Cancer Insights through Macropinocytosis: A Role for Sorting Nexins?

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1 Introduction

Macropinocytosis is an actin-driven endocytic process, whereby membrane ruffles fold back onto the plasma membrane to form large (> 0.2 μm in diameter) endocytic organelles called macropinosomes (Swanson & Watts, 1995). The rapid and large fluid-carrying capacity of macropinosomes is central to their role in the immune response, possessing great potential for antigen sampling from the environment as the amount of material internalized greatly exceeds that of other endocytic pathways (Norbury, 2006). Studies of oncogenic signaling and cellular responses to growth factors have also implicated macropinocytosis in the molecular mechanisms of cancer and tumorigenesis. Treatment with Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF) and Hepatocyte Growth Factor/Scatter Factor (HGF/SF) - ligands associated with uncontrolled cell proliferation in cancerous tissue - have all been shown to rapidly increase the rate of macropinosome formation and fluid-phase uptake (Kerr & Teasdale, 2009). Cells overexpressing oncogenes have been shown to exhibit elevated levels of macropinocytosis, as well as accelerated spontaneous motility in wound healing assays (Platek *et al*., 2007). This suggests a strong link between the membrane ruffling necessary for macropinosome formation and cell motility regulation, a process crucial in tumour progression and metastasis. As the molecular mechanisms of macropinocytosis become further defined a detailed evaluation of their regulation of immunological and cancerous processes is needed.

Recently, members of the Sorting Nexin (SNX) family have been localized to the dynamic ruffling cell surface and found to be associated with early-stage macropinosomes (Kerr *et al*., 2006; Merino-Trigo *et al*., 2004; J. T. Wang *et al.*, 2010). SNX-PX-BAR proteins form a subset of the SNX family and their lipid-binding (PX) and membrane-curvature sensing (BAR) domain architecture is consistent with a potential role in the dramatic membrane remodeling and trafficking required in the initiation of macropinosome formation. Other SNX-PX-BAR proteins have also been found to interact with regulators of actin remodeling, implicating them not only in macropinocytosis but also cell motility, metastasis, and tumorigenesis. This chapter will outline the outcomes of systematic functional studies into the impact of the SNX-PX-BAR family on macropinocytosis, and the insights this will provide into the molecular mechanisms of cancer biology.

2 Endocytosis

Endocytosis is the cellular process which facilitates the internalisation of nutrients, fluid, and signalling molecules from the extracellular environment (Miaczynska *et al*., 2004). It maintains cellular homeostasis in numerous processes, including the regulation of cell-surface receptors, remodelling of the plasma membrane, and cell motility and migration (Di Fiore & De Camilli, 2001; Jones *et al.*, 2006). To account for this vast diversity of biological roles, there are many variants of endocytosis, each distinct in the specific complement of molecular machinery utilised in organelle formation. The evolution of multiple endocytic pathways allows the cell to specifically regulate the kinetics of internalization for different subsets of cargo and appropriately respond to varying physiological conditions (Figure 1).

2.1 Macropinocytosis

Macropinocytosis is a process first reported by Warren Lewis in 1931 (Swanson & Watts, 1995), which is also clathrin and caveolae-independent. Similar to other clathrin and caveolae-independent pathways, macropinocytosis is cholesterol sensitive (Grimmer *et al*., 2002), but its requirement of dynamin varies depending on cellular conditions and cell type (Altschuler *et al.*, 1998; Bonazzi *et al.*, 2005; Damke *et al.*, 1994; Herskovits *et al.,* 1993; Y. W. Liu *et al*., 2008; Macia *et al*., 2006; Sabharanjak *et al.*, 2002). Unlike Clathrin-Mediated Endocytosis (CME), it is not regulated by the binding of cargo to the receptors which then recruit effector molecules that aide in vesicle formation (Maxfield & McGraw, 2004); instead the activation of Receptor Tyrosine-Kinases (RTK) in response to growth factor treatment drives the actin-mediated evaginations of the plasma membrane, non-selectively engulfing large volumes of fluid to form phase bright macropinosomes larger than 0.2 μm in diameter (Kerr & Teasdale, 2009; Swanson & Watts, 1995). Strikingly, this heterogeneous size range is significantly larger than other endocytic compartments such as Clathrin-Coated Vesicles (CCVs) (85-110 nm), caveolae (55-75 nm), and Clathrin-Independent Carriers/GPI-anchored protein-enriched Early Endosomal Compartments (CLIC/GEECs) (40-80 nm), and the diameter of the macropinosome is the main distinguishing factor from other endocytic pathways (Kirkham *et al*., 2005; Parton & Richards, 2003; Pearse, 1976; Rubenstein *et al*., 1981; Yamada, 1955). Interestingly treatment with millimolar concentrations of the ion exchange inhibitor amiloride inhibits macropinocytosis but not CME (West *et al*., 1989), which has been associated with the lowering of submembranous pH and preventing Rho GTPase signalling and actin remodelling (Koivusalo *et al.*, 2010). This property can be used to define macropinocytosis (Figure 2A) along with the size of the organelle and responsiveness to growth factor stimulation (Kerr & Teasdale, 2009).

Of the known endocytic routes into the cell, phagocytosis is the pathway that most closely resembles macropinocytosis. The actin-mediated extension of the pseudopod required for phagosome formation is structurally similar to the membrane ruffling in macropinocytosis (Swanson, 2008), and macropinosomes are also known as spacious phagosomes due to their morphological similarity to phagosomes without a particle inside (Alpuche-Aranda *et al.*, 1994). The mechanism of formation however is the main distinction between phagocytosis and macropinocytosis, as the former process is initiated by the clustering of cell surface receptors binding the ligands on the bacterium or particle to be phagocytosed (Swanson, 2008). Macropinocytosis however is not directly initiated by cargo or receptor molecules, but is a result of an increase in actin-polymerization on distinct regions of the cell surface leading to membrane ruffling. Evidently, it is the mechanism of formation and the size of the resulting organelle formed that clearly distinguishes macropinocytosis from the other known endocytic pathways. The macropinocytic formation process including the unique complement of protein and lipid molecules that specifically defines this organelle and regulates its formation is still poorly understood.

