rac and Pak1. Amino acid starvation causes inactivation of the serine/threonine kinase mTORC1 and thereby de-represses lysosomal catabolism of macropinocytic cargo. Conversely, amino acids internalized in the fluid phase of macropinosomes or generated through lysosomal catabolism of macropinocytosed proteins activate mTORC1. Note that depicted signaling events may regulate macropinocytosis only in specific cell types or in the context of specific oncogenic mutations. *AMPK* AMP-activated protein kinase, *ARHGEF2* Rho/Rac guanine nucleotide exchange factor 2, *CaMKK2* calcium/calmodulin-dependent protein kinase kinase 2, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *GEF* guanine nucleotide exchange factor, *mTORC1* mechanistic target of rapamycin complex 1, *Pak1* p21-activated kinase 1

Subsequent studies showed that AMPK-mediated activation of Rac can trigger the induction of macropinocytosis (Zhang et al. 2021; Kim et al. 2018). AMPK-mediated activation of Rac has been observed in several cell lines subjected to nutrient deprivation. Glucose starvation increases Rac activity and macropinocytosis induction in prostate cancer cells deficient for PTEN and in non-small cell lung carcinoma cells harboring mutations in K-Ras or EGFR (Kim et al. 2018; Hodakoski et al. 2019). In cancer-associated fibroblasts, glutamine starvation elevates cytosolic calcium levels and thus activation of CaMKK2, leading to AMPK-mediated

activation of Rac. At the same time, the glutamine starvation-mediated increase in cytosolic calcium promotes expression of the Rac GEF ARHGEF2. Both events synergize to promote Rac-mediated macropinocytosis (Zhang et al. 2021). These findings reveal a principle by which macropinocytic activity is coupled to cellular metabolic state.

Regulation of Macropinocytic Cargo Degradation by mTORC1

Many functions of AMPK are antagonized by the serine/threonine kinase mechanistic target of rapamycin complex 1 (mTORC1), which is the central coordinator of cellular metabolism and growth (Saxton and Sabatini 2017; Kim and Guan 2019; González and Hall 2017). In mammalian cells, mTORC1 functions as a coincidence detector for amino acid levels and growth factor signals. These upstream inputs converge on two small GTPases that reside on lysosomal membranes—Rag and Rheb. Rag GTPases are activated by sufficient intracellular abundance of several amino acids including leucine, arginine, and methionine. Rheb is activated by growth factor signaling and suppressed by various stresses. Concerted action of Rag and Rheb leads to activation of mTORC1: Rag recruits mTORC1 to lysosomal membranes, where mTORC1 binds to and is activated by Rheb. mTORC1 is also regulated by AMPK, which represses Rheb and thereby antagonizes mTORC1 activation (Inoki et al. 2003).

As the central regulator of lysosomal protein degradation, mTORC1 plays an important role in controlling the intracellular fate of macropinocytic cargo (Fig. 8.3) (Lawrence and Zoncu 2019; Palm and Thompson 2017). Even highly macropinocytic cells degrade extracellular proteins inefficiently when residing in nutrient-rich environments where mTORC1 is active. Upon nutrient starvation, the ensuing inactivation of mTORC1 de-represses lysosomal catabolism and thereby enhances the degradation of macropinocytosed proteins (Palm et al. 2015). Because AMPK suppresses mTORC1 signaling, cells might respond to a decline in energy levels with concerted upregulation of macropinocytosis and lysosomal catabolic activity. Conceivably, this principle allows cells to tap into the copious nutrient stores of extracellular proteins during starvation, while preventing their energetically wasteful degradation under nutrient-replete conditions.

Regulation of mTORC1 by Macropinocytosis

While nutrient sensors regulate macropinosome formation, nutrient uptake through macropinocytosis in turn influences metabolic signaling. Cells take up exogenous free amino acids through plasma membrane transporters, which leads to activation of mTORC1. Moreover, cells internalize free amino acids as solutes contained in the fluid phase of macropinosomes, which can contribute to mTORC1 activation

(Yoshida et al. 2015; Charpentier et al. 2020). However, macropinocytosis can also promote mTORC1 activation by supplying an entirely different source of amino acids—extracellular proteins, whose degradation in the lysosome generates free amino acids intracellularly. Through uptake and lysosomal catabolism of extracellular proteins, macropinocytic cells sustain mTORC1 activity even in environments where free amino acids are scarce (Palm et al. 2015). mTORC1 activation by macropinocytosed proteins is antagonized by the GATOR2 complex, which communicates cytosolic levels of several amino acids to mTORC1 (Hesketh et al. 2020). Interestingly, genetic deletion of GATOR2 has opposite effects on activation of mTORC1 by amino acids generated through lysosomal catabolism of macropinocytosed proteins and by amino acids taken up via plasma membrane transporters, suggesting that mTORC1 senses these two nutrient sources through distinct mechanisms.

Similarities and Differences between Macropinocytosis and Autophagy

The regulation of macropinocytosis shares mechanistic similarities with the induction of another vesicle trafficking pathway, macroautophagy (hereafter referred to as autophagy) (Florey and Overholtzer 2019). Autophagy, or "self-eating", is a degradative pathway that delivers intracellular constituents to the lysosome (Xie and Klionsky 2007; Mizushima 2007). During this process, an isolating membrane, the phagophore, sequesters cytosolic contents such as proteins, macromolecular complexes, and even whole organelles into a double-membrane vesicle, the autophagosome. Subsequent autophagosome fusion with the lysosome initiates degradation of autophagic cargo by lysosomal hydrolases. In nutrient-rich conditions, cells usually display low levels of basal autophagy, but autophagy is rapidly upregulated in response to nutrient starvation (Saxton and Sabatini 2017; Kim and Guan 2019). The formation of autophagosomes is initiated by the Unc-51 like autophagy activating kinase 1 and 2 (Ulk1/2), the mammalian homologs of yeast Atg1. The kinase activity of Ulk1/2 is suppressed by mTORC1 and promoted by AMPK (Kim et al. 2011; Egan et al. 2011). Consequently, upon nutrient starvation the ensuing shift from mTORC1 to AMPK signaling leads to activation of Ulk1/2 and subsequent initiation of autophagy.

Cells can respond to starvation by upregulating autophagy and macropinocytosis, thereby increasing delivery of macromolecules from intracellular and extracellular sources, respectively, to the lysosome (Lawrence and Zoncu 2019). The mechanisms of macropinocytosis induction during starvation are incompletely understood, but this process does not require Ulk1/2 (Palm et al. 2015). Thus, AMPK and mTORC1 regulate macropinocytosis and autophagy through distinct molecular processes. Nevertheless, both vesicle trafficking pathways converge on the lysosome, where co-regulation might take place. Interestingly, growth factor stimulation has opposite

effects on macropinocytosis and autophagy. Growth factors trigger macropinocytosis through activation of Ras and PI3-kinase but suppress autophagy through activation of mTORC1. Conceivably, these similarities and differences in the regulation of macropinocytosis and autophagy reflect their metabolic functions. Macropinocytosis supplies exogenous nutrients, which support net biomass formation and cell growth (Commisso et al. 2013; Palm et al. 2015; Kamphorst et al. 2015; Kim et al. 2018). By contrast, autophagy degrades intracellular components, which can support cell survival during limited periods of starvation but eventually results in cellular atrophy (Lum et al. 2005). This may explain why nutrient sensors promote autophagy as well as macropinocytosis to sustain cell survival during starvation, whereas growth factors trigger macropinocytosis while suppressing autophagy to promote cell growth.

Induction of Macropinocytosis by Signaling Pathways that Regulate Development and Tissue Homeostasis

Transcriptional Macropinocytosis Regulation by Hippo Signaling

In addition to growth factor-regulated and nutrient-sensing signaling pathways, several developmental signaling pathways have emerged as regulators of macropinocytosis (Fig. 8.4). The Hippo signaling pathway regulates cell proliferation and cell fate in metazoan organisms to control organ growth, repair, and regeneration (Ma et al. 2019; Totaro et al. 2018). Hippo signaling converges on regulation of the transcriptional coactivators yes-associated protein 1 (Yap) and transcriptional coactivator with PDZ-binding motif (Taz), which promote expression of genes that increase cell proliferation and growth while suppressing apoptosis. Hippo signaling integrates a variety of cues from a cell's tissue environment, including signals from adherens junctions, extracellular matrix attachment, and mechanical forces. Hippo signaling also responds to metabolic environment (Koo and Guan 2018). Long-term deprivation of leucine increases macropinocytosis in pancreatic cancer cells by promoting nuclear translocation and thus transcriptional activity of Yap and Taz (King et al. 2020). Genetically activating Hippo signaling through deletion of the negative pathway regulator Nf2 also enhances macropinocytosis (Chiasson-MacKenzie et al. 2018). Mechanistically, Yap/Taz upregulate transcription of the TAM receptor Axl, an RTK that can activate PI3-kinase and thus trigger macropinocytosis. The ligand of Axl, Gas6, recognizes phosphatidylserines on necrotic cells and promotes their macropinocytic uptake. This suggests that by upregulating expression of Axl, Yap/Taz allow starved cancer cells to feed on necrotic debris as a nutrient source (King et al. 2020).

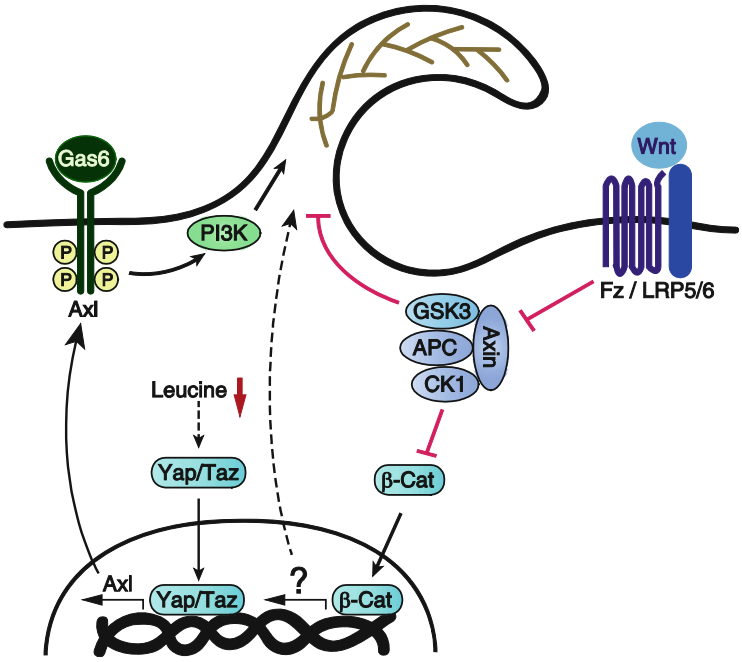

Fig. 8.4 Regulation of macropinocytosis by the Hippo and canonical Wnt signaling pathways. Leucine deprivation promotes nuclear translocation of the transcriptional regulators Yap and Taz, which induce expression of the receptor tyrosine kinase Axl. Activation of Axl by its ligand Gas6 induces macropinocytosis through PI3-kinase. Binding of Wnt ligands to their receptor Fz/Lrp5/6 inhibits the β-catenin destruction complex, resulting in stabilization and nuclear accumulation of β-catenin, which enhances macropinocytosis through unknown transcriptional changes. By repressing the β-catenin destruction complex subunit GSK3, Wnts also promote macropinocytosis acutely. Note that depicted signaling events may regulate macropinocytosis only in specific cell types or in the context of specific oncogenic mutations. *APC* adenomatous polyposis coli, *Axl* Axl receptor tyrosine kinase, *CK1* casein kinase 1, *Fz* Frizzled, *Gas6* growth arrest specific 6, *GSK3* glycogen synthase kinase 3, *Lrp5/6* low-density lipoprotein receptor-related protein 5/6, *Taz* transcriptional coactivator with PDZ-binding motif, *Yap* yes-associated protein 1

Transcriptional and Acute Macropinocytosis Regulation by Canonical Wnt Signaling

Canonical Wnt signaling is another developmental signaling pathway that regulates macropinocytosis. Wnts are secreted signaling proteins that spread through tissues to coordinate development, homeostasis, and stem cell maintenance (Zhan et al. 2017; Nusse and Clevers 2017). Binding of Wnt ligands to their receptor on receiving cells, a heterodimer of Frizzled (Fz) and LDL receptor-related protein 5/6 (Lrp5/6), initiates an intracellular signaling cascade that leads to stabilization and nuclear accumulation of β-catenin. There, β-catenin recruits transcriptional coactivators to induce expression of Wnt target genes. Wnt stabilizes β-catenin by inhibiting the

β-catenin destruction complex containing glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), as well as the adaptor proteins axin and adenomatous polyposis coli (APC). A genetic screen for genes that modulate cellular entry of Bacillus Calmette-Guerin, which relies on macropinocytosis, identified a role for canonical Wnt signaling in this endocytic process (Redelman-Sidi et al. 2018). Persistent activation of Wnt signaling by genetic or pharmacological suppression of β-catenin destruction complex components enhances macropinocytic activity (Albrecht et al. 2020; Redelman-Sidi et al. 2018). Stimulation of cells with Wnt ligands or inhibition of the negative Wnt regulator GSK3 also promotes macropinocytosis (Albrecht et al. 2020; Tejeda-Muñoz et al. 2019). Thus, Wnts can induce macropinocytosis acutely, reminiscent of the timescale at which growth factors act. The molecular mechanisms through which persistent and acute activation of Wnt signaling induce macropinocytosis are incompletely understood, but they involve, at least in part, Pak1-mediated remodeling of the actin cytoskeleton (Tejeda-Muñoz et al. 2019). This raises the intriguing possibility that Wnts converge with growth factors on common downstream effectors to induce macropinocytosis.

Regulation of Macropinocytosis by Oncogenic Signaling

Since its discovery in the early twentieth century, macropinocytosis has been recognized as a characteristic of many cancer cells (Lewis 1937). Due to the ease with which cancer cells can be cultured and manipulated, they have become a popular system in which to study molecular mechanisms and pathophysiological functions of macropinocytosis. At the same time, efforts to understand how genetic alterations in cancer drive macropinocytosis have yielded important insights into the signaling pathways that regulate macropinocytosis in normal cells (Zhang and Commisso 2019). Case in point, a pioneering study of the molecular mechanisms by which Ras causes cellular transformation identified Ras as the first signaling protein to induce macropinocytosis: Microinjection of H-Ras protein into quiescent fibroblasts rapidly triggered membrane ruffling and macropinocytosis, reminiscent of the cellular response to growth factor stimulation. Intriguingly, an oncogenic Ras variant stimulated macropinocytosis much more potently than wild-type Ras, suggesting that macropinocytosis contributed to the transforming properties of oncogenic Ras alleles (Bar-Sagi and Feramisco 1986). This finding has been confirmed in many cell types, and expression of oncogenic Ras variants has become a common tool to increase macropinocytic activity for mechanistic studies (Commisso et al. 2013; Amyere et al. 2000; Porat-Shliom et al. 2008).

Following the discovery that macropinocytosis is triggered by activated Ras, an increasing number of oncogenes have been implicated in its regulation. For example, oncogenic v-Src, which was discovered in Rous sarcoma virus and is derived from cellular Src, triggers macropinocytosis in the absence of growth factor stimulation (Veithen et al. 1996). We now understand that the hallmark of cancer cells to sustain growth factor signaling cell-autonomously is fundamentally linked to high

macropinocytic activity (Commisso 2019). Consequently, cancer-associated mutations in the proto-oncogenes and tumor suppressors which comprise growth factor signaling pathways promote macropinocytosis in the absence of extrinsic stimuli. Activating mutations in Ras and PIK3CA are among the most common oncogenic events in cancer, and their role in the initiation of macropinocytosis is understood in mechanistic detail. In addition, cancer cells dysregulate growth factor signaling pathways through alterations at multiple additional steps, including activation of autocrine signaling loops by cancer cell-secreted growth factors as well as activating mutations in RTKs and various downstream effectors including Rac and Pak1. Negative pathway regulators, such as the lipid phosphatase PTEN and the Ras GAP Nf1, are potent tumor suppressors and frequently lost in cancer (Sanchez-Vega et al. 2018; Hanahan and Weinberg 2011). For many cancer-associated mutations in components of growth factor signaling pathways, the effects on macropinocytic activity remain to be established experimentally, but they likely contribute to the prevalence of macropinocytosis in transformed cells.