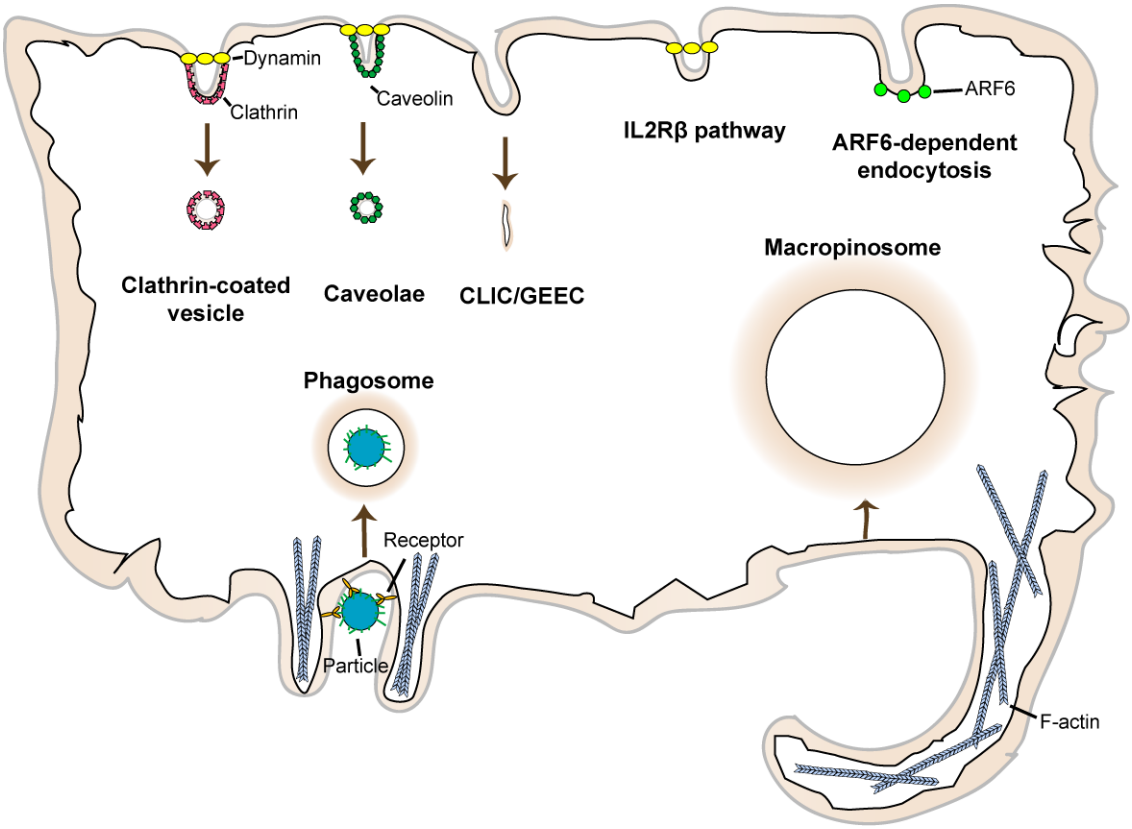

Figure 1: Variants of Endocytosis. The internalization of cargo, receptors, particles or fluid into mammalian cells can occur via a wide variety of pathways. Clathrin-coated vesicles are formed via clathrin-mediated endocytosis, whose scission from the plasma membrane is mediated by dynamin. Caveolae are caveolin-dependent, and also require dynamin in many cell types to be excised from the cell surface (Kirkham & Parton, 2005). The CLIC/GEEC pathway has been observed to form ring-like or tubular structures in a clathrin and caveolae-independent manner; cholesterol levels though, have been shown to affect their formation (Kirkham *et al*., 2005). Internalisation of the beta subunit of the Interleukin 2 Receptor (IL2Rβ) has been found to occur through a clathrin and caveolae-independent pathway distinct from CLIC/GEECs, and dependent upon dynamin activity (Lamaze *et al*., 2001). ARF6-dependent endocytosis is also clathrin and caveolae-independent, and does not rely on dynamin activity (Naslavsky *et al*., 2004). Phagocytosis requires the binding of cell surface receptors to a particle prior to its internalization, and macropinocytosis forms macropinosomes as a result of actin-mediated plasma membrane ruffles.

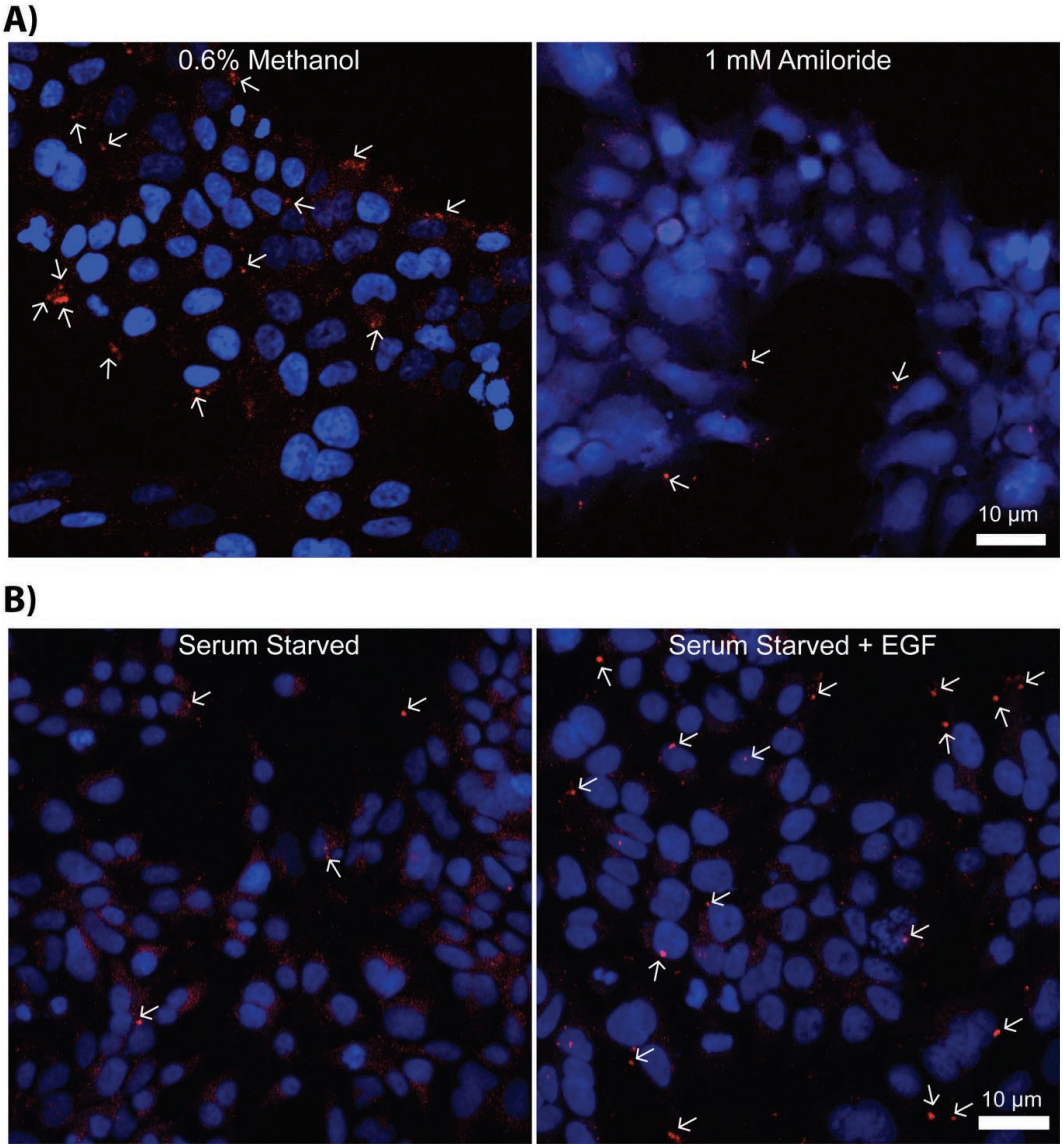

Figure 2: Regulation of macropinocytosis. A) HEK-Flp-In cell monolayers were treated with 1 mM Amiloride or carrier (0.6% Methanol) for 30 minutes at 37°C prior to pulsing with the fluid-phase marker dextran-TR for 5 minutes at 37°C in the continued absence of the drug or carrier. B) HEK-Flp-In cell monolayers were serum-starved for 16 hours and incubated with 100 µg/mL dextran-TR in the presence or absence of 100 ng/mL EGF for 5 minutes at 37°C. Dextran-TR in red and DAPI-positive nuclei shown in blue. White arrows point to macropinosomes > 0.2 µm in diameter and above the baseline threshold for fluorescence intensity. Scale bar = 10 µm.

3 Physiological Implications of Macropinocytosis

In order to drive the formation of large macropinosomes, macropinocytosis must utilise a significant portion of the available membrane within the cell to internalize large volumes of fluid. This would require coordinated membrane trafficking events to recycle the internalized membrane back to the cell surface, which is by no means energetically favourable. From an evolutionary perspective, this energetically-expensive endocytic pathway must provide a selective advantage in order to be preserved; indeed macropinocytosis has been implicated across a wide array of physiological processes, including immune antigen sampling and cellular signalling.

The rapid and large fluid-carrying capacity of macropinosomes is central to their role in the immune response, possessing great potential for antigen sampling from the environment. Antigen capture occurs via internalization of extracellular fluid by antigen-presenting cells, after which Major Histocompatibility Complex (MHC) II molecules form stable MHC-antigen complexes and present the antigenic peptides on the cell surface (Lanzavecchia, 1996). This process is accelerated by macropinocytosis as the amount of material internalized through this form of endocytosis greatly exceeds that of other endocytic pathways. This is evident in dendritic cells, which are professional antigen-presenting cells (Mempel *et al*., 2004; Sallusto *et al.*, 1995). In their immature state, dendritic cells readily undergo macropinocytosis, and can internalize over 40% of their cell volume every hour in order to sample as much of their immediate environment as possible before presenting antigens to T cells post maturation (Larsen *et al.*, 1990; Norbury, 2006; Sallusto *et al.*, 1995). The concomitant down-regulation of macropinocytosis along with dendritic cell maturation signifies its relevance and specificity towards the antigen-sampling process. Recent studies have directly implicated a significant role for dendritic cells in cancer immunotherapy, where the cells' expansive antigen-sampling capacity was applied towards the capture of tumour antigens that subsequently generated tumour-specific cytotoxic T lymphocytes (Diamond *et al*., 2011; Fuertes *et al*., 2011).

Studies of oncogenic signalling and growth factor stimulation have also implicated macropinocytosis in these processes. Cells overexpressing oncogenes have been shown to exhibit elevated levels of macropinocytosis (Amyere *et al*., 2002; Amyere *et al*., 2000; Kasahara *et al.*, 2007). Accelerated spontaneous motility in wound healing assays have also been observed in these cells, which suggests a link between the membrane ruffling necessary for macropinosome formation and cell motility regulation, a process crucial in tumour progression and metastasis (Platek *et al.*, 2004; Platek *et al*., 2007). In conjunction with this, treatment with EGF (Figure 2B), PDGF and HGF/SF, all associated with uncontrolled cell proliferation in cancerous tissue, have been shown to rapidly increase the rate of macropinosome formation and its associated fluid-phase uptake (Dowrick *et al.*, 1993; Haigler *et al.*, 1979; Kerr *et al.*, 2006; Sun *et al.*, 2003).

The complement of growth factors associated with the upregulation of macropinocytosis varies depending on cell type. Recombinant human Macrophage Colony-Stimulating Factor (rM-CSF) and PhorbolMyristate Acetate (PMA) have been shown to stimulate macropinocytosis in bone marrow-derived mouse macrophages (Racoosin & Swanson, 1989, 1992; Swanson, 1989). HGF/SF has been shown to increase ruffling and macropinocytosis in Madin-Darby Canine Kidney (MDCK) cells, EGF rapidly stimulates pinocytosis in A-431 and Human Embryonic Kidney (HEK) 293 cells, and PDGF has a similar effect in NIH/3T3 cells (Anton *et al*., 2003; Dowrick *et al*., 1993; Haigler *et al*., 1979; Kerr *et al*., 2006). Not all cell types however require the addition of growth factors or external stimuli to carry

out macropinocytosis. Immature dendritic cells for example, display constitutively active macropinocytosis to facilitate their antigen-sampling function, and oncogene-activated Rat-1 fibroblasts consistently maintain a high level of fluid uptake (Amyere *et al*., 2000; Sallusto *et al.*, 1995). The difference between transiently-regulated macropinocytosis and its constitutive variants has not been definitively characterized, but may be a result of the differential regulation of common signalling pathways. The fact that the oncogenes overexpressed in constitutive macropinocytosis, such as v-Src, c-Src, K-Ras and H-Ras, are all downstream of growth factor binding, supports such speculation (Amyere *et al.*, 2002; Amyere *et al*., 2000; Kasahara *et al*., 2007; Porat-Shliom *et al.*, 2007).

The Src and Ras oncogenes implicated in macropinocytosis are known to induce the PI(3)K signalling cascade, translocating the p85 subunit of PI(3)K to the plasma membrane followed by its p110 subunit (Amyere *et al.*, 2002). This is consistent with the necessity of PI(3)K activity for macropinocytosis, as treatment with the PI(3)K inhibitors wortmannin and LY294002 abrogates macropinosome formation (Amyere *et al*., 2000; Araki *et al*., 1996). Activated class IA PI(3)Ks phosphorylate Phosphatidylinositols (PI) at the 3'position of the inositol ring (Lindmo & Stenmark, 2006) and its substrates are concentrated at the plasma membrane. PI(3)K activity may therefore be responsible for the transition from $PI(4,5)P_2$ to $PI(3,4,5)P_3$ on the plasma membrane as the ruffle closes to form the macropinosome (Porat-Shliom *et al*., 2007). This localized increase in the levels of $PI(3,4,5)P_3$ on the cell surface acts as a docking site for many downstream targets (Vermeer *et al.*, 2006), including Phosphoinositide-specific Phospholipase-C (PI-PLC). PI-PLC catalyses the formation of Inositol 1,4,5-triphosphate (IP_3) and diacylglycerol from $PI(4,5)P_2$ which in turn activates Protein Kinase C (PKC) and actin polymerization (Amyere *et al.,* 2002). $PI(3,4,5)P_3$ also recruits Vav, a Guanine Exchange Factor (GEF) which activates Cdc42 and Rac1. ADP-ribosylation factor-6 (ARF-6) has also been implicated in Rac-1 activation, as well as regulating Phosphatidylinositol-4-Phosphate 5 Kinase (PIP5K) activity to catalyze the production of $PI(4,5)P_2$ (Boshans *et al.,* 2000; Honda *et al.*, 1999). Guanine Exchange Factors such as EFA6, general receptor for phosphoinositides-1 (GRP1), and Arf-GEP100 have been linked in their regulation of ARF-6 activity, as the cycling between GDP or GTP-bound states is crucial for its function (Niedergang *et al.*, 2003; Someya *et al.*, 2001).