Beyond the discoveries concerning growth factor signaling pathways, cancer genetics have been instrumental for the identification of new regulators of macropinocytosis. Hippo and Wnt signaling were first identified as macropinocytosis regulators through studies in cancer cells where these developmental signaling pathways are dysregulated (Redelman-Sidi et al. 2018; King et al. 2020; Tejeda-Muñoz et al. 2019). Similarly, the study of cancer cell adaptations to nutrient starvation identified the first links between metabolism and macropinocytosis. For example, a subset of pancreatic cancer cells displays low basal macropinocytic activity, despite expressing activated K-Ras variants. In these cells, macropinocytosis is upregulated by glutamine starvation, which triggers secretion of EGF to potentiate RTK signaling (Lee et al. 2019). In K-Ras-transformed cells, macropinocytosis is also regulated by bicarbonate levels. Oncogenic Ras signaling increases intracellular bicarbonate levels through upregulation of plasma membrane bicarbonate transporters or, in response to hypoxia, by expression of carbonic anhydrase (Garcia-Bermudez et al. 2021; Ramirez et al. 2019). Clearly, the number of seemingly unrelated processes and signaling pathways that modulate macropinocytosis in cancer cells is still expanding rapidly, and this chapter was limited to a discussion of major themes. While cancer genetics continue to identify new regulators of macropinocytosis, tumor biology is spearheading the study of macropinocytosis in vivo. Physiological roles of macropinocytosis remain unclear in many cell types, but elegant in vivo studies in tumors have established the importance of macropinocytic nutrient uptake for cancer cell metabolism (Commisso et al. 2013; Kamphorst et al. 2015; Lee et al. 2019; Davidson et al. 2017). Beyond their importance for cancer biology, these studies provide a conceptual framework in which to investigate regulation and functions of macropinocytosis in physiological contexts.

Concluding Remarks

A unique feature of macropinocytosis among the different endocytic processes is its intricate regulation by non-cell-autonomous and cell-intrinsic signaling pathways. The central role of growth factor signaling in macropinocytosis initiation is understood in molecular detail. In addition, several functionally diverse signaling pathways have emerged as novel players, including nutrient-sensing and developmental signaling pathways. In contrast to this complexity of upstream regulators, a comparatively small set of signaling proteins appears to regulate the molecular events that orchestrate actin-driven membrane ruffling and macropinosome formation. A key challenge for the field is thus to clarify whether the different upstream signaling pathways converge on a few common effectors or whether they act through distinct mechanisms. Clearly, multiple signaling pathways increase a cell's macropinocytic activity by enhancing growth factor—RTK signaling, for example by promoting autocrine growth factor stimulation or increasing expression of RTKs (King et al. 2020; Lee et al. 2019). Other signaling pathways increase the activity of the actin regulators Rac and Pak1 acutely or enhance expression of upstream regulators such as the Rac GEF ARHGEF2 (Albrecht et al. 2020; Zhang et al. 2021). These processes are not mutually exclusive; transcriptional mechanisms might set the threshold or amplitude at which an acute stimulus can induce macropinocytosis.

Another open question is the impact of cellular and genetic context in which a signaling event leads to the induction of macropinocytosis. Many studies that led to identification of new macropinocytosis regulators were conducted in cancer cell lines of epithelial origin. This requires consideration, because many carcinoma cells harbor mutations that activate the Ras and PI3-kinase signaling pathways. Consequently, a specific signaling event may not be sufficient to initiate macropinocytosis but rather require oncogenic dysregulation of growth factor signaling. For example, activation of macropinocytosis by AMPK in prostate cancer cells depends on deletion of PTEN, which increases PI3-kinase signaling (Kim et al. 2018). Increased macropinocytosis in response to a rise in intracellular bicarbonate levels occurs in pancreatic cancer cells in the context of activating K-Ras mutations (Garcia-Bermudez et al. 2021; Ramirez et al. 2019). These findings suggest that oncogenic dysregulation of growth factor signaling sensitizes cancer cells to the induction of macropinocytosis by other stimuli. It may be worthwhile to ask whether these processes are co-opted by cancer cells and normally integrate inputs from growth factors with other stimuli to regulate macropinocytosis in physiological contexts.

In conclusion, rapid progress in understanding the complex signaling networks that regulate macropinocytosis provides a framework in which to study emerging functions of macropinocytosis, for example, in nutrient uptake and cell growth. At the same time, characterizing the functions of macropinocytosis will help to understand why specific signaling pathways regulate macropinocytosis in different cell types and physiological contexts. Together, these lines of research will lead to a deeper understanding of the complex regulatory principles and diverse functions of macropinocytosis in mammalian cells.

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Chapter 9
Wnt, GSK3, and Macropinocytosis

Nydia Tejeda-Muñoz and Edward M. De Robertis

Abstract Here we review the regulation of macropinocytosis by Wnt growth factor signaling. Canonical Wnt signaling is normally thought of as a regulator of nuclear β-catenin, but emerging results indicate that there is much more than β-catenin to the Wnt pathway. Macropinocytosis is transiently regulated by EGF-RTK-Ras-PI3K signaling. Recent studies show that Wnt signaling provides for sustained acquisition of nutrients by macropinocytosis. Endocytosis of Wnt-Lrp6-Fz receptor complexes triggers the sequestration of GSK3 and components of the cytosolic destruction complex such as Axin1 inside multivesicular bodies (MVBs) through the action of the ESCRT machinery. Wnt macropinocytosis can be induced both by the transcriptional loop of stabilized β-catenin, and by the inhibition of GSK3 even in the absence of new protein synthesis. The cell is poised for macropinocytosis, and all it requires for triggering of Pak1 and the actin machinery is the inhibition of GSK3. Striking lysosomal acidification, which requires macropinocytosis, is induced by GSK3 chemical inhibitors or Wnt protein. Wnt-induced macropinocytosis requires the ESCRT machinery that forms MVBs. In cancer cells, mutations in the tumor suppressors APC and Axin1 result in extensive macropinocytosis, which can be reversed by restoring wild-type protein. In basal cellular conditions, GSK3 functions to constitutively repress macropinocytosis.

Keywords Endocytosis · Multivesicular bodies · Macropinocytosis · GSK3 · ESCRT · Wnt-STOP · Lysosome regulation · Colorectal cancer · Hepatocellular carcinoma

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Introduction

The activation of canonical Wnt pathway is a driving force in many human cancers, especially colorectal, hepatocellular, and mammary carcinomas (MacDonald et al. 2009; Nusse and Clevers 2017; Galluzzi et al. 2019). Wnt causes the stabilization and nuclear transport of newly synthesized transcriptional regulator β-catenin (Fig. 9.1), and the generally accepted view in the field is that the canonical effects

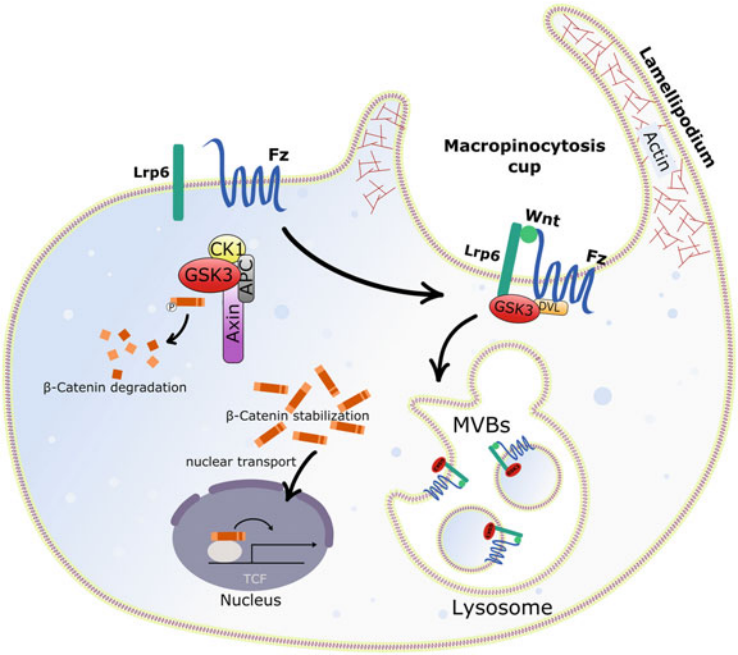

Fig. 9.1 Canonical Wnt signaling triggers the formation of large macropinocytosis cups and the ingestion of large amounts of extracellular macromolecule nutrient packages. The plasma membrane is trafficked into MVBs/late endosomes that engulf the receptor complexes, including cytosolic GSK3 (in red), through the action of the ESCRT machinery that forms intraluminal MVB vesicles. The sequestration of GSK3 is a required step in the activation of the canonical Wnt pathway. Interfering with the ESCRT machinery blocks both Wnt signaling and macropinocytosis. When the Wnt ligand binds to the Fz and LRP6 co-receptors, cytosolic GSK3 binds to the receptors, Axin1, and Dishevelled (DVL), all of which are GSK3 substrates. After endocytosis, GSK3 is sequestered inside endosomes and trafficked, together with extracellular proteins into lysosomes. The sequestration of GSK3 and of the β-catenin destruction complex, is required for Wnt-induced macropinocytosis. The sequestration of GSK3 inhibits its capacity to phosphorylate β-catenin, allowing β-catenin to accumulate in the cytoplasm and eventually transport it to the nucleus. Once in the nucleus, β-catenin binds to TCF transcription factors and activates the transcriptional expression of many downstream genes such as c-Myc, cyclin D1, Axin2, and others. The sequestration of cytosolic GSK3 by Wnt signaling also leads to the stabilization of many cellular proteins in a phenomenon known as Wnt-STabilization Of Proteins (Wnt-STOP), which has become an entire subfield in Wnt signaling research. Diagram based on findings reported in Taelman et al. (2010) and Tejeda-Muñoz et al. (2019)

of Wnt growth factors are caused by the transcription of β-catenin target genes. Here we review recent results that indicate Wnt is a regulator of many other cellular physiological activities, such as macropinocytosis, endosome trafficking, protein stability, and lysosomal activity. Some of these regulatory responses take place within minutes and do not require new protein synthesis, indicating that there is much more to Wnt beyond the well-established transcriptional role of β-catenin. The main conclusion that emerges from these studies is that in basal cell conditions the activity of the key protein kinase GSK3, which is inhibited by Wnt pathway activation, normally represses the actin machinery that orchestrates macropinocytosis.

Endosomal Sequestration of GSK3 during Wnt Signaling

As indicated in Fig. 9.1, Wnt signals through the stabilization of the transcriptional activator β-catenin, which is transported into the nucleus. In the nucleus, it accumulates and binds to TCF/LEF transcriptional factors on promoters and activates many Wnt target genes such as c-myc, cyclin D1, and Axin2 (Nusse and Clevers 2017). In the absence of Wnt, newly translated β-catenin is rapidly degraded by a cytosolic destruction complex consisting of the tumor suppressor proteins Axin1 and Adenomatous Polyposis Coli (APC), and the enzymes Casein Kinase 1 (CK1) and Glycogen Synthase Kinase 3 (GSK3), which phosphorylate β-catenin, generating a phosphodegron that is recognized by polyubiquitin ligases leading to β-catenin degradation in proteasomes (Fig. 9.1) (MacDonald et al. 2009). The activity of GSK3 is highly regulated and is a key player in the ancient Wnt signaling pathway that patterns the body of all animals (Loh et al. 2016). Wnt growth factors bind to their cell surface co-receptors LDL receptor-related protein 6 (Lrp6) and Frizzled (Fz) in the canonical pathway (Fig. 9.1). Formation of this trimeric complex recruits Dishevelled (Dvl) and Axin to the plasma membrane in what is known as the Lrp6 signalosome (Bilic et al. 2007). Lrp6 contains multiple GSK3 phosphorylation sites that may competitively decrease GSK3 activity locally (Cselenyi et al. 2008). However, this inhibition is only transient, and to achieve a sustained Wnt signal endocytosis of the Wnt receptor complex into multivesicular bodies (MVBs) is required (Fig. 9.1) (Taelman et al. 2010).

The activated Wnt-Lrp6-Fz receptor complexes are endocytosed into the cell and, as they enter the late endosome, are translocated together with GSK3 inside the intraluminal vesicles (ILVs) of MVBs (Taelman et al. 2010; Vinyoles et al. 2014). Lrp6, Fz, Axin1, and Dvl are all substrates of GSK3, and the normally cytosolic GSK3 enzyme becomes sequestered together with its substrates inside the ILVs of MVBs (Fig. 9.1). In this way, GSK3 and Axin1 become separated from its cytosolic substrates by two membranes, the MVB/lysosome limiting membrane and the ILV membrane (Fig. 9.1).

The sequestration of GSK3 in MVB vesicles is essential for Wnt signaling. The invagination of ILVs in late endosomes is a required step for any plasma membrane

protein to reach the lysosome during membrane trafficking. This outside–inside vesicle formation requires a molecular machinery of many proteins known as Endosomal Sorting Complexes Required for Transport (ESCRTs). Blocking MVB formation prevents Wnt/β-catenin signaling, as shown by interfering with machinery components such as HRS/Vps27 and Vps4 (Taelman et al. 2010), as well as the late endosome regulator Rab7 (Dobrowolski et al. 2012). In general, targeting of activated plasma membrane receptors to MVB/lysosomes downregulates signaling, but in the case of Wnt the sequestration of GSK3 and Axin1 generates the signal.

Wnt signaling not only stabilizes β-catenin but also many other substrates phosphorylated by GSK3. Up to 20% of the human proteome contains three or more GSK3 sites in a row (consensus S/TXXXS/T), and the addition of Wnt prolongs the total half-life of HeLa cell proteins by 25% (Taelman et al. 2010). This phenomenon, in which GSK3 substrate proteins are stabilized by Wnt, is designated Wnt-STabilization Of Proteins, or Wnt-STOP (Acebron et al. 2014; Koch et al. 2015). Since Wnt signaling is maximal at cell cycle phases G2 and M, Wnt-STOP plays an important role in achieving an increase in cell size in preparation for cell division (Acebron et al. 2014). Wnt-STOP has also been proposed to affect chromosomal stability, endolysosomal biogenesis, and proper mitosis (Huang et al. 2015; Ploper et al. 2015; Lin et al. 2020). Thus, there is much more to Wnt/GSK3 signaling than the transcriptional control by β-catenin.

Macropinocytosis

Pinocytosis (Gr., *pinein*, to drink) is a clathrin-independent endocytic mechanism first described by Warren Lewis (1931). It is a non-receptor-mediated actin-driven process that requires the activation of p21-activated kinase-1 (Pak1) (Doherty and McMahon 2009; Dhamawardhane et al. 2000), which leads to the formation of plasma membrane ruffles, actin tent poles, and macropinocytic cups that internalize extracellular fluid (Condon et al. 2018; Swanson 2018). In general, receptor-mediated endocytosis leads to the formation of small vesicles visible by electron microscopy of less than 100 nm, formed by the clathrin or caveolin machineries, called micropinocytosis (Nichols and Lippincott-Schwartz 2001; Doherty and McMahon 2009). The term macropinocytosis is currently used to designate actin-driven pinocytic vesicles of more than 200 nm.

From an experimental point of view, the modern standard defining macropinocytosis experimentally is the endocytosis of Tetramethylrhodamine-dextran (TMR-dextran 70 kDa) that has a hydrated diameter greater than 200 nm (TMR-dextran 70 kDa) (Commisso et al. 2014). As shown in Fig. 9.2, the machinery that drives macropinocytic cups can be blocked by a derivative of Amiloride known as ethyl-isopropyl amiloride (EIPA). Uptake of TMR-Dextran that is sensitive to EIPA constitutes the modern gold standard for macropinocytosis (Commisso et al. 2013).

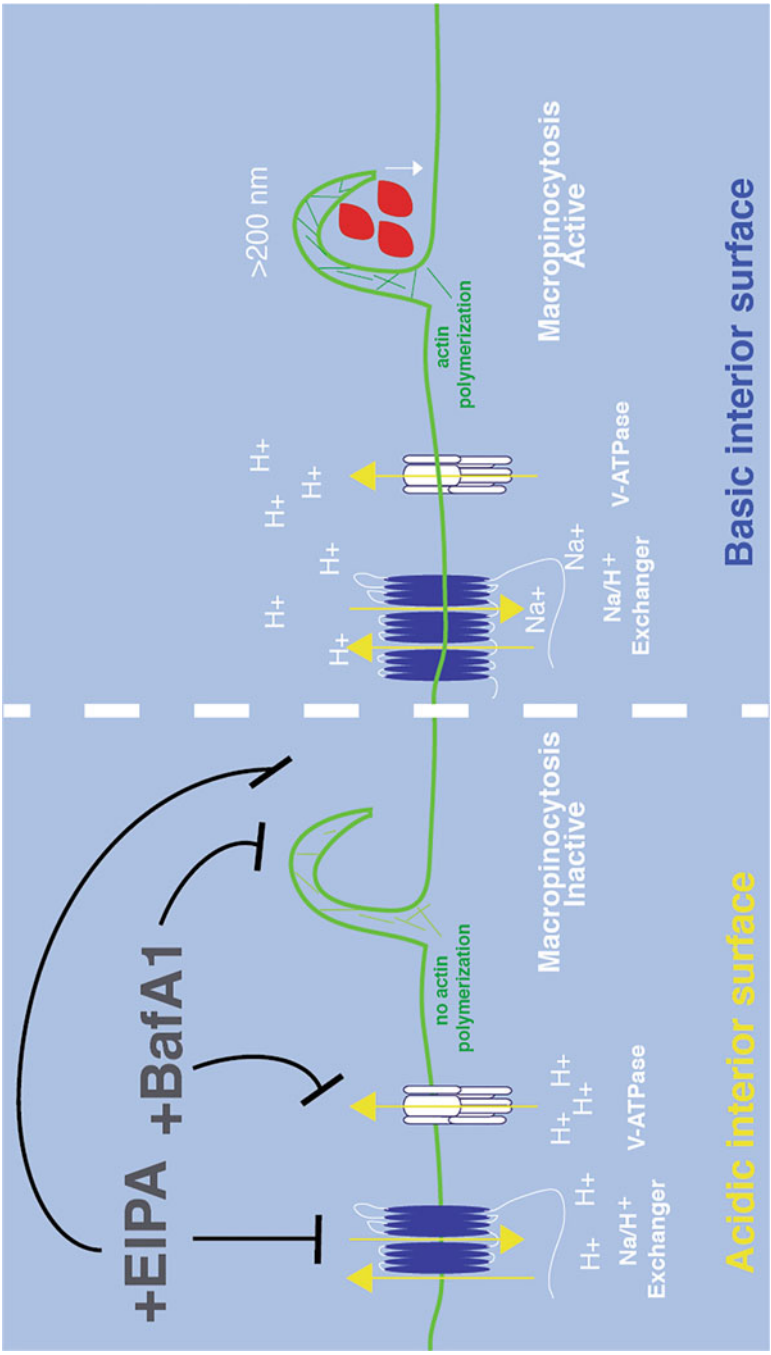

Fig. 9.2 Regulation of micropinocytosis by proton exchangers. Macropinocytosis is an actin-driven process by which extracellular concentrated packages of amino acids (from proteins) and sugars (from glycoproteins) can nourish the cell. The amount of building blocks available as macromolecules far exceeds the amount of free amino acids in circulating plasma (Palm 2019). However, under basal circumstances, cells prefer to use amino acid transporters. Experimentally,

EIPA inhibits the plasma membrane Na^+/H^+ exchange pump, resulting in a more acidic submembranous cytoplasm that inhibits the actin polymerization required for macropinocytosis (Koivusalo et al. 2010) (Fig. 9.2). Importantly, Amiloride, which also can inhibit micropinocytosis, albeit at higher doses, has been used in medical practice as a common diuretic for many decades.