4 Molecular Regulation of Macropinocyosis

The cellular signalling that regulates macropinocytosis must activate downstream pathways that comprise of a unique complement of protein and lipids specific to macropinosome formation. Although there is a relative paucity of information regarding the components unique to macropinocytosis, the molecules known to be involved in this endocytic pathway can be broadly categorized into actin modulators that regulate membrane ruffling, membrane trafficking regulators including members of the RabGTPase family, phosphoinositides that affect the membrane composition of the macropinosome, and the phosphoinositide-binding Phox-homology (PX) domain and Sorting Nexin proteins.

4.1 Actin modulators and membrane ruffling

Membrane ruffling is the initial step of macropinocytosis (Amyere *et al*., 2002), whereby dynamic cell surface ultrastructures lengthen into membrane extensions that reattach to the cell membrane while capturing extracellular fluid (Francis *et al.*, 1993; West *et al*., 2000). This enveloping action of the mem-

brane ruffle then forms the macropinosome. The mechanism by which amiloride inhibits macropinosome formation is proposed to be the inhibition of cell ruffling (Swanson & Watts, 1995) as cytoplasm acidification within macrophages has been shown to abrogate cell ruffling while alkalinization induced hyper-ruffling (Heuser, 1989). Inhibitors of actin-polymerization, which is crucial to membrane ruffling, have also been shown to abrogate macropinocytosis (Hacker *et al*., 1997; Mettlen *et al*., 2006).

Actin polymerization involves the addition of monomeric actin (G-actin) into polar filaments (F-actin) through either the fast growing (barbed) or slower growing (pointed) ends (Millard *et al.*, 2004). The Arp2/3 complex consists of 2 actin-related proteins (Arp) Arp2 and Arp3 in complex with the Arp2/3 Complex Components (ARPC) ARPC1, 2, 3, 4, and 5 in 1:1 stochiometry with each other (Machesky *et al*., 1994; Millard *et al*., 2004). The Arp2/3 complex is able to drive actin polymerization, where actin monomers bind to and hydrolyze ATP in order to be added onto the fast growing barbed end of F-actin. Arp2/3 is also able to initiate *de novo* actin nucleation, by binding to the pointed ends of F-actin and producing barbed-end filament seeds from which new actin branches may extend (Goley & Welch, 2006; Millard *et al.*, 2004). Given that actin polymerization is required for the membrane ruffling necessary for macropinocytosis, it is unsurprising that the Arp2/3 complex has been implicated in macropinosome formation (Insall *et al.*, 2001). The *de novo* formation of actin branches would push out into the plasma membrane and create membrane ruffles, which upon folding back onto the cell surface form macropinosomes.

The activation of the Rho GuanosineTriphosphatases (GTPases) Cdc42, and Rac1 from their GDP to GTP-bound forms, is important in mediating the actin polymerization necessary for membrane ruffling (West *et al.*, 2000). GTP-bound Cdc42 and $PI(4,5)P_2$ synergistically activate Neuronal Wiskott-Aldrich Syndrome protein (N-WASP), by binding to its CRIB domain and basic region respectively (Higgs & Pollard, 2000; Rohatgi *et al*., 2000). The autoinhibitory conformation of N-WASP is then relieved, exposing the VCA domain that has been shown to be necessary and sufficient to activate the Arp2/3 complex (Machesky *et al*., 1999; Rohatgi *et al*., 1999). The adaptor proteins Nck and Growth factor Receptor-Bound protein 2 (Grb2) have been shown to activate N-WASP as well, binding to the proline-rich domain of N-WASP through their respective Src Homology 3 (SH3) domains (Carlier *et al*., 2000; Rohatgi *et al.,* 2001). The additional presence of a Src Homology 2 (SH2) domain on both Nck and Grb2 allows them to associate directly with activated RTKs, providing a link between these upstream signals and N-WASP activation (Buday, 1999). WASP-Interacting SH3 protein (WISH) has also been shown to bind to and activate N-WASP, enhancing N-WASP induced Arp2/3 complex activation independent of Cdc42 (Fukuoka *et al.,* 2001). The G-actin-binding Profilin is another protein implicated in enhancing Arp2/3 complex activation through its interactions with N-WASP and WASP-family Verpolin-homologous protein (WAVE) (Miki *et al*., 1998; Suetsugu *et al.*, 1998;Yang *et al*., 2000). Evidently N-WASP activation is very specifically coordinated, and there is evidence to suggest it is negatively regulated by WASP-Interacting Protein (WIP). WIP retards N-WASP/Cdc42-induced actin polymerization through the Arp2/3 complex, and is involved in stabilizing actin filaments (Martinez-Quiles *et al*., 2001).

Rac1 has also been shown to be a regulator of macropinocytosis. It is necessary for growth-factor-induced membrane ruffling, and its microinjection rapidly stimulates actin accumulation at the cell surface (Ridley *et al*., 1992); microinjection of dominant-negative Rac1 also inhibits macropinocytosis in immature dendritic cells (West *et al*., 2000). Moreover transient Rac1 activation has been temporally associated with membrane ruffle closure, and its deactivation precedes the formation of the macropinosome cup (Yoshida *et al*., 2009). Rac1 activation has also been linked to WAVE2, as WAVE2 defi-

cient embryonic fibroblasts suffer from severe defects in cell growth, motility, and Rac1-mediated actin polymerization (Yan *et al.*, 2003). Rac1 does not directly associate with WAVE2 however, instead interacting with multi-protein WAVE2 complexes that include Abi1, Nck-associated protein 1 (Nap1), Specifically Rac1-Associated protein 1 (Sra1), Insulin Receptor Substrate protein 53 (IRSp53) and HSPC300 (Abou-Kheir *et al*., 2008; Gautreau *et al.*, 2004). Proteins within this WAVE2 complex all translocate to the tips of membrane protrusions following the injection of constitutively-active Rac1; moreover Sra1 and Nap1 have been reported as the components of the WAVE2 complex that interact directly with Rac1, as their siRNA-mediated depletion inhibits the formation of Rac1-dependent lamellipodia in response to growth factor treatment (Steffen *et al*., 2004). Recently IRSp53 has been implicated in stabilizing the interaction of Rac1 with Abi1 and WAVE2; IRSp53 forms a complex with Abi1 and WAVE2 in a Rac1 activation-dependent manner, and depletion of IRSp53 reduces Rac1 association with the other proteins in the complex (Abou-Kheir *et al.*, 2008). Abi1 has been shown to bind to the WHD domain of WAVE2, thus facilitating the activation of the Arp2/3 complex through WAVE2 (Innocenti *et al.*, 2004). Consistent with the role of Abi1 in actin polymerization, Abi1 has also been observed to regulate WAVE2 in Rac1-dependent macropinocytosis (Innocenti *et al.*, 2005). Evidently each member of the WAVE2 protein complex plays a role in mediating the Rac1-dependent mechanisms that ultimately lead to membrane ruffling and macropinocytosis.

Despite the fact that membrane ruffling is required for macropinocytosis, ruffling itself is not sufficient for macropinosome formation. The microinjection of dominant-negative Rac1 and inhibition of Rac1, Rho and Cdc42 activity through the *Clostridium difficile* B toxin abrogated macropinocytosis without significantly affecting membrane ruffling (West *et al.*, 2000). These observations indicate that downstream effectors of Rho GTPases are required in regulating macropinosome formation following membrane ruffling, and PAK1 is one such candidate. Both Cdc42 and Rac1 can activate PAK1 by binding to its p21-Binding Domain (PBD) (Hoppe & Swanson, 2004), and PAK1 has been shown to be necessary for PDGF-induced macropinocytosis in fibroblast cells (Dharmawardhane *et al.*, 2000). Significantly, PAK1 phosphorylates Carboxyl-terminal Binding pProtein 3/Brefeldin-A-ADP-Ribosylated Substrate (CtBP3/BARS) at serine 147, and this phosphorylation is essential for EGFR-stimulated macropinocytosis but not membrane ruffling. Moreover, CtBP3/BARS is translocated to the macropinocytic cup in response to EGF treatment, and overexpression of its dominant negative mutant inhibited the fission of these cups from the plasma membrane. This suggests that downstream of Rac1 and Cdc42, PAK1-phosphorylation of CtBP3/BARS is involved in the formation and fission of the macropinosome from the plasma membrane (Liberali et al., 2008).

4.2 Phosphoinositides

Given that the initiation of macropinocytosis is PI(3)K-dependent, it logically follows that the phosphoinositides generated by PI(3)K activity are involved in this process. Phosphoinositides result from the phosphorylation of phosphatidylinositol at different positions along the inositol ring (Lindmo & Stenmark, 2006), and different phosphoinositide species crucial for the formation and maturation of macropinosomes. In A431 cells, $PI(4,5)P_2$ levels on membrane ruffles are more than double the amount present on planar membranes, rapidly dropping just prior to macropinosome closure. Conversely, $PI(3,4,5)P_3$ levels increases locally at the site of macropinosome formation and peaks when the macropinosome closes (Araki *et al*., 2007). A similar elevation in $PI(3,4,5)P_3$ levels on the membrane ruffles was

also observed in HeLa cells (Porat-Shliom *et al*., 2007) and in macrophages the subsequent drop in $PI(3,4,5)P_3$ levels coincided with the accumulation of PI(3)P (Yoshida *et al.*, 2009).

The phosphoinositide-metabolising enzymes that regulate these phosphoinositide transitions are all potential candidates in this. PI is converted to PI(3)P or PI(4)P through the actions of vacuolar protein sorting (Vps) 34-p150 and PI(4)KIIα respectively (Krauss & Haucke, 2007). $PI(4,5)P_2$ synthesis from PI(4)P at the plasma membrane is predominantly regulated by PIP5K (Ishihara *et al.*, 1996; Ishihara *et al*., 1998), or it can be formed from dephosphorylating $PI(3,4,5)P_3$ through the 3-phosphatase activity of Phosphatase and Tensin homolog (PTEN) (Wishart & Dixon, 2002); $PI(3,4,5)P_3$ is synthesized from $PI(4,5)P_2$ by the action of class I PI(3)K (Krauss & Haucke, 2007). The conversion of $PI(3,4,5)P_3$ to PI(3)P on the macropinosome body may be the result of the sequential dephosphorylation of $PI(3,4,5)P_3$ as catalysed by 4 and 5-phosphatases. Src Homology 2 domain-containing Inositol 5-Phosphatase (SHIP) 1 and 2 are potential 5-phosphatase candidates, dephosphorylating $PI(3,4,5)P_3$ to $PI(3,4)P_2$ at the cell surface. Type I and II 4-phosphatases are then able to catalyse the conversion between $PI(3,4)P_2$ and PI(3)P (Krauss & Haucke, 2007). Alternatively $PI(3,4,5)P_3$ may simply be lost from the macropinosome membrane and VPS34-p150 drives the *de novo* synthesis of PI(3)P from PI (Zerial & McBride, 2001). The levels of $PI(3,4,5)P_3$ within the cell has been shown to be crucial for macropinosome formation, as PTEN or a lipid phosphatase deficient mutant PTEN(G129E) overexpression significantly decreased or increased macropinocytosis respectively (Wang *et al.*, 2010).

The synthesis of $PI(3,5)P_2$ from PI(3)P is catalysed by PIKfyve (Sbrissa *et al*., 1999; Shisheva *et al*., 1999), and has recently been implicated in the intracellular maturation of the macropinosome (Kerr *et al.*, 2010). Disruption of PIKfyve activity through either overexpression of the catalytically inactive PIKfyve mutant or using a specific inhibitor of PIKfyve catalytic activity YM201636 (Jefferies *et al*., 2008), both inhibited macropinosome fusion with the late endosomes/lysosomes (Kerr *et al.*, 2010). Given that PI(3)P can be found on early-stage macropinosomes, this data indicates that PIKfyve regulates its conversion to $PI(3,5)P_2$ on macropinosomes in order to facilitate the fusion of the organelle with the late endosome/lysosomes.

4.3 RabGTPases

Following the transition from $PI(4,5)P_2$ to $PI(3,4,5)P_3$ on macropinosome membranes, Rab5 recruitment has been observed prior to $PI(3,4,5)P_3$ loss (Porat-Shliom *et al*., 2007). Rab5 belongs to the Rab family of GTPases that play crucial roles in membrane trafficking, and has been known to regulate endosome fusion, motility of endosomes along microtubules, and early endosomal identity (Zerial & McBride, 2001).

The Rab5 signal cascade leading to membrane docking and fusion is well understood. The Rabaptin-5/Rabex-5 complex is recruited onto early endosomes through Rabaptin-5 activity, and activates Rab5-GTP through the nucleotide exchange function of Rabex-5. Rab5-GTP then interacts with Vps34-p150, a PI(3)K which produces PI(3)P (Zerial & McBride, 2001). At this stage of maturation, macropinosomes become rich in PI(3)P, and consequently proteins containing FYVE or Phox homology (PX) domains, are able to bind (Stenmark *et al*., 1996; Xu *et al.*, 2001). These include EEA1, and the Rab5 effector Rabankyrin-5, both of which possess FYVE domains (Hamasaki *et al*., 2004; Schnatwinkel *et al*., 2004). EEA1 is known to form oligomeric complexes with a t-SNARE required for endosome fusion, Syntaxin 13, in the presence of NSF (McBride *et al.*, 1999). Both EEA1 and Rabankyrin-5 interact directly with Rab5 in a GTP-dependent fashion as well as binding to PI(3)P, and overexpression and siRNA-mediated depletion of Rabankyrin-5 increases and decreases the number of macropinosomes formed

respectively (Schnatwinkel *et al*., 2004). Although PI(3)P is not involved in macropinosome formation, inhibition of PI(3)P synthesis through 3-methyladenine treatment inhibited homotypicmacropinosome-macropinosome fusion (Araki *et al*., 2006). This process is likely to be mediated by the Rab5 pathway, as the constitutively active Rab5 mutant Rab5(Q79L) results in homotypic fusion leading to swollen endosomes whose size resemble early macropinosomes (Stenmark *et al*., 1994).