More recently, it has been found that vacuolar ATPase (V-ATPase) located in the plasma membrane, which also pumps out protons to the extracellular space, is required for macropinocytosis induced by Ras activation (Ramirez et al. 2019). The activity of V-ATPase can be inhibited by Bafilomycin A1, and similar results in the inhibition of the macropinocytosis actin machinery (Fig. 9.2). However, Bafilomycin A1 also inhibits the acidification of intracellular endosomes/MVBs, which are required for sustained macropinocytosis (Dobrowolski et al. 2012; Tejeda-Muñoz et al. 2019).

RTK Growth Factor Signaling and Macropinocytosis

Some cells such as macrophages display constitutive macropinocytosis, drinking one-third of their volume per hour (Lewis 1931) and internalizing their entire plasma membrane every 33 min (Steinman et al. 1976). Importantly, treatment of cells with receptor tyrosine kinases (RTK) growth factors such as epidermal growth factor (EGF) can trigger transient macropinocytosis for about 10 min (Haigler et al. 1979; West et al. 1989). A new field in cancer research was opened up by the realization that point mutations that cause activation of Kras allowed sustained macropinocytosis in pancreatic ductal adenocarcinoma (PDAC) and other cancers (Commisso et al. 2013; Ramirez et al. 2019). Through macropinocytosis, serum proteins and a host of extracellular glycoproteins enter the cellular fluid compartment to be either recycled out of the cell or directed to lysosomes for degradation in order to generate key metabolites that fuel cell growth and proliferation (Commisso et al. 2013; Palm and Thompson 2017; Hodakoski et al. 2019; King et al. 2020).

Fig. 9.2 (continued) macropinocytosis incorporation of extracellular macromolecules can be blocked by EIPA or Amiloride. These drugs inhibit macropinocytosis by blocking the plasma membrane Na^+/H^+ exchanger, resulting in the acidification of the submembranous cortical cytoplasm (left panel). Under these acidic conditions, the actin remodeling required for macropinocytosis is prevented. Amiloride is a diuretic that has been used in clinical practice for over 50 years. Bafilomycin A1 (BafA1), an inhibitor of the vacuolar ATPase (V-ATPase), can also inhibit macropinocytosis by blocking proton pumping to the extracellular space. The panel on the right shows that when the interior surface under the plasma membrane has a more basic pH, macropinocytosis of particles of >200 nm can take place after stimulation by RTK/Ras or Wnt. Large macromolecules (in red) are ingested indiscriminately via engulfment by membrane folds and cups. In the case of Wnt macropinocytosis, these molecules are directed into lysosomes, which increase greatly in catabolic activity and the release of nutrients

It is known that Ras activation and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) are important regulators of macropinosome formation; members of the Ras superfamily of small guanosine triphosphatases (GTPases), Ras, Rac, Cdc42, Arf6, Rab5, and others have been associated with plasma membrane ruffle formation and macropinocytic activity (Bar-Sagi and Feramisco 1986; Swanson 2018; Overmeyer et al. 2008; Recouvreux and Commisso 2017). In addition, membrane phospholipids in particular phosphatidylinositol (PI), PI4P, PI5P, PI(4,5)P2, PI(3,4,5) P3, phospholipid kinases, and phosphatases that interconvert them (such as the tumor suppressor PTEN) are also important players in the spatiotemporal regulation of macropinocytosis (Araki et al. 1996; Clark et al. 2014; Marques et al. 2017). Additionally, it has been proposed that destabilization of adherens junctions by Wnt signalling activation could increase cytoplasmic β-catenin and further activate Wnt signaling (Vlad-Fiegen et al. 2012). We can expect that during the progression of tumors initiated by Wnt pathway activation there will be synergistic effects at the level of macropinocytosis with other oncogenic mutations such as Ras, RTKs, PI3K, and loss of PTEN.

The Thompson laboratory identified a role for the transcriptional coactivators YAP and TAZ in activating the TEAD family of transcription factors, which promote transcriptional activation of the Axl receptor tyrosine kinase that in turn activates PI3K/Akt and stimulates macropinocytosis (King et al. 2020). Interestingly, it is known that YAP/TAZ is also stabilized by Wnt family ligands. YAP/TAZ stability has been shown to be under the regulation of components of the Wnt destruction complex and stabilized by Wnt in a fashion similar to β-catenin (Azzolin et al. 2012). However, unlike Wnt signaling, the effects of YAP/TAZ on macropinocytosis are purely transcriptional (King et al. 2020).

Canonical Wnt and Macropinocytosis

Recent investigations have led to the realization that one of the main effects of the Wnt growth factors is to trigger the rapid and sustained activation of macropinocytosis, and that macropinocytosis is essential for canonical Wnt signaling (Albrecht et al. 2018; Redelman-Sidi et al. 2018; Tejeda-Muñoz et al. 2019; Albrecht et al. 2020). During a study by Albrecht et al. 2018, we found that addition of Wnt3a to a variety of cell lines induced, within minutes, the formation of large MVBs that sequestered cytosolic GSK3. These correlated with a massive increase in endocytosis of extracellular BSA (Bovine Serum Albumin). Using BSA-DeQuenched (BSA-DQ), a tracer that only fluoresces after BSA is degraded by proteolytic enzymes inside lysosomes, a striking Wnt-induced increase in the digestion of the extracellular proteins was found (Albrecht et al. 2018). This suggested that Wnt had, within a few minutes of treatment, an important effect on cellular metabolism.

Using a different approach, Redelman-Sidi et al. (2018), studied the requirements for the phagocytosis of Bacillus Calmette-Guérin (BCG), which is used as a bacterial treatment for bladder cancer. Phagocytosis and macropinocytosis are actin-driven

processes that share many common elements (Bloomfield and Kay 2016; Buckley and King 2017). Pinocytosis is drinking by cells, while phagocytosis is eating by cells. It was found that siRNAs that increase Wnt signaling (such as knockdown of Kremen1 or Dkk1) resulted in increased BCG uptake and macropinocytosis in many cancers (Redelman-Sidi et al. 2018). This Wnt-driven macropinocytosis was transcriptionally downstream of β-catenin and, like RTK-Ras-PI3K macropinocytosis, was entirely dependent on the activity of Pak-1.

Mutation of the tumor suppressor APC is found in most colorectal cancers (CRC). Since APC, like Axin1, is a component of the β-catenin destruction complex, its mutation results in increased canonical Wnt signaling. Using a doxycycline-inducible shAPC mouse model, it was shown that APC depletion increased phagocytosis of the bacterial microbiome (Redelman-Sidi et al. 2018). Increased translocation of bacteria from the lumen into the epithelium of the colon is thought to be an important determinant in colon cancer. Furthermore, intracolonic instillation of TMR-dextran showed an increase of macropinocytosis in vivo, which could be blocked by EIPA (Redelman-Sidi et al. 2018).

In a parallel study, we found that Wnt3a caused a major rearrangement of the actin cytoskeleton (Fig. 9.3a and b). In the presence of Wnt, F-actin was seen to encircle membrane vesicles forming at the plasma membrane (Tejeda-Muñoz et al. 2019). Using membrane-targeted GFP, live visualization of macropinocytosis vesicles could be filmed forming in the apical region on cells in which Wnt signaling is activated but, interestingly, not in the basolateral region of adjoining cells (Fig. 9.3c and d; note arrowhead and arrow) (Albrecht et al. 2020). The Wnt-induced vesicles resulted in increased TMR-dextran macropinocytosis that could be inhibited by EIPA. Similar macropinocytosis could also be induced by other Wnt-stimulating agents such as overexpression of Dishevelled, Frizzled 8, or a dominant-negative form of the Wnt inhibitor Axin (Tejeda-Muñoz et al. 2019).

Taken together, these studies demonstrate that canonical Wnt signaling induces macropinocytosis.

Macropinocytosis is Induced by Loss-of-Function of APC and Axin1

It is known that 85% of colorectal carcinomas (CRC) are initiated by mutations in APC that increase the level of β-catenin (Kinzler and Vogelstein 1996). Familial adenopolyposis is a disease caused by heterozygous truncations in APC. Patients develop hundreds to thousands of benign polyps that progress into cancer after acquiring multiple additional mutations, usually starting with activated Kras (Segditsas and Tomlinson 2006). A very useful cell culture model system has been established for the CRC cell line, SW480, which has constitutive Wnt/β-catenin that can be suppressed by stable transduction with low levels of wild-type APC (Faux et al. 2004). A similar system exists in the case of hepatocellular carcinoma (HCC), in which the Alexander cell line (Alexander et al. 1976; Satoh et al. 2000) that had

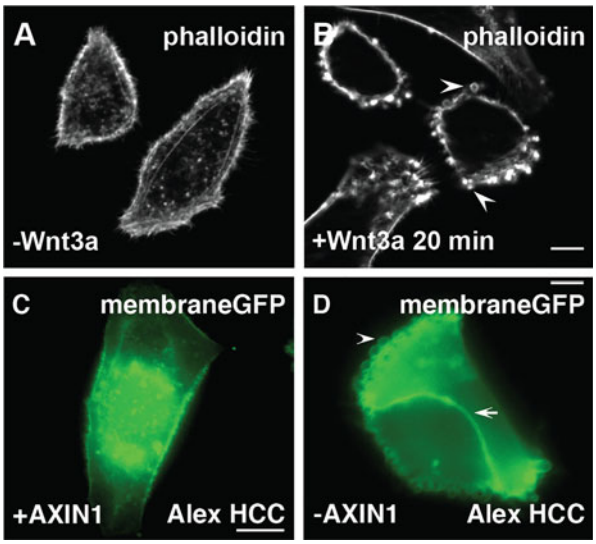

Fig. 9.3 Wnt signaling induces the formation of macropinocytic vesicles. (**a** and **b**) Wnt3a induces the rapid rearrangement of the cortical F-actin cytoskeleton, visualized here by phalloidin staining of HeLa cells. In the absence of Wnt, actin is found in stress fibers, the cell cortex, and filopodia. After addition of Wnt3a for 20 min, the actin machinery surrounds large macropinocytic vesicles indicated by arrowheads. (**c** and **d**) Alexander HCC±Axin1 cells provide an ideal model system to study macropinocytosis in cancer because the two cell lines are identical except for the presence or absence of exon 4 of the scaffold protein Axin1 that binds to GSK3. In Axin1 minus cells, Wnt signaling is activated and this is repressed by reconstitution with wild type Axin1 in the Axin1 plus cells. Note that labeling of the plasma membrane with plasma membrane-targeted GFP shows macropinocytotic vesicles in the apical region (arrowhead) and not in the basolateral region between cells (arrow). Studies with Alexander HCC±Axin1 cells demonstrate that the Wnt destruction complex plays a fundamental role in the regulation of macropinocytosis. (**a**) and (**b**) are reproduced from Tejeda-Muñoz et al. (2019) (with permission from Proceedings of the National Academy of Sciences and Creative Commons), and (**c**) and (**d**) are from Albrecht et al. (2020) (with permission of Cell Reports, Copyright Elsevier)

been kept in culture for 40 years could be reconstituted with full length of Axin1 (Albrecht et al. 2020). Both in SW480±APC and in HCC±Axin1, Wnt-induced macropinocytosis was eliminated by reconstitution with the corresponding tumor suppressor gene at physiological levels (Tejeda-Muñoz et al. 2019; Albrecht et al. 2020). This indicates that the Wnt destruction complex plays a fundamental role in macropinocytosis.

SW480 cells lacking APC have high levels of nuclear β-catenin (Fig. 9.4a). Interestingly, treatment of these cells for only 2 h with EIPA or Amiloride greatly inhibited nuclear β-catenin accumulation (Fig. 9.4b) as well as a β-catenin activation reporter Luciferase assay (Fig. 9.4c). This shows that continuous macropinocytosis is required for canonical Wnt signaling in these cancer cells.

As first found by Taelman et al. (2010), Wnt signaling through MVB sequestration can be blocked by transfecting a dominant-negative Vps4-EQ point mutation that inactivates the ATPase activity of this ESCRT protein. Blocking MVB

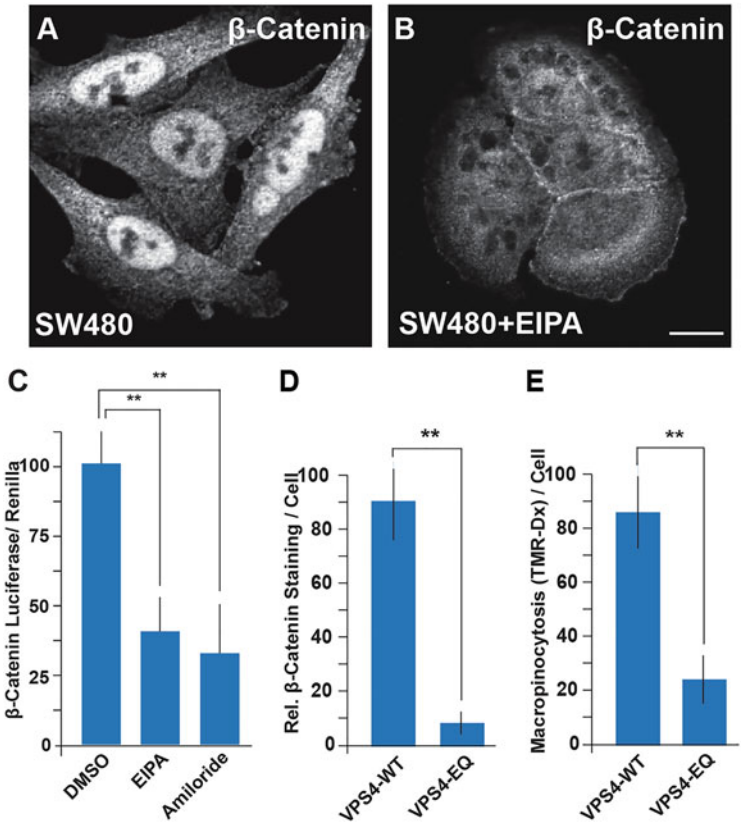

Fig. 9.4 Nuclear accumulation of β-catenin in colorectal cancer cells requires macropinocytosis and the MVB biogenesis ESCRT component Vps4. (**a** and **b**) Inhibition of micropinocytosis with EIPA for only 2 hours greatly decreases nuclear β-catenin accumulation in SW480 CRC cells lacking APC. (**c**) EIPA or Amiloride treatment inhibited BAR (β-catenin activity reporter) Luciferase/Renilla signaling in transfected SW480 cells within 2 hours of treatment. As in the case of nuclear localization, canonical Wnt signaling requires continued macropinocytosis. (**d**) Inhibiting the ESCRT machinery by Vps4-EQ transfection, but not by Vps4-WT, decreases β-catenin levels in SW480 cells, showing the requirement of MVB formation for nuclear β-catenin accumulation, which is the hallmark of canonical Wnt activation. (**e**) Macropinocytosis of TMR-dextran 70 k in CRC cells mutant for APC requires an active ESCRT machinery. Treatments that interfere with the ESCRT machinery block β-catenin stabilization and macropinocytosis. Vps4 is an ATPase involved in the final stage of intraluminal vesicle closure during MVB formation. The point mutation Vps4-EQ acts as a dominant-negative that inhibits the ESCRT machinery and prevents MVB formation. Reproduced from Tejeda-Muñoz et al. (2019), with permission from Proceedings of the National Academy of Sciences and Creative Commons

formation with Vps4-EQ resulted in lower levels of β-catenin accumulation in SW480 CRC cells (Fig 9.4d). In addition, macropinocytosis of TMR-dextran was inhibited by interfering with the MVB machinery (Fig. 9.4e). These experiments show that membrane trafficking through MVB/endosomes is required for sustained macropinocytosis and Wnt/β-catenin signaling (Tejeda-Muñoz et al. 2019).