It was discovered that endosomes undergo a Rab5 to Rab7 conversion from early to late endosomes (Rink *et al*., 2005), and this transition has also been observed on macropinosomes (Kerr *et al.*, 2006). Rab7 is a late endosome marker that has been linked to macropinocytosis and is primarily responsible for early to late endosome cargo trafficking as well as homotypic fusion between late endosomes (Pfeffer, 2003). The conversion from Rab5 to Rab7 on macropinosomes suggests that at least in some model systems, macropinosomes exhibit a maturation process from early to late endosomal structures, reminiscent of classical endosomes. Rab7 works in a similar GTPase-dependent manner as Rab5, and localizes to macropinosomes later in the maturation process - Rab7-positive macropinosomes often co-label with Lysosomal Glycoprotein A (LGP-A) which initiates their merge into the tubular late endosomal/lysosomal compartment. This late stage in maturation can also be marked by an accumulation of Lysosomal-Associated Membrane Protein 1 (LAMP1) on the macropinosome, an indication that the organelle is starting to fuse with the late endosome/lysosome system (Egami & Araki, 2009). It is there where macropinosome contents are presumably degraded and its membrane content is recycled (Racoosin & Swanson, 1993).

Rab7-Interacting Lysosomal Protein (RILP) is a crucial part of Rab7-mediated lysosomal degradation of endosomal contents (Colucci *et al*., 2005). RILP binds to Rab7-GTP and recruits dynein-dynactin motor complexes, transporting Rab7-positive compartments towards the lysosomes (Jordens *et al*., 2001). RILP is also known to interact with another member of the Rab family, Rab34 (Wang & Hong, 2005), which has been shown to be localized at membrane ruffles and its overexpression increases macropinocytosis (Sun *et al.*, 2003). However a more recent study disputed the reported role of Rab34 in fluid-phase uptake, as its overexpression in HeLa cells did not result in a difference in the rate of fluid-phase uptake. Furthermore, its localization was found to be at the Golgi instead of on membrane ruffles and Rab34 depletion resulted in defective secretion from the Golgi to the plasma membrane (Goldenberg *et al.*, 2007). The role of Rab34 in macropinocytosis is therefore controversial.

Recently Rab21 was found to associate with early-stage macropinosomes in RAW264 macrophages. Rab21 localizes to the macropinosome in a GTPase-dependent manner, and its temporal recruitment follows the loss of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ from the macropinosome membrane. Moreover Rab21 enrichment on the macropinosome follows Rab5 but precedes Rab7 recruitment, dissociating from the organelle prior to the recruitment of the late endosome/lysosome marker LAMP-1. The spatiotemporal distribution of Rab21 during macropinocytosis implicates its involvement in early stage macropinocytosis, but unlike Rab5, Rab21 overexpression does not elevate macropinosome formation (Egami & Araki, 2009; Schnatwinkel et al., 2004).

4.4 PX domain proteins and Sorting Nexins

The lack of knowledge regarding molecules that specifically regulate macropinosome formation has hindered investigation into this endocytic pathway, highlighting the need to examine novel sets of candidate proteins for their role in macropinocytosis. The PX domain family is an ideal set for this purpose, spanning across forty-nine proteins in the mammalian genome, many of which have been reported to bind to a

wide variety of phosphoinositides in a diverse array of membrane and protein trafficking events (Teasdale & Collins, 2012). Twelve of the PX-domain proteins also contain a C-terminal Bin/Amphiphysin/Rvs (BAR) domain, which is involved in homo and heterodimerization, detecting membrane curvature and tabulation (Habermann, 2004; Itoh & De Camilli, 2006; Peter *et al.*, 2004; Zimmerberg & McLaughlin, 2004). It has also been demonstrated that the PX and BAR domains cooperate in the coincidence detection of curved membranes rich in specific phosphoinositides (Carlton *et al*., 2004; J. G. Carlton & Cullen, 2005), and together are involved in the endosomal localization of SNXs (Liu *et al.*, 2006). SNX1, SNX2, SNX4, SNX5, SNX6, SNX7, SNX8, SNX9, SNX18, SNX30, SNX32 and SNX33 all contain PX and BAR domains, and together they form the SNX-PX-BAR family.

SNX5 was the first member of the SNX-PX-BAR family identified to be involved in macropinocytosis. EGF treatment is known to upregulate macropinocytosis (Haigler *et al.*, 1979), and SNX5 is transiently recruited to the plasma membrane in response to EGF (Merino-Trigo *et al.*, 2004). This is likely due to the elevation in $PI(3,4)P_2$ on the plasma membrane following EGF treatment, to reflect the $PI(3,4)P_2$-specificity of the PX domain of SNX5 as determined by liposome binding assays (Merino-Trigo *et al*., 2004). Following its cell-surface translocation, SNX5 can be localized to discrete subdomains of the macropinosome along with Rab5, SNX1 and EEA1 (Kerr *et al*., 2006). SNX5 is recruited early in the macropinocytic process, with a temporal association with macropinosomes that overlaps with that of Rab5 but precedes Rab7 recruitment. Once on the macropinosome, SNX5 forms extensive microtubule-dependent tubules that depart from the macropinosome body. This extensive tubulation removes a significant portion of the limiting membrane of the macropinosome, changing the organelle's shape and volume. This mechanism is speculated to be responsible for recycling and trafficking components of the macropinosome (Kerr *et al*., 2006). Within primary bone-marrow derived mouse macrophages, depletion of SNX5 significantly decreased the size and number of macropinosomes formed (Lim *et al.*, 2012).

The lipid-binding and membrane tubulating capacity of SNX5 has been implicated in the early stages of macropinocytosis (Kerr *et al.*, 2006), and this could be correlated to its PX-BAR domain architecture. It logically follows then that other members of the SNX-PX-BAR family could also be involved in macropinocytosis. When comparing the sequence similarity of SNX5 to the other 11 human members of the SNX-PX-BAR family using a bioinformatics approach, the SNX-PX-BAR family can be split into three subgroups or classes as previously described (Seet & Hong, 2006). Class 1 includes SNX5 and the 4 most closely related proteins by amino acid sequence similarity - SNX1, SNX2, SNX5, SNX6, and SNX32. The level of sequence homology between the proteins separates class 1 from class 2, which comprises of SNX4, SNX7, SNX8, and SNX30. Class 3 can also be referred to as the SH3-PX-BAR subgroup, as all three members of this class (SNX9, SNX18, and SNX33) share an N-terminal SH3 domain. Given that the regulation of macropinocytosis varies in response to different cellular conditions across different cell types, it is important to validate candidates using functional screens in consistent cell models. A systematic gain-of-function screen was carried out for each member of the SNX-PX-BAR family, where the number of macropinosomes formed by cells overexpressing the candidate proteins were imaged and computationally analysed (Wang *et al.*, 2010). Upon transient overexpression in HEK-Flp-In cells, SNX1, SNX5, SNX9, SNX18, and SNX33 were all able to independently elevate the frequency at which macropinocytosis occurred (Figure 3).