Rapid Macropinocytosis Induction by Wnt in the Absence of New Protein Synthesis

Macropinocytosis induction by Wnt is a rapid process and, remarkably, takes place even in the absence of new protein synthesis, as it can occur in the presence of cycloheximide (Tejeda-Muñoz et al. 2019; Albrecht et al. 2020). Thus, the actin machinery is poised for macropinocytosis and does not require synthesis of new β-catenin. The Wnt transcriptional loop can also trigger macropinocytosis (Redelman-Sidi et al. 2018; Albrecht et al. 2020), but the immediate activation of macropinocytosis must use a different mechanism to activate Pak1 and the actin machinery (Albrecht et al. 2020).

GSK3 Represses Macropinocytosis

How can canonical Wnt signaling have such diverse effects on cellular physiology as the stabilization of β-catenin, Wnt-STOP, MVB membrane trafficking, and macropinocytosis? We realized that the common link was the activity of GSK3 and its regulation by the destruction complex. Inhibition of GSK3 with Lithium chloride (LiCl) or CHIR9901 resulted in macropinocytosis that could be blocked by EIPA (Albrecht et al. 2020). Wnt signaling normally results from the sequestration of cytosolic GSK3 in MVB/lysosomes (Taelman et al. 2010; Albrecht et al. 2018, 2020), but chemical inhibitors of GSK3 can achieve the same result.

The destruction machinery, through APC and Axin1, plays a crucial role in GSK3 regulation. The Alexander HCC cell line only lacks exon 4 of Axin1, which is the part of the Axin1 scaffolding protein that contains its GSK3 binding sites. Lack of this region confers macropinocytosis and the ability to grow at the expense of 3% BSA in the absence of serum (Albrecht et al. 2020). While Axin1 is indispensable for the phosphorylation of β-catenin by GSK3, it is not yet known how many of the other Wnt-STOP substrates require Axin1. Perhaps much of the cytosolic GSK3 is bound to its substrates or inactivated by phosphorylation in Ser 9 (Cohen and Frame 2001), and a large fraction of the cytosolic GSK3 requires activation by Axin1. GSK3 phosphorylates Axin1 keeping it in an active open form (Kim et al. 2013). Conversely, Axin1 binds Protein Phosphatase 2A (PP2A) in close proximity to the GSK3 binding domain (Satoh et al. 2000). In turn, PP2A dephosphorylates GSK3 at Ser 9, keeping it in the active form (Kim et al. 2013). The main conclusion that emerges from the GSK3 inhibition experiments is that the complex of GSK3 and Axin1 serves to repress macropinocytosis in basal cell conditions. When Wnt signaling takes place, Axin1 and GSK3 are translocated into the MVB/late endosome compartment and macropinocytosis is initiated.

Endocytosis and Canonical Wnt Signaling

In general, receptor-mediated endocytosis takes place through micropinocytosis of small vesicles. However, in the Wnt field there is controversy whether Lrp6-Frizzled receptors are dependent on endocytosis via clathrin or caveolin (Blitzer and Nusse 2006; Yamamoto et al. 2006; Rim et al. 2020). We had proposed that Wnt-Lrp6-Fz micropinocytosis might first decrease GSK3 in the cytosol to sufficient levels to allow the initiation of macropinocytosis (Tejeda-Muñoz et al. 2019; Albrecht et al. 2020). Here we modify our model to circumvent the micropinocytosis mechanism debated by other laboratories. In our revised model in Fig. 9.1, the Wnt receptor complex has been placed within the macropinocytic cup, where it might trigger local inhibition of GSK3 activity in the plasma membrane signalosome (Cselenyi et al. 2008) sufficient to activate Pak1-mediated macropinocytosis.

Once macropinocytosis is trigged, MVB sequestration of the destruction complex by the ESCRT machinery, recently validated by Lrp6-APEX2 interactome data (Colozza et al. 2020), is required in order to sustain low cytosolic levels of GSK3 and membrane trafficking flux into lysosomes (Fig. 9.1). How Pak1 is activated by GSK3 inhibition remains to be determined, but Pak1-4, DOCKs (guanine nucleotide exchange factors that activate CDC42), and Ras proteins contain putative GSK3 phosphorylation sites that could be stabilized by Wnt-STOP (Taelman et al. 2010). In addition, Kras has been found to interact with Lrp6-APEX2 after only 5 minutes of Wnt3a addition in HeLa cells (Colozza et al. 2020), providing another possible pathway for the activation of Pak1 by Wnt.

Wnt-induced Macropinocytosis Increases Lysosomal Acidification and Activity

Oncogenic transformation causes changes in lysosomal volume and subcellular localization (Kirkegaard and Jäättelä 2009; Kallunki et al. 2013). Many cancer cells increase the number of lysosomes and autolysosomes to maintain homeostasis by increasing degradation and recycling of macromolecules to maintain cell proliferation in order to survive stressful conditions (Kroemer and Jäättelä 2005; Cardone et al. 2005; Zhitomirsky and Assaraf 2016). Recently, it was reported that Wnt signaling increases the acidification and activity of lysosomes (Albrecht et al. 2020).

An unexpected discovery was that Wnt is a major regulator of lysosomal activity. SiR-Lysosome is a cell permeable reagent that contains a peptide that binds exclusively to the activated form of cathepsin D in lysosomes (Marciniszyn et al. 1976). As shown in Fig. 9.5a and c, addition of Wnt3a greatly increased the amount of active cathepsin D in lysosomes. Notably, treatment with the GSK3 inhibitor, LiCl, increased active lysosomes to a comparable degree (Fig. 9.5b and d), suggesting that treatment with Wnt protein is able to inhibit most of the GSK3 activity responsible for lysosome regulation. Lysosome activation was eliminated by treatment by EIPA,

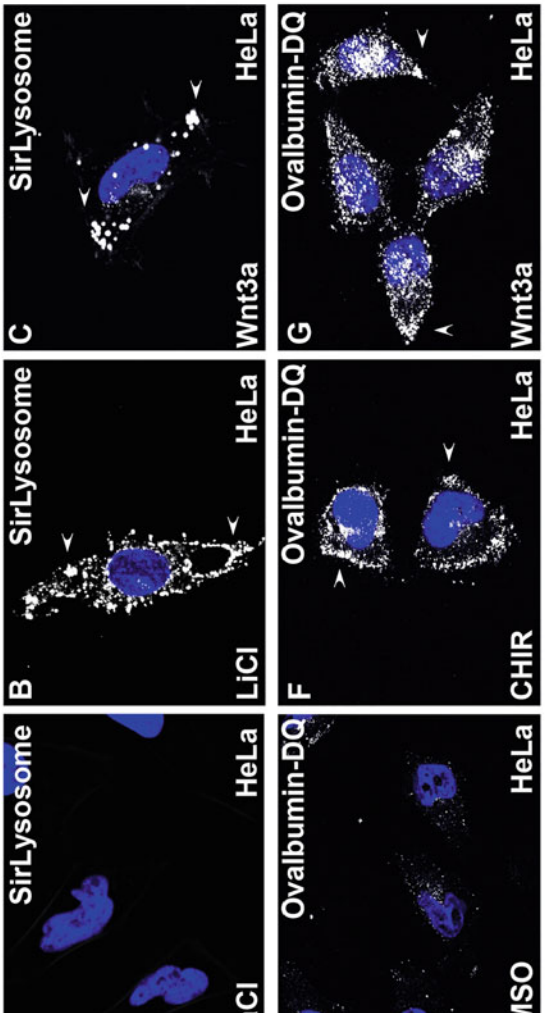

Fig. 9.5 Lysosome activity is strongly increased by treatment for 20 minutes with Wnt3a or GSK3 inhibitors. (**a–c**) SiR-Lysosome is a cell-permeable probe that specifically binds to the activated form of cathepsin D in lysosomes. Note that Wnt3a or GSK3 inhibition with LiCl contain more lysosomes with active cathepsin **d** (arrowheads) when compared to NaCl treated cells used as a control for LiCl. (**d**) Quantification of active cathepsin D. (**e–g**) Ovalbumin-DQ was added as an extracellular protein and is a tracer that fluoresces in lysosomes only after being cleaved by proteases; note that the GSK3 inhibitor CHIR (CHIR9901) and Wnt increase the degradation of extracellular proteins engulfed through non-receptor-mediated endocytosis (since ovalbumin is a non-mammalian protein there is no ovalbumin specific receptor). (**h**) Quantification of ovalbumin-DQ cell fluorescence; note that the GSK3 inhibitor and Wnt increase lysosome catabolism to similar levels. Reproduced from Albrecht et al. (2020) with permission of Cell Reports Copyright Elsevier

indicating that this process requires macropinocytosis (Albrecht et al. 2020). Increases in lysosomal enzymatic activity were also found using a β-glucosidase substrate, BSA-DQ, or ovalbumin-DQ (Fig. 9.5e–h). Using a pH ratiometric lysosensor probe, preexisting lysosomes became much more acidic with Wnt treatment, through a macropinocytosis-mediated mechanism since it was blocked by EIPA (Albrecht et al. 2020). The results suggest a major metabolic rearrangement after Wnt treatment, in which extracellular macromolecules are ingested and, instead of being recycled to the outside, are directed to the lysosome for degradation into its elementary components. There remains much more to be learned about membrane trafficking regulation by Wnt and GSK3.

The lysosome may represent a point of vulnerability for targeting the progression of Wnt-driven cancers. Lysosomes can be targeted with lysosomotropic drugs that accumulate in and inhibit lysosomes such as Chloroquine, Hydroxychloroquine, their newer potent derivative dimeric quinacrine DQ661 (Rebecca et al. 2017), and LLOME (L-leucyl-L-leucine O-methyl ester) (Skowyra et al. 2018). Arsenic trioxide (As_2O_3) destabilizes lysosomes (Miele et al. 2009). Cytotoxic nanoparticles are taken up by macropinocytosis, for example, Abraxane (an albumin-conjugated Taxol derivative currently approved for breast and pancreatic cancers) (Miele et al. 2009). Lysosomes and cancer are hot topics, and the new connections between the Wnt β-catenin destruction machinery and MVB/lysosome trafficking are a promising area for cancer therapeutic interventions.

Conclusions

Macropinocytosis has emerged as a major pathway for the cellular acquisition of rich packages of nutrients in the form of extracellular macromolecules (Palm et al. 2017). Its role in cancer is particularly important and extends to many tumors beyond pancreatic ductal carcinoma. Although it has been known for many years to be transiently activated by the EGF-RTK-Ras-PI3K pathway, macropinocytosis has now been found to be also activated, in a more sustained manner, by canonical Wnt signaling. Both pathways require the activation of Pak1. Although Wnt signaling exerts its transcriptional effects through the stabilization of nuclear β-catenin, it also has many other effects such as macropinocytosis, Wnt-STOP, endosomal membrane trafficking, and the regulation of lysosomal activity. The destruction complex containing the tumor suppressors Axin1 and APC plays an important role by promoting the activity of GSK3. This key cellular serine/threonine kinase is involved in many cellular processes (polarity, metabolism, cell cycle, gene expression, embryonic development, and oncogenesis). The activity of GSK3 is not only regulated by MVB sequestration as emphasized here but also by multiple phosphorylations (Cohen and Frame 2001). The most important ones are the inhibitory phosphorylation at Ser 9 of GSK3β and Ser 21 of GSK3α which can be mediated

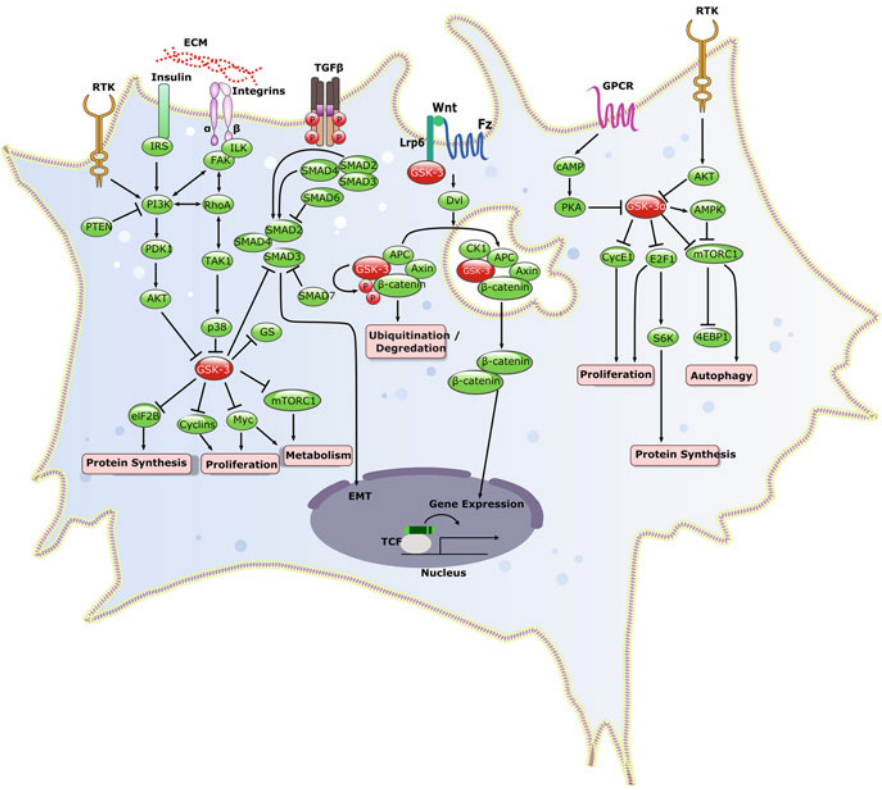

Fig. 9.6 GSK3 activity is regulated by many signaling pathways in addition to canonical Wnt. While the Wnt-Lrp6-Fz receptor complex causes GSK3 sequestration in MVB/lysosomes, many other pathways also affect the activity of GSK3, principally through phosphorylation at Ser 9 of GSK3β and Ser 21 of GSK3α. In turn, GSK3 phosphorylates many target proteins regulating their activity and degradation through Wnt-STOP. GSK3 was first recognized as a central mediator of the insulin pathway, as a key kinase that phosphorylates the glycogen synthase enzyme, inhibiting the incorporation of glucose into glycogen. Subsequent studies revealed that, in addition to glucose metabolism, GSK3 is a critical regulator of multiple signaling pathways including RTK/Ras/PI3K/PTEN, AKT/GSK3/mTORC1, GPCR/cAMP/PKA, and others. The pathways in which GSK3 acts as a key regulator, when dysregulated, have been implicated in the pathogenesis of human diseases such as diabetes, Alzheimer's disease, bipolar disorder, and cancer. This explains why GSK3 has emerged as an important target for drug development. Diagram inspired by Lal et al. (2015)

by PKA, AKT, PKC, and S6K. As depicted in Fig. 9.6, GSK3 is involved in many signaling pathways, and understanding its regulation will open new windows in cell physiology, stem cells, and cancer.

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Chapter 10
KRAS Addiction Promotes Cancer Cell Adaptation in Harsh Microenvironment Through Macropinocytosis

Laetitia Seguin

Abstract KRAS is the most frequently mutated oncogene in cancer and despite intensive studies, attempts to develop effective therapies targeting KRAS or its downstream signaling have failed mostly due to the complexity of KRAS activation and function in cancer initiation and progression. Over the years, KRAS has been involved in several biological processes including cell survival, proliferation, and metabolism by promoting not only a favorable tumor environment but also a cell-microenvironment dialog to allow cancer cells to adapt to tumor microenvironment scarcity. One of the mechanisms involved in this adaption is KRAS-mediated macropinocytosis. Macropinocytosis is an evolutionarily conserved, large-scale, and nonselective form of endocytosis involving actin-driven cell membrane remodeling to engulf large amounts of extracellular fluids and proteins from the local environment. While macropinocytosis process has been known for decades, recent gain interest due to its regulation of KRAS-driven tumor growth in adverse microenvironments. By promoting extracellular protein and other macromolecules internalization, macropinocytosis provides a survival mechanism under nutrient scarce conditions and the potential for unrestricted tumor growth. Thus, a better understanding of macropinocytotic process is needed to develop alternative therapeutic strategies.

Keywords KRAS addiction · Integrin · Microenvironment sensing · Macropinocytosis

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KRAS Addiction in Cancer

KRAS, a Multifaceted Protein

KRAS is one of the most frequently mutated genes across human cancers, with the highest incidence in pancreatic (>75%), colorectal (50%) and lung (30%) adenocarcinomas, and is associated with proliferative, aggressive tumors, and resistance to cancer therapeutics (Bos 1989). KRAS protein, originally identified in Kirsten sarcoma virus for its capacity to transform normal cells, is a small guanosine triphosphatase (small protein G) functioning as a molecular switch on the inner side of the plasma membrane by cycling between two conformational states: an active guanosine triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP)-bound state. Activation of upstream signaling pathways results in the recruitment of Guanosine Exchange Factors (GEFs), such as SOS1 and SOS2 that catalyze the release of GDP from KRAS thus promoting KRAS activation. The intrinsic GTPase activity of KRAS is enhanced by GTPase-Accelerating Proteins (GAPs) to promote KRAS inactivation (Bos et al. 2007). However, *KRAS* mutations lead to the impairment of GTP hydrolysis and cause GAP insensitivity locking KRAS in its active conformation state. Oncogenic (or active wild-type) KRAS interacts with several effector proteins, such as RAF, PI3K, and RALGDS, and initiates several downstream signaling cascades involved in multiple phenotypes, including loss of contact inhibition, altered metabolism, and uncontrolled proliferation (Liu et al. 2019; Pylayeva-Gupta et al. 2011).

KRAS-driven Cancer Heterogeneity, a Hint to Understand KRAS Addiction

Since its discovery in the early 1980s, KRAS protein its downstream signaling has been extensively studied due to its clinical relevance as an oncogenic driver in cancer pathogenesis. Despite intensive efforts and different strategies investigated, KRAS-driven cancers are refractive to therapeutics and treatment options (Papke and Der 2017). Alternative approaches to combatting KRAS-mutant cancers are clearly needed. Multiple intrinsic and acquired resistance mechanisms have been described such as increased KRAS protein level or change in prenylation for KRAS-direct targeting and activation of alternative pathways for KRAS-mediated downstream signaling inhibition approaches. Another resistance mechanism is the tumor intra- and inter-heterogeneity that influences intrinsic and adaptive drug response. Only half of the tumors harboring oncogenic KRAS are dependent on KRAS to survive and proliferate. This notion, called KRAS-dependency or KRAS-addiction is a type of oncogene addiction, a state in which cancer cells depend on signaling from a single oncogene to survive. Knowing that KRAS mutation and overexpression are not sufficient to confer KRAS-addiction (Ito et al. 2020), a better understanding of

KRAS-dependency could lead to new therapeutic alternatives. Integrative approaches including gene signature approach to analyze KRAS-dependent pathways and loss-of-function genetic screens in KRAS-dependent and independent cells have been conducted to screen for KRAS synthetic lethal relationships and determine genes that are essential for KRAS-driven cancer proliferation and survival. Several studies have already highlighted selective vulnerabilities for KRAS-addicted tumors such as TBK1, GATA2, SHP2, XPO1, and STK33, that may represent therapeutic candidates to treat these tumors (Barbie et al. 2009; Kumar et al. 2012; Ruess et al. 2018; Kim et al. 2016; Scholl et al. 2009). However, conflicting results and profound heterogeneity of KRAS mutant cancers complicate uniform identification of KRAS synthetic lethal targets (Aguirre and Hahn 2018).

The underpinnings that drive KRAS mutant cancer heterogeneity are poorly characterized and result of multiples molecular events taking place during tumor initiation and progression.

Most KRAS mutations occur at codons 12, 13, and 61 with single amino acid substitutions leading to KRAS constant activation. Until recently, it was admitted that different activated mutations cause identical effects on KRAS function. However, several evidences have demonstrated that each KRAS mutant isoform has specific functional properties leading to distinct downstream signaling impacting tumor cell biological behavior and clinical outcomes (Renaud et al. 2016). $KRAS^{G12C}$ mutant favors ERK downstream signaling, $KRAS^{G12V}$ activates RalA/B signaling and $KRAS^{G12D}$ triggers AKT pathway (Muñoz-Maldonado et al. 2019). Intriguingly, the frequency of *KRAS* single point mutation varies in different cancer types suggesting a different role in cancer development and progression. Interestingly, KRAS-addiction depends on tissue of origin (Yuan et al. 2018). Indeed, pancreatic cancer cells appear to be strongly dependent on KRAS signaling for tumor initiation and progression in vitro and in vivo as deletion of KRAS promotes pancreatic tumor regression (Brummelkamp et al. 2002), while KRAS-addiction heterogeneity has been described in lung adenocarcinoma, in which oncogenic KRAS promotes tumorigenesis but secondary mutations can contribute to KRAS-independency (Yuan et al. 2018). Large-scale cancer genome sequencing highlighted a wide spectrum of co-occurring genetic alterations that may alter KRAS-addiction. In lung cancer, mutation in *STK11/LKB1*, *TP53*, *CDKN2A/B*, and *KEAP1* characterize three major KRAS-driven lung cancer subtypes with distinct biology and therapeutic vulnerabilities (Skoulidis et al. 2015).

Transcriptomic analysis of KRAS-driven cancers also highlighted gene signatures involved in KRAS-addiction escape. For instance, the expression of proteins involved in epithelial–mesenchymal transition (EMT) promotes KRAS-independency (Singh et al. 2009). More particularly, it has been shown that the YAP/Tead2 complex drives cell proliferation independently of KRAS activation leading to KRAS-addiction escape (Kapoor et al. 2014).

KRAS-driven cancer heterogeneity is a challenge in the discovery of new therapeutic alternatives. Thus, understanding why cells are addicted or not to oncogenic KRAS has become the holy grail to explore alternative opportunities for targeting

KRAS-driven cancers and additional biomarkers are necessary to identify patients with KRAS-addicted tumors.

KRAS Localization, a Critical Aspect of KRAS Addiction

One hypothesis of KRAS-addiction is KRAS spatial organization at the plasma membrane. While it has been known for decades that KRAS signaling strongly depends on its enrichment at the plasma membrane through a farnesylated and polybasic targeting sequence within its hypervariable region (Hancock et al. 1990; Willumsen et al. 1984; Jackson et al. 1994; Sperlich et al. 2016), attempts to inhibit KRAS localization with post-translational farnesylation inhibitors (farnesyl transferase inhibitors or FTIs) have failed in the clinic due to alternative isoprenylation of KRAS in the presence of these inhibitors (Downward 2003). A better understanding of the events required for KRAS localization at the plasma membrane is critical to developing alternative approaches. It has been shown that KRAS must dimerize and assemble into transient cholesterol-independent nanoclusters for proper biological activities by increasing the effective local concentration, thus proximity, of KRAS-binding partners (Muratcioglu et al. 2015; Ambrogio et al. 2018). The spatial distribution of KRAS is modulated by its expression level and by its activated state as recent works have shown that KRAS-GTP nanoclusters are spatially distinct from KRAS-GDP nanoclusters (Plowman et al. 2005; Prior et al. 2003). KRAS nanoclusters are dynamic and depend on high local concentration of specific lipids mostly phosphatidic acid (PA) and phosphatidylserine (PS) and on the constitution of the polybasic sequence. Depletion of PA, PS, or amino acid change of the polybasic domain results in failure of KRAS nanocluster formation and modulates KRAS functions (Hancock 2003), providing an explanation for the ability of KRAS to drive several signaling outputs. Besides plasma membrane constitution, the regulation of dynamic spatial cycle of KRAS in the cell has been highlighted to be critical for KRAS localization and multiple KRAS partners are involved in this process. Indeed, the GDI-like solubilizing factor GMP phosphodiesterase6-δ binding (PDEδ) sustains KRAS dynamic and spatial organization by facilitating its diffusion in the cytoplasm (Zimmermann et al. 2013). Mechanistically, PDEδ sequesters KRAS preventing its binding to endomembrane and thereby enhancing its diffusion in the cytoplasm and recycling from the endosome to shuttled back to the plasma membrane via vesicular transport. Genetic and pharmacologic inhibition of PDEδ impairs KRAS enrichment at the plasma membrane and decreases proliferation of KRAS-addicted cancers in vitro and in vivo (Schmick et al. 2014). Several evidences have shown that the calcium-sensing protein calmodulin Ca^{2+}/CaM, by interacting selectively with KRAS, redirects it from the plasma membrane to intracellular membrane and thereby modulates KRAS effectors activation, inhibiting MAPK signaling to favor PI3K/AKT signaling (Sperlich et al. 2016; Wang et al. 2015). This Ca^{2+}/CaM-KRAS interaction has also been involved in KRAS-mediated tumorigenic phenotype through downregulation of the noncanonical Wnt/Ca^{2+}

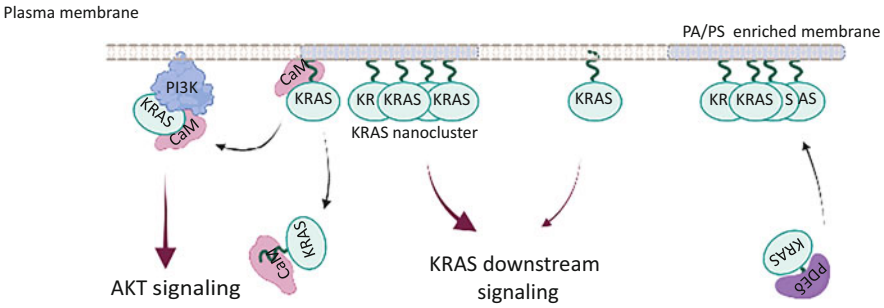

Fig. 10.1 KRAS localization modulates KRAS downstream signaling (Biorender)

signaling (Wang et al. 2015). Among KRAS binding partners, Galectin-3, Nucleophosmin, and Nucleolin interact with KRAS to activate MAPK pathway (Inder et al. 2010). While Nucleolin increases KRAS level at the plasma membrane, Nucleophormin increases KRAS clustering (Inder et al. 2010) and cytoplasmic Galectin-3 increases activated KRAS nanocluster formation (Shalom-Feuerstein et al. 2008). Knowing that Galectin-3, Nucleophosmin, and nucleolin are highly expressed in many cancers, investigating their role in KRAS-addiction could provide new therapeutic perspectives. *Altogether, these results point out the critical role of KRAS dynamic nanocluster formation that allows KRAS interaction with different partners promoting KRAS-driven specific downstream signaling (Fig. 10.1).*

Tumor Microenvironment, a Clandestine Modulator of KRAS Addiction

While some interesting approaches have been investigated, several discrepancies between studies have been highlighted in terms of KRAS dependency. These inconsistencies may be explained by off-target effects and the efficiency of the different silencing systems used to inhibit KRAS. Another explanation may be that KRAS addiction depends on the cellular microenvironment. Indeed, differences in KRAS addiction has been shown in 2D and in 3D culture system in vitro and evidences highlight a stronger KRAS addiction in 3D culture (Fujita-Sato et al. 2015). While in monolayer conditions, the growth of cancer cell lines such as A549 and PANC1 was relatively resistant to KRAS depletion, in anchorage-independent culture conditions, KRAS depletion resulted in striking growth suppression (Fujita-Sato et al. 2015), reflecting the different degrees of KRAS requirements in different environments. It is now admitted that 2D cell culture condition oversimplifies the tumor microenvironment complexity while in 3D-spheroid conditions, cancer cells are exposed to oxygen, nutrients, and metabolites diffusion gradient leading to microenvironment heterogeneity with hypoxic and nutrient-poor areas that better mimic in vivo tumor growth conditions. In addition, 2D cell culture condition

changes cell–cell and cell–matrix interactions and thus cell behavior. For instance, it has been shown that in matrix-adherent cells, KRAS is recruited into membrane nanoclusters by a variety of cell surface receptors that serve as interaction platforms to drive cell survival and proliferation. The redundancy in receptors capable of mediating KRAS clustering may explain why matrix-adherent cells can easily switch dependence between pathways when one is inhibited by a given targeted therapeutic. However, in the absence of matrix adhesion, epithelial cell surface receptors poorly cluster, as it has been demonstrated for EGFR (Gao et al. 2015). Therefore, in anchorage-independent growth, a hallmark of KRAS-driven transformation, KRAS clustering may be mediated by fewer partners. Thus, the use of an in vitro anchorage-independent culture model may identify more relevant in vivo signaling pathways downstream of KRAS. It has been recently shown that integrin αvβ3 interacts with KRAS to form a complex required for KRAS-addicted cancer cells survival in 3D culture but also in vivo (Seguin et al. 2014). *Altogether, these results suggest that tumor microenvironment impacts KRAS-addiction, the more drastic the microenvironment, the more addicted to KRAS the cancer cells.*

Macropinocytosis and Cancer Cell Adaptation

Macropinocytosis, an Advantage for KRAS-Addicted Cell Promoting Survival

Tumor microenvironment is highly heterogeneous with dense extracellular matrix resulting in high interstitial pressure and nutrient- and/or oxygen-deprived regions due to defective vasculature (Weis and Cheresh 2011; Denko 2008). To sustain KRAS-mediated unrestricted proliferation, cancer cells adapt to nutrient-constrain microenvironments by rewiring their metabolism and by using unconventional energy sources. Not only KRAS promotes a metabolic reprogramming of tumor cells, shifting them toward an anabolic metabolism called the Warburg effect, it also maintains redox balance and increases autophagy and macropinocytosis to recycle and scavenge essential nutrients important for cell growth and survival (Bryant et al. 2014). More particularly, KRAS-driven macropinocytosis encourages a cancer cell opportunistic behavior whereby they do not rely on any particular nutrient to proliferate, thus allowing them to grow upon nutrient-deprived microenvironment. Indeed, in nutrient-poor conditions, KRAS-driven cancer cells rely on extracellular macromolecules rather than amino acids via macropinocytosis to maintain intracellular amino acid levels required for cell proliferation. Albumin is the most abundant plasma protein, and its internalization through KRAS-mediated macropinocytosis, followed by lysosomal degradation and amino acid release bypasses the lack of glucose, glutamine, or amino acid such as arginine, leucine, and proline within the microenvironment in vitro and in vivo (Hodakoski et al. 2019; Commisso et al. 2013; Palm et al. 2015; Kamphorst et al. 2015; Olivares et al. 2017). Besides

albumin, ATP is one of the major biochemical constituents of the tumor microenvironment (Cao et al. 2019; Wang et al. 2017). Indeed, extracellular ATP concentrations are 1000 times higher in the tumor than in normal tissue. As fast cancer cell proliferation increases energy demand and ATP consumption, one way to supplement this extra energy needs is to internalize extracellular ATP via macropinocytosis (Wang et al. 2017). Dividing cells acquire lipids and fatty acids required to reproduce their membrane either through macropinocytosis or de novo synthesis. KRAS-driven cancer increases lipids and fatty acid scavenging possibly making them more resistant to hypoxic or scarce conditions (Florey and Overholtzer 2019). Extracellular matrix (ECM) is also a main component of the tumor microenvironment. Indeed, the tumor ECM is more abundant, denser, and stiffer than in normal tissue and can account for up to 60% of the tumor mass. KRAS-mediated macropinocytosis of ECM proteins, including fibronectin, laminin, and collagen represent a source of amino acids in cancer tissues (Olivares et al. 2017; Davidson et al. 2017; Muranen et al. 2017; Yamazaki et al. 2020). While, common knowledge was that oncogenic KRAS-driven cancer cells exhibit a constitutive macropinocytosis initiated by constant activation of the PI3K/AKT pathway, recent extensive studies have highlighted a much more complex reality. KRAS mutation influences KRAS-mediated downstream signaling, thus KRAS functions. The $KRAS^{G12R}$ mutation, rare in lung and colorectal cancer represents the third more common mutation in pancreatic cancer. $KRAS^{G12R}$-driven cancer cells are impaired in PI3K signaling and macropinocytosis (Hobbs et al. 2019). This study not only highlights the critical role of the PI3K pathway in macropinocytosis but also the importance of KRAS binding partners to favor specific KRAS-mediated functions. Furthermore, nutrient availability within the microenvironment activates KRAS-mediated macropinocytosis in a subtype of KRAS-driven pancreatic cancer cells. Indeed, glutamine starvation induces macropinocytosis via EGFR signaling induction and Pak activation (Lee et al. 2019). These results provide evidence that some KRAS-driven cancer cells integrate metabolic input to regulate macropinocytosis depending on nutrient availability. Therefore, elucidating the mechanisms required for those cells to adapt quickly to tumor environment scarcity has become critical to better understand KRAS-driven tumor behavior. Cancer cells carrying oncogenic KRAS crosstalk with the microenvironment by driving a selective dependence on the surfaceome and more particularly on integrin signaling to promote cancer progression. Indeed, not only oncogenic KRAS upregulates integrin expression (Martinko et al. 2018), it also interacts with integrin αvβ3 to promote KRAS-driven microenvironmental stress tolerance. Mechanistically, in a 3D-nutrient poor microenvironment, Galectin-3 by directly binding to integrin αvβ3 drives its clustering leading to KRAS recruitment at the plasma membrane favoring KRAS-mediated AKT signaling promoting macropinocytosis and redox balance, thereby allowing cell survival and proliferation despite diverse environmental stresses such as nutrient deprivation and hypoxia. Pharmacologic inhibition of Galectin-3 disrupts KRAS/integrin αvβ3 complex and decreases cancer cell survival in vivo in $KRAS^{G12D}$ mice and in patient-derived xenografts by inhibiting macropinocytosis and strongly inducing accumulation of cellular ROS (Seguin et al. 2017). However, whether

macropinocytosis is required to maintain redox has still to be investigated. The intra- and inter-heterogeneity of integrin αvβ3 expressions observed in KRAS-driven cancers could represent a biomarker to discriminate KRAS-addicted tumors and explain at least in part why cancer cells harboring oncogenic KRAS are heterogeneous in terms of macropinocytosis potential. *Altogether, these results suggest that KRAS drives a cancer cell/tumor microenvironment dialogue to promote cell adaption in harsh microenvironment.*