SNX1 has been observed to interact and form heterodimers with SNX5 by several groups (Kerr *etal*., 2006; H. Liu *et al.*, 2006) despite limited evidence to the contrary (Wassmer *et al*., 2007). Like SNX5, SNX1 overexpression also changes the frequency of macropinosome formation, suggesting that

the two proteins are acting in complex as part of a common mechanism in macropinocytosis. This hypothesis is further substantiated by the colocalization of SNX1 and 5 on newly formed macropinosomes, indicating their recruitment early in the formation process.

Apart from SNX1 and SNX5, the remaining hits from the gain-of-function screen constitute the SH3 subgroup of the SNX-PX-BAR family - SNX9, SNX18 and SNX33. SNX9 possesses arguably the strongest link to macropinocytosis, as it has been mechanistically linked to macropinosome formation by virtue of its role in actin assembly. SNX9 has been reported to interact with N-WASP through its N-terminal SH3 domain (Shin *et al*., 2007; Yarar *et al.*, 2007). This interaction is thought to drive N-WASP activation along with the binding of $PI(4,5)P_2$, as SNX9 also binds to the PI(4)P-5 kinases Iα, Iβ and Iγ to regulate $PI(4,5)P_2$ synthesis (Shin *et al*., 2008; Yarar *et al.*, 2007). As well as regulating N-WASP-mediated Arp2/3 complex activation, SNX9 has been shown to directly interact with the Arp2/3 complex to drive actin nucleation and membrane ruffling (Shin *et al*., 2008; Yarar *et al.*, 2007). SNX9 can be found on F-actin rich membrane ruffles and tubules (Yarar *et al.*, 2007), and its PX-BAR unit drives extensive membrane tubulation within both *in vitro* liposomes and *in vivo* (Shin *et al.*, 2008). The scission of these tubules occurs through the interaction between the SH3 domain of SNX9 and dynamin 2, as overexpressing the PX-BAR unit of SNX9 alone results in extensive tubules unable to undergo membrane scission for vesicle formation (Haberg *et al*., 2008).

Similar to SNX9, the PX-BAR unit of SNX18 induces extensive membrane tubulation; however in HeLa cells, SNX18 does not colocalise with SNX9, instead being found on endocytic vesicles positive for adaptor protein complex 1 (AP1) but devoid of clathrin (Haberg *et al.*, 2008). This suggests a possible functional divergence between SNX9 and SNX18, although this divergence appears to vary depending on cell type. Within NIH3T3 cells, SNX18 interacts with N-WASP (Park *et al*., 2010), and also associates with actin and Rac1 in linear filamentous structures near the surface of HEK-Flp-In cells (Figure 4). The association between SNX18, N-WASP, $PI(4,5)P_2$, and actin machinery in HEK Flp-In cells mirrors that of SNX9 and points to a potential mechanism for their roles in macropinocytosis (Haberg *et al*., 2008). Recently SNX18 has also been observed to impact developing spinal motor neurons, as its expression level in the embryonic spinal cord is downregulated as the motor neurons mature (Nakazawa *et al.*, 2011). SNX33 has been shown to interact with both SNX9 and WASP (Zhang *et al.*, 2009), and its involvement in phagosome maturation is conserved across mammalian and *C.elegans* cell systems (Almendinger *et al.*, 2011).

The interaction between SNX9, SNX18, and SNX33 with N-WASP indicates that actin modulation is the mechanism by which these proteins are able to upregulate macropinosome formation. These direct interactions with actin regulating molecules have not been observed for the remainder of the SNX-PX-BAR family, and appears to be contingent on the presence of a SH3 domain. The roles played by SNX1 and SNX5 in promoting macropinocytosis are likely a result of accelerated membrane trafficking and turnover at the cell surface, although this has not been definitively demonstrated.

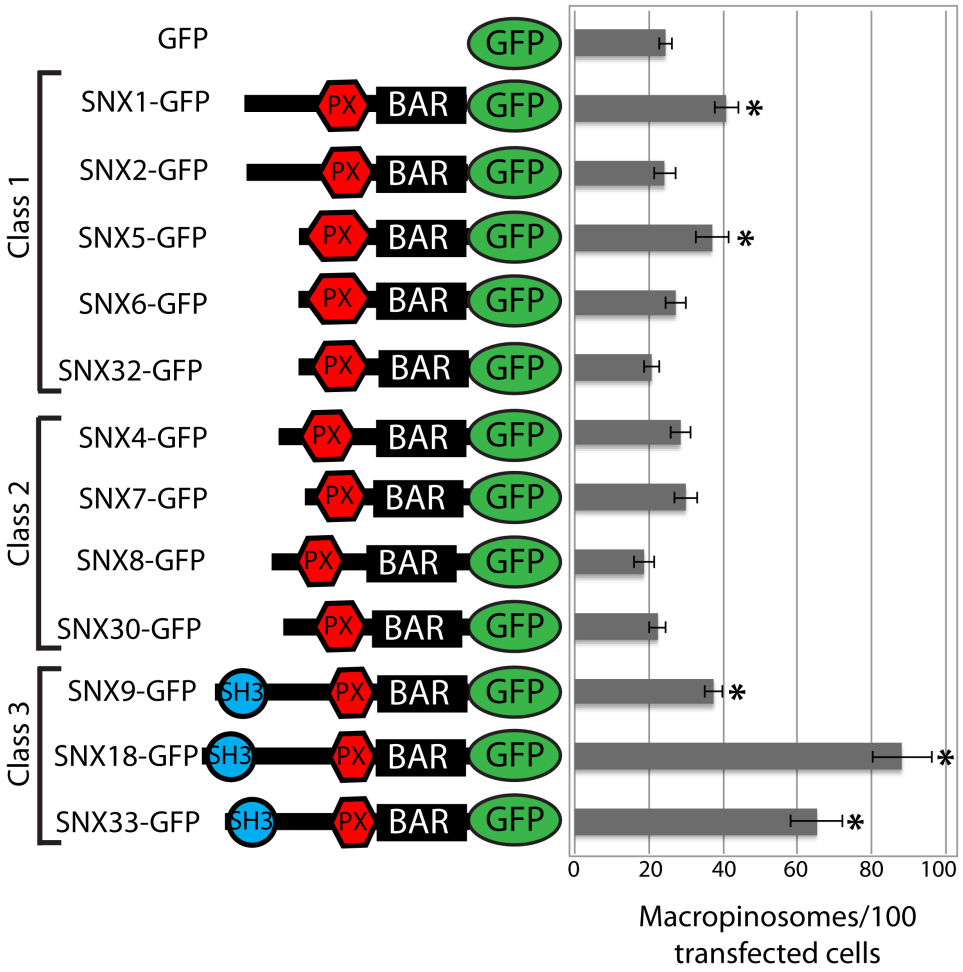

Figure 3: The SNX-PX-BAR family is involved in macropinosome formation. HEK-Flp-In cells transiently overexpressing GFP tagged members of the SNX-PX-BAR family (organized according to sequence similarity (Seet & Hong, 2006)) were assayed for macropinosome formation. The mean number of macropinosomes/100 transfected cells was quantitated over 8 replicates of 500 transfected cells for each condition. * denotes statistical significance ($p<0.05$) using the Student's T-test, performing pairwise analyses relative to cells transfected with GFP alone. Error bars denote S.E.M. Adapted from (Wang *et al*., 2010).