Integrin, a Key Mediator of KRAS-Driven Macropinocytosis

Integrins are highly diversified class of key extracellular matrix (ECM) adhesion receptors that sense microenvironmental changes and have a profound impact on cell ability to survive in specific locations by triggering a range of cellular responses such as cell adhesion, migration, proliferation, survival, and differentiation (Seguin et al. 2015). They consist of two distinct transmembrane subunits (α and β), which connect the intracellular cytoskeleton and the pericellular ECM. In mammals, 18 α and 8 β integrin subunits have been identified that combine to form 24 different heterodimers. Not only a given integrin can bind to multiple ligands, but also a single ligand can recognize multiple integrins, therefore, Spatio-temporal patterns of integrin versus ligand expression ultimately determine how a cell senses and responds to its environment (Moreno-Layseca et al. 2019). Integrins signal through the cell membrane in a bidirectional manner and the mechanisms involved in integrin activation have been extensively studied over the past few decades. It is now well established that integrin functions are regulated through multiple mechanisms including conformational changes, protein–protein interaction, and trafficking (Moreno-Layseca et al. 2019). Basically, the "inside out" signaling promotes integrin conformational change and controls its affinity for ECM ligands. The interaction between the integrin and its specific ligand triggers recruitment of protein complexes to the integrin cytoplasmic tails to promote integrin-mediated signaling pathways ("outside-in" signaling). It is now apparent that similar to several transmembrane receptors, numerous intracellular, extracellular, or transmembrane integrin binding partners' fine tunes integrin functions. Integrin signaling and adhesion dynamics are regulated through several endocytic routes including macropinocytosis (Moreno-Layseca et al. 2019). Indeed, during growth factor-induced cell migration, macropinocytosis promotes a fast spatial and temporal redistribution of β1 and β3 integrins from dorsal ruffles to ventral surface. (Gu et al. 2011) While it is well known that integrins are upregulated in cancer (Seguin et al. 2015) and that aberrant integrin functions trigger cancer progression and metastasis (Seguin et al. 2015), recent evidences have shown that integrin promotes cancer cell survival in harsh microenvironment including hypoxia, anchorage-independence, or nutrient-deprived microenvironment by inducing alternative survival pathways (Seguin et al. 2014; Seguin et al. 2017; Skuli et al. 2009; Cosset et al. 2017). Interestingly, a given integrin can trigger multiple cell survival

mechanisms. Indeed, in melanoma cells, hypoxia induces αvβ3 expressions leading to tumor cells adhesion and migration (Cowden Dahl et al. 2005), while in pancreatic and lung cancer anchorage independence or nutrient starvation promote integrin αvβ3-mediated macropinocytosis (Seguin et al. 2017), and in glioblastoma integrin αvβ3 drives cell survival under glucose deprivation through GLUT4 upregulation (Cosset et al. 2017). These results suggest that the diversity of integrin-mediated cell survival mechanisms is contextual and may depend on integrin binding partners. Among those, tertraspanin, syndecan, and galectin have been involved in several endocytotic processes including macropinocytosis.

- Tetraspanins are small, membrane-spanning proteins known to form tetraspanin-enriched microdomains at the plasma membrane that organize transmembrane proteins, such as integrins, immunoglobulin (Ig)-domain-containing proteins, growth factors, and cytokine receptors. They are involved in integrin-mediated through two main mechanisms. By organizing the plasma membrane, they bring multiple proteins to close proximity to the integrins and therefore modulating integrin-mediated cellular response. (Berditchevski 2001) For example, it has been shown that tetraspanin TM4SF promotes PKC recruitment to integrin β1 and influences cell adhesion and spreading. (Zhang et al. 2001a; Zhang et al. 2001b) In addition, multiple evidences have shown that tertaspanins interact with integrin to promote cell migration and metastasis by modulating integrin-dependent adhesion activities through their internalization (Berditchevski 2001; Boucheix and Rubinstein 2001; Serru et al. 1999; Hood and Cheresh 2002; Ivaska et al. 2002). Indeed, CD81 interacts with β1 integrin leading to the small GTPase RAC1 activation and membrane protrusion morphogenesis involved in macropinocytosis (Quast et al. 2011) suggesting the involvement of tetraspanins in the regulation of cytoskeletal actin dynamics (Detchokul et al. 2014).
- The syndecan family consists of four transmembrane heparan sulfate proteoglycans mainly present on the cell surface. While syndecans 1, 2, and 3 are tissue specific, syndecan 4 is ubiquitously expressed. Studies have shown that syndecans interact with integrins via their ectodomains to support focal-adhesion formation (Morgan et al. 2007). Two syndecans, syndecan 1 and syndecan 4, have been extensively studied over the past two decades, and differences in integrin association have been demonstrated (Morgan et al. 2007). Although both syndecans bind to integrin α2β1, αvβ3, and α6β4, syndecan 1 binds to αvβ5 and syndecan 4 to α5β1 and α6β1 specifically suggesting a cell response specificity depending on the microenvironment. Syndecans drive directional cell migration and focal adhesion dynamic by modulating integrin trafficking through spatio-temporal activation of the small GTPases Rac1, RhoA, RhoG, and Arf6. (Brooks et al. 2012) Indeed, syndecan-4-mediated Arf6 activation control differential recycling of αvβ3 and α5β1 integrins by activating α5β1 integrin recycling to the plasma membrane and suppressing αvβ3 trafficking, thus modulating focal-adhesion turnover and cell migration (Moreno-Layseca et al. 2019; Morgan et al. 2013). While syndecan-1 has been involved in integrins β1 and β3-mediated adhesion and migration regulation, a recent report has shown that in pancreatic

cancer, KRAS-mediated macropinocytosis requires Syndecan-1 recycling (Yao et al. 2019). Interestingly, oncogenic KRAS not only increases syndecan-1 expression but also integrin signaling with an upregulation of integrins β1, β3, and β4. However, the role of integrins within this context has still to be determined.

- Galectins, a family of β-galactoside-binding lectins influence tumor cell behavior by binding to carbohydrates on the extracellular domain of integrins and regulating their clustering. Several galectins have recently been identified to interact with integrins. Galectin-1, which promotes lung cancer metastasis by potentiating integrin α6β4 and Notch1/Jagged2 signaling and Galectin-3, which induces integrin β3-mediated anchorage-independence and drug resistance (Seguin et al. 2015). Recently, Galectin-3 has been described as an essential protein in multiple endocytic mechanisms. Not only Galectin-3 is involved in lysosome repair and removal, (Jia et al. 2020) it also plays a critical role in CD44 recycling via clathrin-independent endocytosis (Lakshminarayan et al. 2014) and in integrin-mediated macropinocytosis (Seguin et al. 2017).

  Altogether these results demonstrate that integrin partners may modulate integrin-mediated microenvironment sensing and cellular response highlighting the importance of the interplay between cancer cell and microenvironment (Fig. 10.2).

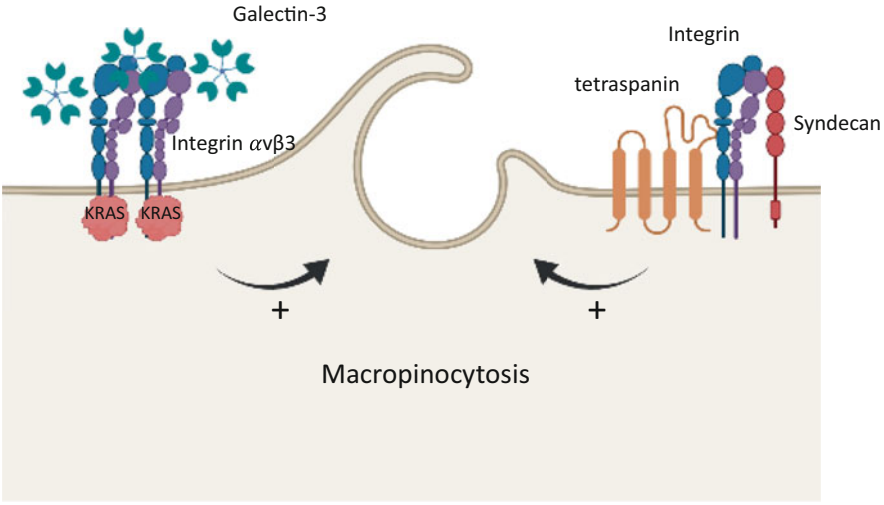

Fig. 10.2 Integrin partners are involved in macropinocytosis (Biorender)

Integrin Trafficking, a Modulator of Cell Metabolism

Energy balance is fundamental to maintain unrestricted tumor growth; thus cancer cells must adapt their metabolism to different environmental challenges. Two master regulators of cell growth are involved in cellular nutrient sensing and metabolism adaptation: the 5′-adenosine monophosphate-activated protein kinase (AMPK) and one of its downstream targets the mammalian target of rapamycin (mTOR). Sophisticated mechanisms including energy level, amino acid concentration, and oxygen level regulate the dynamic between AMPK and mTOR (Gleason et al. 2007). Basically, under nutrient-replete conditions, AMPK is inhibited leading to mTOR activation allowing cells to use free amino acid from transporters to maintain cellular energy demand for cell proliferation (Palm et al. 2017). In nutrient-poor conditions, AMPK is activated leading to mTOR inhibition and activation of alternative energy sources and more particularly macropinocytosis to promote not only cell survival but also unrestricted cell proliferation (Vara-Ciruelos et al. 2019). As integrins are central players in microenvironment sensing, integrin functions are highly regulated through metabolic cues (Ata and Antonescu 2017). Indeed, crosstalk of integrin and AMPK/mTOR signaling is essential to promote cell proliferation. While ligand-bound integrin induces mTOR activation and cell proliferation under nutrient-supplied condition (Moreno-Layseca et al. 2019), a withdrawal in nutrients activates AMPK that in turn inhibits mTOR (Gleason et al. 2007; Vara-Ciruelos et al. 2019). AMPK activation increases ligand-bound integrin internalization through macropinocytosis and inhibits its recycling in favor of its degradation. The amino acids released activate the mTOR pathway to promote tumor growth. Interestingly, this phenomenon is not unique to cancer cells as it has been shown recently that breast normal cells under growth factor or serum starvation and mTOR inhibition, uptake soluble laminin through β4 integrin-mediated macropinocytosis, which in turn returns mTORC1 activity to avoid excess uptake of extracellular proteins (Muranen et al. 2017). Those results have been validated in vivo where mammary epithelial cells in dietary restricted mice increase laminin uptake from the extracellular matrix (Muranen et al. 2017). Altogether, these results suggest that ligand-bound integrin internalization is controlled by nutrient availability and that the uptake of ECM components, regulated by integrin trafficking under nutrient-deficient conditions could provide a source of nutrients for cells. To promote unconstraint cell proliferation oncogenic KRAS has been shown to activate mTOR pathway (Rodriguez-Viciana et al. 1994). However, under nutrient-starved conditions, oncogenic KRAS bypass mTOR inhibition and associated cell death by driving AMPK activation and macropinocytosis to promote cell survival and proliferation (Palm et al. 2015). In fact, macropinocytosis promotes mTOR inhibition resistance in a subset of pancreatic tumor cells (Michalopoulou et al. 2020). Several stress conditions can activate AMPK such as an increased in intracellular calcium (Vara-Ciruelos et al. 2019; Hawley et al. 2005; Woods et al. 2005; Zhang et al. 2017) or a glucose starvation (Zhang et al. 2017), two conditions that favor KRAS-addicted cell survival. Interestingly, AMPK is critical for KRAS-driven lung cancer

development through the induction of lysosomes (Eichner et al. 2019). Those results reflect a dependence on AMPK for survival only in cancer cells that are metabolically compromised and energetically stressed. Altogether, these results demonstrate that cancer cells sense their microenvironment and adapt to nutrient scarcity.

Cancer cells face a challenge to sustain uncontrolled proliferation under conditions of limited nutrient availability. While KRAS functions have been extensively studied, this oncogene has emerged as a key parameter in supporting tumor growth under nutrient stress microenvironment through several metabolic pathways allowing cancer cell metabolic reprogramming and promoting a pro-tumorigenic microenvironment. Not all cancer cells use the same energy source to maintain a high rate of proliferation, in fact, depending on tissue of origin, cancer cells will favor specific nutrient utilization, therefore, representing a specific vulnerability (Mayers et al. 2016). By modulating macropinocytosis, oncogenic KRAS abrogates this metabolic dependency and encourages an opportunistic uptake of unspecific extracellular nutrients providing a survival advantage of KRAS-addicted tumor cells. This adaptation to the scarcity of the microenvironment requires a dialogue between cancer cells and the surrounding stroma through transmembrane receptors and more particularly integrins. Fine-tuning of this cancer cell/microenvironment communication is orchestrated by integrin's partners, thus, their differential expression may define at least in part why some cells are addicted to KRAS to survive in nutrient-poor microenvironments.

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Chapter 11
KRAS^{G12R}-Independent Macropinocytosis in Pancreatic Cancer

G. Aaron Hobbs and Channing J. Der

Abstract Macropinocytosis is a critical route of nutrient acquisition in pancreatic cancer cells. Constitutive macropinocytosis is promoted by mutant KRAS, which activates the PI3Kα lipid kinase and RAC1, to drive membrane ruffling, macropinosome uptake and processing. However, our recent study on the KRAS^{G12R} mutant indicated the presence of a KRAS-independent mode of macropinocytosis in pancreatic cancer cell lines, thereby increasing the complexity of this process. We found that *KRAS^{G12R}*-mutant cell lines promote macropinocytosis independent of KRAS activity using PI3Kγ and RAC1, highlighting the convergence of regulation on RAC signaling. While macropinocytosis has been proposed to be a therapeutic target for the treatment of pancreatic cancer, our studies have underscored how little we understand about the activation and regulation of this metabolic process. Therefore, this review seeks to highlight the differences in macropinocytosis regulation in the two cellular subtypes while also highlighting the features that make the KRAS^{G12R} mutant atypical.

Keywords KRAS · Macropinocytosis · Mutant-specific signaling · PI3K · Pancreatic cancer · Metabolism

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Introduction

Macropinocytosis is a regulated form of endocytosis that mediates the nonselective uptake of solute molecules, nutrients, and antigens. Originally described in 1931, macropinocytosis is a process often associated with dendritic cells, where internalized antigens are presented by the major histocompatibility complexes (Lewis 1931; Norbury et al. 1995). However, constitutively active mutants of RAS and RAC1 small GTPases can promote macropinocytosis in non-dendritic cells (Bar-Sagi and Feramisco 1986; Ridley et al. 1992), where the SRC tyrosine and PI3K lipid kinases are activated to promote macropinocytosis (Veithen et al. 1998). Once activated, PI3K promotes the activity of RAC1, which promotes membrane ruffling and macropinocytosis (Kotani et al. 1995; Nobes et al. 1995). As PI3K is a downstream effector of RAS, these data cemented RAC1 as the terminal node in RAS-PI3K-RACGEF-RAC1-mediated constitutive macropinocytosis.

In the decades that followed the initial discovery of RAS-dependent macropinocytosis, there has been considerable effort to define the signaling machinery that promotes KRAS-dependent macropinocytosis. As such, numerous additional RAS effectors have been shown to be necessary for RAS-mediated macropinocytosis (Hobbs and Der 2020; Recouvreux and Commisso 2017). However, to our surprise, we observed that one specific KRAS-mutant protein, harboring a glycine-to-arginine substitution at codon 12 ($KRAS^{G12R}$), was uniquely defective in promoting KRAS-dependent constitutive macropinocytosis (Hobbs et al. 2020). Despite the inability of $KRAS^{G12R}$ to promote macropinocytosis, $KRAS^{G12R}$-mutant PDAC cell lines displayed robust levels of macropinocytosis. Therefore, this review highlights the current understanding of KRAS-dependent constitutive macropinocytosis and describes how the $KRAS^{G12R}$ mutant uncovered evidence of a novel mechanism of KRAS-independent constitutive macropinocytosis.

KRAS-Dependent Constitutive Macropinocytosis in Pancreatic Cancer

The early mechanistic studies on macropinocytosis regulation described RAS as a key protein for promoting macropinocytosis in fibroblasts and other model cell systems (Bar-Sagi and Feramisco 1986; Ridley et al. 1992). Later, constitutive macropinocytosis was shown to be a key source of macronutrients in pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer (Commisso et al. 2013; Commisso et al. 2014).

Mutational activation of KRAS is found in over 95% of PDAC patients (Prior et al. 2020). Utilizing $KRAS^{G12C}$- or $KRAS^{G12V}$-mutant pancreatic cancer cell lines, macropinocytosis was determined to be dependent on KRAS protein expression (Commisso et al. 2013). Additionally, Commisso *et al* determined that the internalized proteins were degraded and glutamine, not glucose, fueled the citric acid cycle

in this context. As glutamine deprivation in PDAC cell lines promotes macropinocytosis (Lee et al. 2019), and glutamine has been described as a key metabolite in the tricarboxylic acid cycle (Hui et al. 2017), constitutive macropinocytosis appears to overcome the low levels of glutamine in PDAC tumors. Finally, KRAS-mutant tumor growth was reduced when inhibiting macropinocytosis using a nonselective inhibitor of macropinocytosis (ethylisopropyl amiloride; EIPA) in mouse xenograft models. Importantly, the use of BxPC-3 PDAC cells, which are *KRAS*^{WT} but *BRAF*-mutant (Chen et al. 2016), did not undergo measurable constitutive macropinocytosis and tumor xenografts were unresponsive to EIPA treatment (Commisso et al. 2013). These studies provided strong evidence that targeting KRAS-driven macropinocytosis could be an effective therapeutic strategy in pancreatic cancer.

Several recent studies have provided additional details into the increasingly complex mechanism of mutant KRAS-dependent macropinocytosis. The sodium bicarbonate-coupled transporter channel protein SLC4A7 has been implicated as a key driver of macropinocytosis (Ramirez et al. 2019). The expression of SLC4A7 is increased by mutant KRAS signaling. SLC4A7 indirectly activates soluble adenylyl cyclase (sAC), which is activated by bicarbonate, and activated sAC activates protein kinase A (PKA). This signaling pathway promotes cholesterol accumulation at the membrane via the activation of the vacuolar ATPase (V-ATPase) and leads to RAC1 activation (Ramirez et al. 2019). While RAC1 has been previously shown to promote macropinocytosis (Ridley et al. 1992), this report provided an alternative mechanism for KRAS-mediated RAC1 localization and activation. Previously, RAS-mediated RAC1 activation via PI3K and the RAC guanine nucleotide exchange factor (GEF) TIAM1 had been described in fibroblasts (Lambert et al. 2002), although it is unclear whether this pathway plays a direct role in pancreas cells.

Separately, mutant-KRAS activity was shown to promote the expression of pleckstrin and Sec7 domain-containing 4 (PSD4), an ADP-ribosylation factor (ARF) GEF. PSD4-activated ARF6 promotes membrane recycling and endocytosis (Prigent et al. 2003) and increases the membrane localization of syndecan 1 (SDC1), a transmembrane proteoglycan that modulates growth factor binding, among other roles (Szatmari and Dobra 2013). At the plasma membrane, SDC1 can recruit and activate RAC1 (Yao et al. 2019). Finally, a third study implicated RIN1 as a KRAS effector necessary for macropinocytosis. RIN1 is a GEF for the endocytosis regulating GTPase RAB5, which was also determined to be necessary for KRAS-dependent macropinocytosis (Kelly et al. 2020). Activated RAB5 has been shown to activate RAC1 in some contexts (Sandri et al. 2012). While these studies highlight the multitude of signals necessary to promote constitutive macropinocytosis by mutant KRAS, the activation of RAC1 at the plasma membrane is a common factor in all of these studies (reviewed in Hobbs and Der 2020). However, due to the specifics of each study, it is unclear whether these pathways represent multiple possible mechanisms or whether they are part of the same overall mechanism that regulates macropinocytosis.

While these studies demonstrated that mutant KRAS can promote constitutive macropinocytosis in PDAC, our studies on $KRAS^{G12R}$ indicated that a KRAS-independent mechanism of macropinocytosis also exists. In our study highlighting macropinocytosis in PDAC cell lines harboring the atypical $KRAS^{G12R}$ mutation, which is the third most common *KRAS* mutation in PDAC, ablation of KRAS had no effect on constitutive macropinocytosis (Hobbs et al. 2020). In the context of $KRAS^{G12R}$, PI3Kγ, an isoform that is primarily expressed in hematopoietic cells but is also expressed in acinar cells of the pancreas (Lupia et al. 2004), significantly contributed to maintaining constitutive macropinocytosis. However, in agreement with Ramirez *et al* and Yao *et al*, genetic silencing of *RAC1* reduced macropinocytosis in $KRAS^{G12R}$ PDAC, highlighting the convergence on RAC signaling for macropinocytosis.

Finally, the transcriptional coactivators Yap/Taz have been implicated in promoting macropinocytosis in *Kras*-mutant murine PDAC cells. By removing the essential amino acid leucine from the culture medium, Yap/Taz translocated to the nucleus and promoted the expression of the TAM (Tyro-Axl-Mer) receptor tyrosine kinase Axl. Axl promoted macropinocytosis via activation of PI3K signaling (King et al. 2020). Here, the role of Kras activity in promoting macropinocytosis was not explicit because inducing the nuclear localization of YAP or TAZ was sufficient to promote macropinocytosis in the $KRAS^{WT}$ BRAF-mutant BxPC-3 PDAC cell line, suggesting that this mode of macropinocytosis may be KRAS independent. In agreement, addition of platelet-derived growth factor (PDGF) to mouse embryonic fibroblasts (MEFs) deficient in all Ras genes (RAS-less MEFs) also induced macropinocytosis, suggesting a mechanism that does not require RAS function (Palm et al. 2017). Conversely, KRAS expression was necessary to promote the EGFR-dependent upregulation of macropinocytosis after glutamine deprivation, which was termed inducible macropinocytosis (Lee and Commisso 2020; Lee et al. 2019). Taken together, these studies suggest that there are at least two distinct mechanisms of constitutive macropinocytosis, KRAS-dependent and KRAS-independent macropinocytosis.

The Atypical $KRAS^{G12R}$ Mutant Has Disrupted Regulator Interactions

Of the six possible single-base nucleotide substitutions that are possible at codon 12 in *KRAS*, the $KRAS^{G12R}$ mutation is the least common in cancer, accounting for just ~1.5% of all *KRAS* mutations (Prior et al. 2020). Yet despite being rare overall, $KRAS^{G12R}$ is the third most prevalent *KRAS* mutation in PDAC (16.7%) (Fig. 11.1a and b). Further, this mutant is enriched in PDAC relative to the general mutation frequencies found in PDAC. The predicted mutational frequency of the $KRAS^{G12R}$ substitution in PDAC is 5.2%, and the three-fold higher actual occurrence provides support that key biological properties of this mutant favor its role as a potent cancer

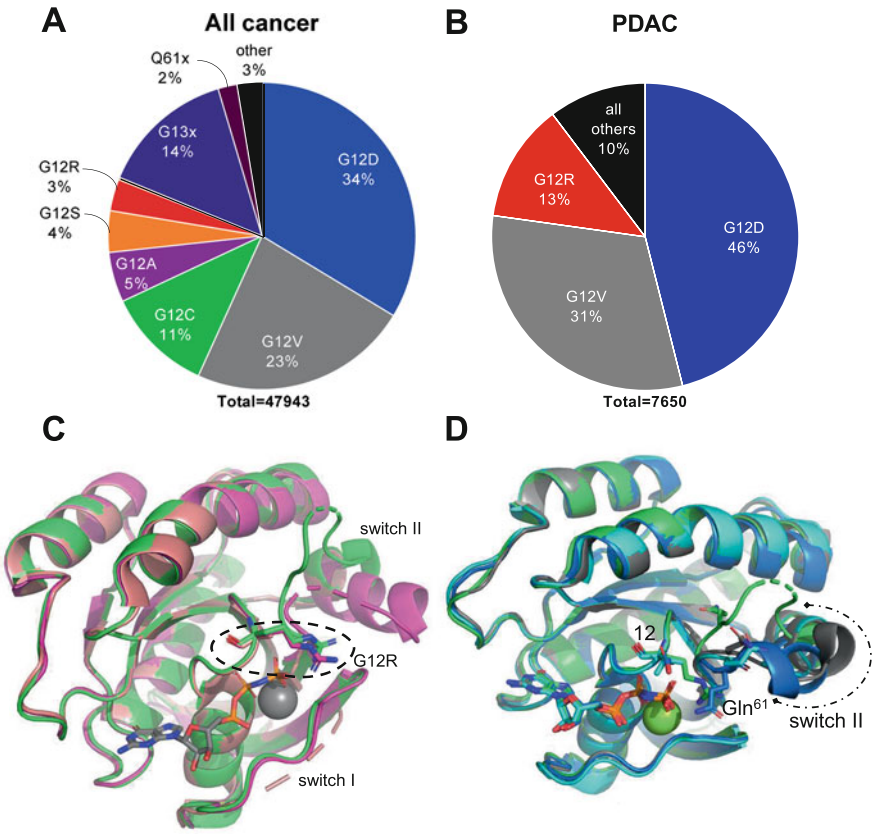


Fig. 11.1 The pancreatic cancer-associated mutant KRAS^{G12R} is atypical. (**a**) The relative frequency of KRAS mutations in cancer. Other represents nearly 150 different unique mutations in KRAS that occur at low frequency. (**b**) The KRAS mutation frequency in PDAC. Data compiled from COSMIC v.94. (**c**) Cartoon overlay of the three molecules in the asymmetric unit from the KRAS^{G12R} crystal structure (PDB: 6CU6). Each color represents a different molecule in the asymmetric unit. Switch I and switch II are indicated, and G12R is circled and shown in sticks. (**d**) A cartoon overlay of the crystal structures of multiple KRAS proteins. Gray, wildtype; blue, KRAS^{G12D} (5USJ), cyan, KRAS^{G12V} (6GOE), green, KRAS^{G12R} (6CU6). The sidechains for codon 12 and Gln^{61} are indicated and switch II is circled. All structures are bound to the GMPPNP analog, and Mg^{2+} is shown as a green sphere

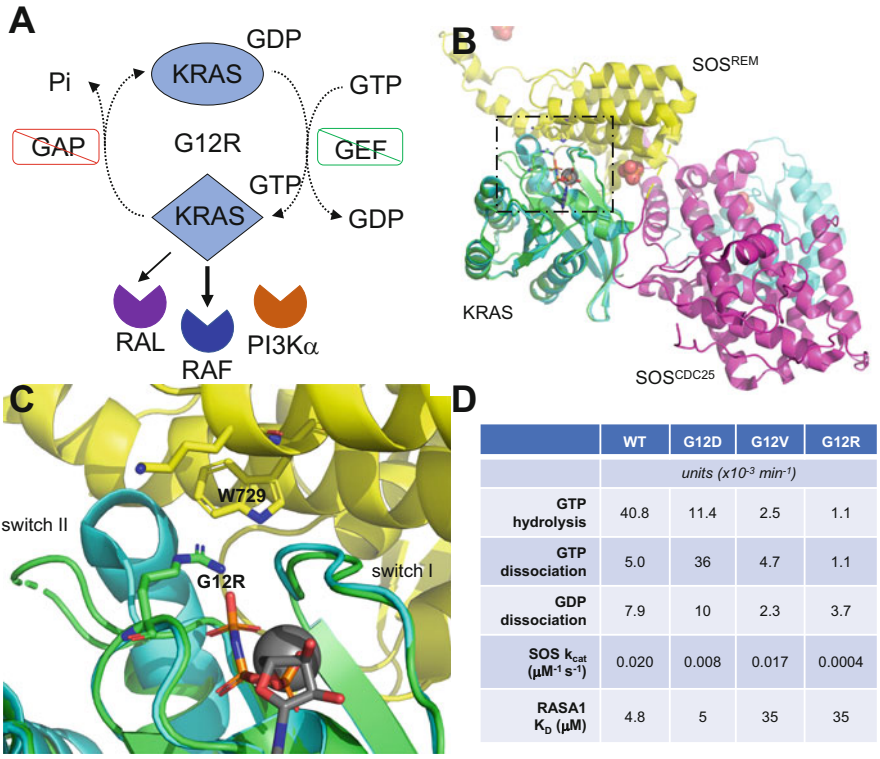
driver in PDAC (Cook et al. 2021). There is now increasing appreciation that different RAS-mutant proteins may have distinct structural, biochemical, and cellular properties, with KRAS^{G12R} standing out as the most unusual codon 12 mutant (Cancer Genome Atlas Research Network. Electronic address & Cancer Genome Atlas Research 2017).

One distinct feature of KRAS^{G12R} is the protein structure. Of the KRAS codon 12 mutants with structural data, only KRAS^{G12R} induces significant structural perturbations. Importantly, there is considerable variability in the three KRAS^{G12R}

molecules within the asymmetric unit, indicative of increased motility in these regions (Fig. 11.1c). In these structures, the helix of switch II, a motif important for regulator and effector binding, shows two possible orientations and residues 60-64 are either not modeled or show significant variability. Part of the rationale for the altered structure of $KRAS^{G12R}$ lies within the size of the arginine side chain (Fig. 11.1d). The arginine side chain folds into the GTP binding pocket of RAS and forms a salt-bridge with the γ phosphate of GTP (Hobbs et al. 2020; Krengel et al. 1990), displacing Gln^{61} from its typical interaction with the γ phosphate. As shown in Fig. 11.1d, the mutated amino acids in $KRAS^{G12D}$ and $KRAS^{G12V}$ are simply smaller and do not interfere with the placement of Gln^{61}, which allows for switch II to form an extended alpha helix. Despite showing some structural variability within the molecules within the asymmetric unit for $KRAS^{G12D}$, switch II is generally more ordered in $KRAS^{G12D}$ compared to $KRAS^{G12R}$.

One consequence of this structural perturbation is the loss of interaction with the RAS GEF SOS1. RAS-specific GEFs stimulate the formation of active GTP-bound KRAS. Consequently, $KRAS^{G12R}$ has the slowest intrinsic GTP hydrolysis and GTP exchange rates of all Gly^{12} mutants (Fig. 11.2a) (Hunter et al. 2015; Krengel et al. 1990). Similar to other codon 12 mutants, $KRAS^{G12R}$ is insensitive to GAP regulation (Hunter et al. 2015; Krengel et al. 1990). However, SOS1 cannot induce nucleotide exchange in $KRAS^{G12R}$ (Hobbs et al. 2020). SOS has two binding sites for RAS molecules, the catalytic site where GDP dissociation is promoted, and the allosteric site where RAS binding is necessary for SOS activity (Margarit et al. 2003). Surprisingly, this exchange defect was observed independent of whether $KRAS^{G12R}$ was GDP- or GTP-bound, possibly due to a charge–charge repulsion between the $KRAS^{G12R}$ sidechain and a lysine residue (aa 728) or a steric clash with a tryptophan reside (aa 729) in SOS (modeled in Fig. 11.2b). Additionally, the disruption of switch II of $KRAS^{G12R}$ (aa 60-76) likely fails to contact key regions within the SOS REM domain (Fig. 11.2c) (Margarit et al. 2003). Together, these disruptions likely account for the inability of SOS to bind to $KRAS^{G12R}$. While $KRAS^{G12R}$ is insensitive to SOS1-mediated exchange, $KRAS^{G12R}$ remains acutely sensitive to the GEF RASGRP1 (Hobbs et al. 2020), suggesting that $KRAS^{G12R}$ utilizes a unique complement of GEFs compared to other KRAS-mutant proteins. However, whether these GEFs regulate KRAS activity in PDAC has not been studied. Finally, a summary of $KRAS^{G12R}$-mediated signaling is presented in Fig. 11.2c, highlighting the insensitivity to GEF and GAP regulation as well as the inability of $KRAS^{G12R}$ to promote PI3Kα activation. The inability to bind to both PI3Kα and SOS1 confirms the structural perturbation in switch II, as observed in the crystal structures of $KRAS^{G12R}$.