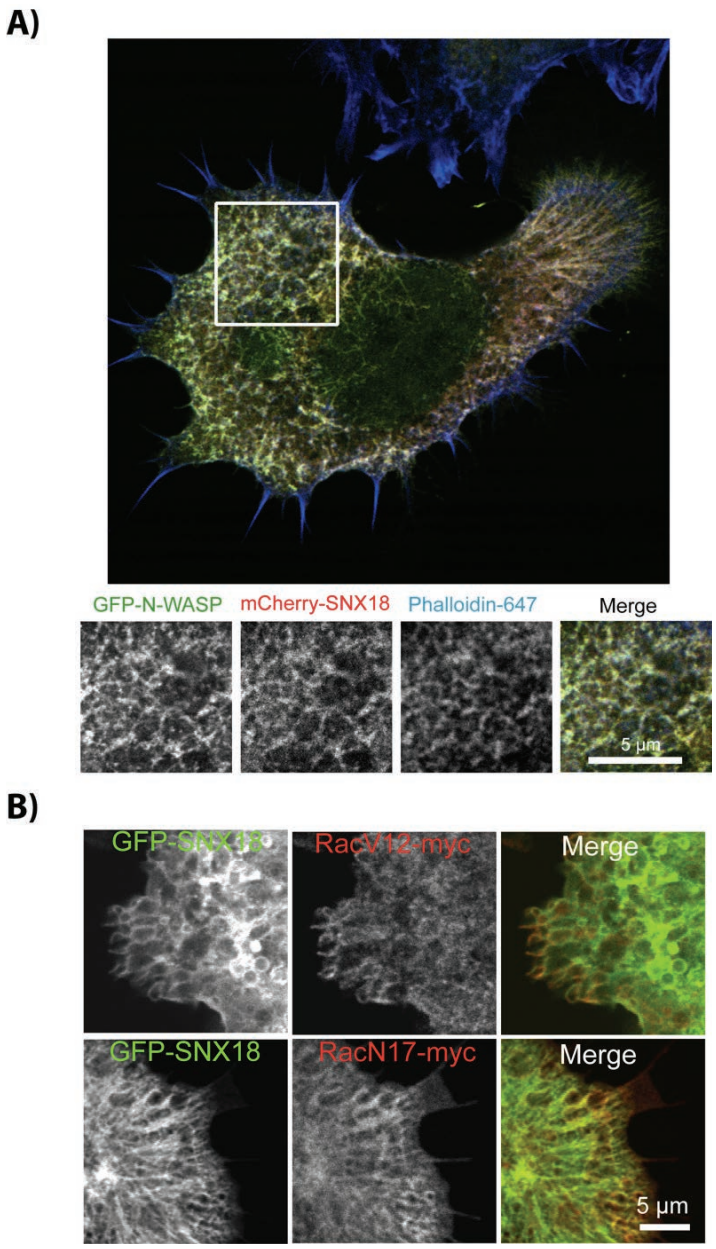

Figure 4: SNX18 associates with N-WASP, Rac1, and actin-positive filaments. A) HEK-Flp-In cells transfected with both pEGFP-N-WASP and pmCherry-SNX18 were fixed in 4% PFA and permeabilized with 0.1% TritonX100. The monolayers were then labelled with Alexa-647-conjugated Phalloidin to stain for filamentous actin-positive structures and mounted onto coverslips. B) HEK-Flp-In cells were transfected together with pEGFP-SNX18 and either myc-RacV12 (constitutively active) or myc-RacN17 (dominant-negative). After fixation with 4% PFA, the cells were permeabilized using 0.1% TritonX100 and labelled with a myc epitope antibody, followed by an Alexa-546-conjugated goat-anti-mouse IgG secondary antibody. Images were captured on the LSM 510 Meta confocal microscope. Scale bar = 5 µm

5 Macropinocytosis– gateway to cancer therapeutics?

Activation of the oncogenes Ras and Src both lead to elevated macropinocytosis (Porat-Shliom *et al.*, 2007; Veithen *et al.*, 1996), which in turn enhances receptor tyrosine kinase signalling (Schmees *et al.*, 2012) and induces metastatic migration (Platek *et al.*, 2004). The sustained activity of Rho GTPasespromote the formation of membrane ruffles (Hoppe & Swanson, 2004) while targeting PAK1 – a key regulator of macropinocytosis (Dharmawardhane *et al.*, 2000) that is highly expressed in ovarian, breast, and bladder cancers (Balasenthil *et al.*, 2004; Ito *et al.*, 2007; Schraml *et al.*, 2003). PI(3)K activity and phosphoinositide regulation are crucial to macropinocytosis, and when PTEN attenuation of PI(3)K signalling is compromised,tumorigenesisis initiated (Li *et al.*, 1997; J. T. Wang *et al.*, 2010). Clearly there is a significant overlap between the mechanisms regulating macropinocytosis and cancer and the insights into the former may help shape our understanding of the latter. The relative paucity in knowledge of the molecules involved in macropinocytosis signifies great potential for the discovery of novel cancer therapeutics and diagnostic markers through the targeting of previously unidentified genes and genetic pathways.

An example of the interconnectedness between the molecular regulation of macropinocytosis, cancer, and future therapeutics, is evidenced by studies revolving around the SNX protein family. Of the SNX proteins involved in the regulation of macropinocytosis, there is a growing body of evidence highlighting potential roles in cancer. SNX1 has been observed to be downregulated in a number of cancers, including ovarian cancer (Ju *et al.*, 2009), gefitinib-sensitive non-small cell lung cancer (Nishimura *et al.*, 2008), and colon cancer (Huang *et al.*, 2011; Nguyen *et al.*, 2006), serving as part of a potential set of molecular diagnostic markers for these classes of aberrant cellular growth. SNX5 has more recently been reported as a marker of papillary thyroid carcinoma (Ara *et al.*, 2012), showing the direct applicability of molecular insight into the SNX family towards cancer diagnostics. The SH3-PX-BAR subfamily comprising of SNX9, SNX18, and SNX33 all interact with actin modulators (Park *et al.*, 2010; Shin *et al.*, 2007; Zhang *et al.*, 2009) and have been shown to be fundamental to the regulation of mitotic progression and cell division (Ma & Chircop, 2012). These cellular events are precisely coordinated by SNX proteins and a plethora of other molecules, which if disrupted can lead directly to metastasis and tumorigenesis. Further research into the SNX protein family, many members of which remain largely unstudied, can help expand our understanding of the molecular networks involved in macropinocytosis, which possesses great potential for breakthroughs in clinical diagnostics and cancer therapeutics.

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