This GEF-related defect implies two possibilities: (1) The reliance of $KRAS^{G12R}$ on RASGRP1, which is generally found in blood cells (Dower et al. 2000; Ebinu et al. 1998) but has also been detected in the islet cells of the pancreas (Taneera et al. 2012), may result in differential localization for active $KRAS^{G12R}$ compared to other KRAS mutants, or (2) SOS1 is not necessary for $KRAS^{G12R}$-mutant PDAC. In agreement with the latter possibility, an allele-specific genetic dependency analysis of PDAC showed that only $KRAS^{G12R}$-mutant PDAC had a reduced dependency for



	WT	G12D	G12V	G12R
	units ($x10^{-3}$ min^{-1})			
GTP hydrolysis	40.8	11.4	2.5	1.1
GTP dissociation	5.0	36	4.7	1.1
GDP dissociation	7.9	10	2.3	3.7
SOS k_{cat} (μM^{-1} s^{-1})	0.020	0.008	0.017	0.0004
RASA1 K_D (μM)	4.8	5	35	35

Fig. 11.2 KRAS^{G12R} is insensitive to GEF-mediated activation and displays allele-specific effector signaling. (**a**) Schematic of KRAS^{G12R} signaling in cells. KRAS^{G12R} is insensitive to GEF- and GAP-mediated regulation and cannot interact with PI3Kα, a key RAS effector. (**b**) The KRAS^{G12R} crystal structure (green) was modeled onto the allosteric RAS molecule (cyan) in the RAS:SOS: RAS crystal structure (SOS^{REM} in yellow, CDC25 domain in magenta). (**c**) Enlarged view of the modeled interaction of RAS^{G12R} with SOS^{REM} domain. In the RAS^{WT}:SOS interaction, RAS switch II interacts with SOS W729, pressing SOS into an activated structure, which promotes exchange in the catalytic RAS molecule. Due to potential steric clashes and/or the disruption of switch II, KRAS^{G12R} cannot activate SOS. (**d**) The biochemical kinetics and binding affinities, including nucleotide dissociation, hydrolysis, GEF-mediated activation and GAP binding affinities, for the most common KRAS mutants in PDAC

GRB2, a protein that connects EGFR signaling to SOS1 (Cook et al. 2021). However, direct inhibition of SOS1 in a variety of cell types, including PDAC and KRAS-mutant colorectal cancer cells, supports a role for SOS1 in RAS-mutant cancers (Hofmann et al. 2021). Hofmann et al generated a novel SOS1 direct inhibitor, which functions by preventing SOS1-mediated RAS activation. The SOS1 inhibitor successfully reduced ERK1/2 phosphorylation as well as cellular transformation in many *RAS*-mutant cell lines. Unsurprisingly, ERK phosphorylation and cellular proliferation were resistant to the BI-3406 SOS inhibitor in the KRAS^{G12R}-expressing cell line. Additionally, isogenic cell lines expressing KRAS^{G13D} and KRAS^{Q61H} were less sensitive to BI-3406, indicating that the

therapeutic benefit of SOS inhibitors may be limited to a subset of KRAS mutations in human cancers. Combined, these observations indicate that SOS1 activity likely plays a role in promoting tumorigenesis in many, but not all, *KRAS*-mutant settings.

$KRAS^{G12R}$ Cannot Bind to p110α and Fails to Promote Macropinocytosis

Another defining feature of $KRAS^{G12R}$ is that it cannot bind to PI3Kα. As such, the finding that $KRAS^{G12R}$ cannot bind PI3Kα, yet is prevalent in PDAC, is perplexing given the studies by Downward and colleagues, who showed that disrupting the ability of KRAS to bind PI3Kα abolished the ability of mutant KRAS to initiate and maintain lung cancer growth, angiogenesis, and metastasis (Gupta et al. 2007; Murillo et al. 2018). Further, Crawford and colleagues found that PI3Kα, but not PI3Kβ, and RAC1 were required for KRAS-mediated pancreatic cancer development in mouse models (Wu et al. 2014).

Due to the inability of $KRAS^{G12R}$ to promote PI3Kα activation, we proposed that $KRAS^{G12R}$ PDAC would display increased sensitivity to MEK/ERK MAPK inhibitors (Hobbs et al. 2020), in part because PI3K activation is a common mechanism to overcome ERK MAPK inhibition (Kun et al. 2021; Wee et al. 2009). Further, PI3K combinations with MEK/ERK inhibitors have proven effective in treatment models but lead to adverse events in the clinic due to the therapeutic toxicity of the combination (Bardia et al. 2020). We surmised that because $KRAS^{G12R}$ was not activating PI3Kα, this cohort would be uniquely susceptible to MEK/ERK monotherapies. While patient-derived xenograft mouse models were responsive to MEK single-agent inhibition, a panel of human organoid cultures did not support MEK/ERK inhibition as a mutation-selective monotherapy (Hobbs et al. 2020). Disappointingly, a phase 2 clinical trial utilizing selumetinib, a MEK inhibitor, showed no significant benefits in progression or survival for the $KRAS^{G12R}$-patient population (Kenney et al. 2021). This study concluded that MEK MAPK monotherapy was unlikely to be successful in this cohort and additional inhibitors would be necessary to achieve meaningful therapeutic benefits.

Humans express four distinct classes of PI3Ks, and class I PI3Ks are effectors of RAS GTPases (Castellano and Downward 2011). Class I PI3K lipid kinases are divided into two subclasses, class IA consists of the p110α, p110β, and p110δ catalytic subunits and class IB consists of the p110γ subunit. Each subclass forms heterodimeric complexes with distinct regulator proteins (class IA, p85; class IB, p101) that stabilize the kinase and regulate localization (Fritsch and Downward 2013). GTP-bound RAS can directly bind with PI3Kα, δ and γ through association with their RAS-binding domains (RBD) (Fritsch et al. 2013). In contrast, PI3Kβ serves as a RAC1 effector (Fritsch et al. 2013). While the four class I PI3K isoforms share the same enzymatic function (Guillermet-Guibert et al. 2008), catalyzing the conversion of phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) into

phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P_3), they are considered distinct in part due to their tissue-specific expression; PI3Kα and PI3Kβ are ubiquitously expressed, while PI3Kδ and PI3Kγ are generally limited to blood and immune cells (Tassi et al. 2007).

While normal pancreas expresses PI3Kα and PI3Kβ, pancreatic cancers have been reported to express all four PI3K isoforms (Baer et al. 2014). Therefore, it is possible that *KRAS^{G12R}*-mutant PDAC compensates for the loss of direct KRAS: PI3Kα binding by the concomitant expression and activation of other PI3K isoforms. In contrast, a tumor-sequencing study reported that *KRAS^{G12R}*-mutant PDAC had a significantly higher prevalence of PI3K pathway mutations compared to other KRAS-mutant tumors (Diehl et al. 2021). However, it is unclear whether the PI3K isoform-specific functions are retained in the cancer setting (Rodon et al. 2013). In support of isoform-specific functions, we showed that KRAS^{G12R}-mutant cells only utilize PI3Kγ for macropinocytosis, and macropinocytosis was insensitive to PI3Kα inhibition in KRAS^{G12R} PDAC cell lines. Further, genetic loss of KRAS^{G12R} expression had no bearing on the overall macropinocytosis levels, indicating a potential mechanism for KRAS-independent macropinocytosis (Hobbs et al. 2020). In agreement with these data, PDGF-induced macropinocytosis in RAS-less MEFs was inhibited by the addition of a pan-PI3K inhibitor, indicating that PI3K signaling alone can promote macropinocytosis independent of KRAS function under certain conditions (Palm et al. 2017). Recently, an additional KRAS-independent macropinocytosis mechanism was reported, which utilized the nutrient sensor AMP-activated protein kinase (AMPK) to promote RAC1 activity, and was triggered by Ca^{2+} signaling when glutamine availability was low (Zhang et al. 2021). While this mechanism was reported in pancreatic cancer-associated fibroblasts, the possibility remains that this mechanism may promote macropinocytosis in other contexts. These studies highlight multiple potential mechanisms for KRAS^{G12R} to overcome its inability to activate PI3Kα.

However, KRAS^{G12D} and KRAS^{G12V}-mutant cell lines appear to utilize multiple PI3K isoforms for macropinocytosis in PDAC, with at least some PI3K isoforms controlled by KRAS function (Hobbs et al. 2020). In our study, the simultaneous genetic ablation of KRAS^{G12D} concurrent with exogenous expression of KRAS^{G12R} failed to rescue macropinocytosis levels (Hobbs et al. 2020). In the context of naturally occurring KRAS^{G12D} PDAC, KRAS-mediated activation of PI3Kα appears necessary for macropinocytosis. These data suggest that the PI3K isoforms have nonredundant mechanisms for promoting RAC1 activation and macropinocytosis in some contexts. However, whether RAC1 is directly activated by PI3Kγ in PDAC and how PI3Kγ is being regulated if not by KRAS^{G12R} are questions that are still unanswered.

While the exact mechanism is unknown, it is clear that macropinocytosis is differently activated in *KRAS^{G12R}*-mutant cell lines. The early studies formally linked mutant RAS activity to macropinocytosis via PI3K and RAC1 activity, and additional studies have added roles for SLC4A7, SDC1, and RIN1. These studies have added increasing depth and complexity to macropinocytosis signaling (Swanson and Yoshida 2019) and have centered on KRAS$^{G12D/V}$-mutant PDAC.

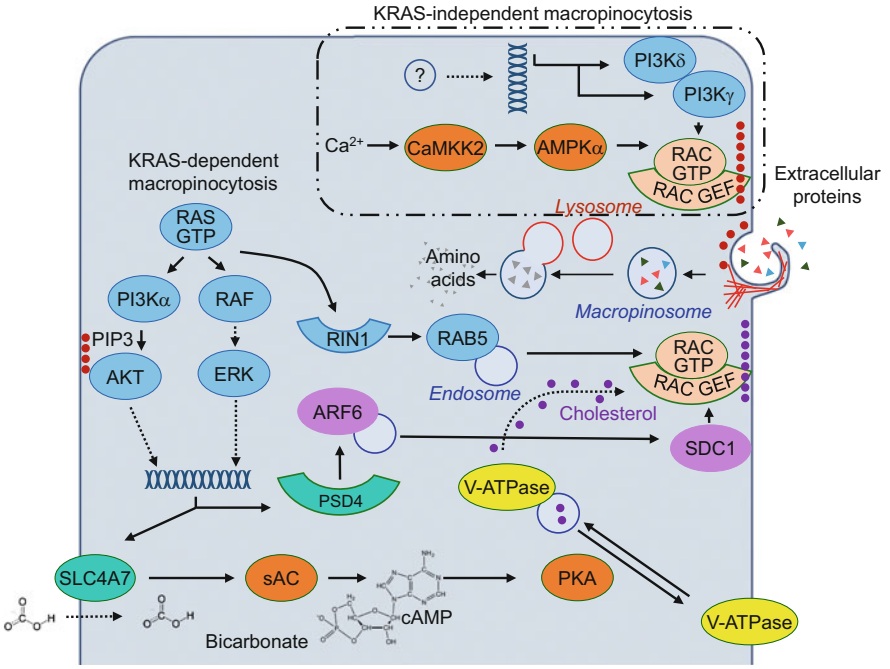


Fig. 11.3 Diagram of the multiple pathways proposed to regulate macropinocytosis in pancreatic cancer. Two main mechanisms of macropinocytosis have been proposed, KRAS-dependent and KRAS-independent macropinocytosis. While each KRAS-dependent macropinocytosis proposal has provided a role for intracellular endosomes, it is unclear if there is a convergence on the same endosome or multiple unique endosomes are required to promote a coordinated response. For KRAS-independent macropinocytosis, it is likely that many additional proteins are involved that coordinate the complex processes that result in macropinocytosis

However, $KRAS^{G12R}$-mutant proteins cannot activate PI3Kα and instead rely on KRAS-independent activity of PI3Kγ alone to promote macropinocytosis, effectively revealing a novel KRAS-independent pathway for the activation of macropinocytosis. The different modes of macropinocytosis are summarized in Fig. 11.3. The unique reliance of $KRAS^{G12R}$ PDAC on PI3Kγ leaves open the possibility that these patients may be susceptible to isoform-specific PI3K therapies given the limited expression of the PI3Kγ and PI3Kδ isoforms in human tissue.

The (Non)redundant Role of PI3K Isoforms in $KRAS^{G12R}$ Macropinocytosis

That PI3K isoforms can have nonredundant functions is not without precedent. The model organism *Dictyostelium discoideum*, which has been used to extensively study macropinocytosis (Zhou et al. 1998), utilizes multiple PI3K isoforms to

promote macropinocytosis. *Dictyostelium* is a model amoeba organism that utilizes macropinocytosis for fluid uptake and nutrient absorption (Bloomfield and Kay 2016). *Dictyostelium* have five PI3K isoforms that appear to play isoform-specific roles in $PI(3,4,5)P_3$ production, macropinocytosis, proliferation, and actin dynamics (Eichinger et al. 2005). In this model system, PI3K1/2 were shown to generate patches of $PI(3,4,5)P_3$ at the plasma membrane, which promoted membrane ruffling. Genetic ablation of *PI3K1/2* resulted in only ~20% total $PI(3,4,5)P_3$ production, a significant decrease in membrane ruffling and a loss of macropinocytosis. However, genetic ablation of PI3K4 showed similar levels of membrane ruffling and overall $PI(3,4,5)P_3$ production but significantly decreased macropinocytosis (Hoeller et al. 2013). Loss of PI3K3 or PI3K5 individually had minimal effects on total $PI(3,4,5)P_3$ levels or macropinocytosis. Taken together, at least in *Dictyostelium*, the PI3K isoforms have nonredundant functions in the context of $PI(3,4,5)P_3$ production and signaling.

While the roles of these additional PI3K isoforms in PDAC are unclear, overexpression of PI3Kδ/γ is clearly a feature of PDAC. The mechanisms that promote upregulation of these isoforms in PDAC and what role they play in tumorigenesis and proliferation, if any, are currently unknown. While PI3Kγ has been shown to play a role in promoting macropinocytosis, macropinocytosis has been observed in other cancers as well, including prostate and breast cancers (Koumakpayi et al. 2011; Reif et al. 2016). Additionally, a subset of lung cancers can utilize macropinocytosis to promote proliferation in the absence of glucose. In this setting, macropinocytosis was regulated by a PI3K-RAC-PAK signaling nexus and macropinocytosis was inhibited by a panPI3K inhibitor (Hodakoski et al. 2019). Interestingly, PI3Kγ overexpression in prostate cancer is associated with disease progression and metastasis (Chung et al. 2020). Thus, it is tempting to overstate the importance of the expression of the dendritic PI3K isoforms in cancer; however, more investigation is necessary on the role of PI3K isoform-specific functions in cancer. Specifically, whether the cancers that overexpress the PI3Kδ/γ isoforms are sensitive to PI3K isoform-specific therapeutic intervention would be of keen interest to researchers and clinicians alike.

Tying it all Together

The role of macropinocytosis in supporting the metabolic needs of cancer cells has been established (Michalopoulou et al. 2016), and PI3K is clearly a key player in this pathway (Veltman et al. 2016). However, many of the studies connecting RAS and PI3K function to macropinocytosis have only been considered in the context of $HRAS^{G12V}$ or $KRAS^{G12D}$ cell lines or mice models (Bilanges et al. 2019; Swanson and King 2019). Additionally, there are few studies that have evaluated the potential role for the additional PI3K isoforms, particularly in pancreatic cancer but in other solid tumors as well. While studies in *Dictyostelium* have provided evidence for nonredundant roles for multiple PI3K isoforms, it is not readily apparent how the

five class I PI3K isoforms in *Dictyostelium* represent the four class I isoforms in human tissue. Further, the expression of PI3Kδ/γ in PDAC was not expected and a role for these isoforms in this tissue is not clear.

It is clear that at least two distinct mechanisms of macropinocytosis are possible (Fig. 11.3). Combining the many studies highlighting KRAS-dependent macropinocytosis indicates numerous similarities in this mode of regulation. Namely, activation of the MEK/ERK MAPK signaling promotes the expression of at least two proteins that coordinate with the endosomal pathways to promote RAC1 activation at the plasma membrane. Further, all KRAS-dependent macropinocytosis studies have implicated endosomal function in regulating this process, whether these proteins are all on the same endosome or unique endosomes is unknown. Finally these studies do not explicitly define the RAC GEF that promotes RAC1 activity.

KRAS-independent macropinocytosis is equally understudied. While we have shown that PI3Kγ is necessary, the role of PI3Kδ overexpression is unclear. Further, RAC1 activity is necessary for KRAS-independent macropinocytosis, yet the RAC GEF is also unclear in this context. It is possible that both mechanisms converge on the same RAC GEF, and a recent study detailing macropinocytosis in cancer-associated fibroblasts suggested that ARHGEF2 was able to promote RAC1-mediated macropinocytosis (Zhang et al. 2021). However, this study did not examine the role of PI3K signaling in the promotion of macropinocytosis.

Whether the PI3K isoforms retain nonredundant functions in a disease setting is unknown, although evidence suggests they may have overlapping roles in some contexts. The studies on $KRAS^{G12R}$, which fails to activate PI3Kα, provide a unique framework to study the signaling and redundancy of the PI3K isoforms, potentially alluding to a mutation-specific therapeutic vulnerability. However, a recent cancer genome sequencing study found that $KRAS^{G12R}$-mutant PDAC had a greater incidence of PI3Kα pathway mutations (Diehl et al. 2021). Thus, the distinct possibility that $KRAS^{G12R}$ overcomes its lack of direct PI3Kα binding by using a multitude of approaches, from PI3Kγ activation, insulin signaling in the pancreas to the yet under-appreciated co-mutations with in the PI3K signaling pathway, exists. In these contexts, determining the unique roles of each PI3K isoform will shed additional light on the functional redundancy of these kinases as well as whether specifically targeting individual PI3K isoforms will provide a therapeutic window in $KRAS^{G12R}$-mutant PDAC. While several isoform-specific PI3K inhibitors have been approved for use in human cancers, many of these inhibitors are used in hematologic cancers, and only a few PI3K isoform-selective inhibitors have been used in solid tumors, such as breast cancer (Hanker et al. 2019). As such, it remains to be seen whether targeting PI3K with an isoform-selective approach in pancreatic cancer will represent a viable direction or a therapeutic dead-end. However, it appears possible that the $KRAS^{G12R}$ mutation represents a unique setting where isoform-specific PI3K inhibitors may have an added advantage.

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