ACTIVATED FC RECEPTOR SUPRAMOLECULAR COMPLEX

by

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ABSTRACT

Phagocytosis is a part of immune response. IgG opsonized particles of greater that 1μm are recognized by Fcγ receptors on the surface of professional phagocytes such as macrophages and neutrophils. IgGs are part of immune system and is a cognate ligand of the Fc receptor. Live Cell Affinity Receptor Chromatography (LARC) was used to capture an activated Fcγ receptor supramolecular complex from the surface of live human neutrophils, by allowing IgG opsonized microbeads to bind to the cell surface. The cells were burst in PBS, collected and digested along side with controls. Isolated FcyR complex was analysed by LC-MS/MS. Fc and control experiment lists of SEQUEST correlated proteins were screened for a total cumulative score of at least 2400 and a minimum of three different peptides. This served as the basis of protein involvement in the FcyR mediated phagocytosis, which were then searched with *iHOP* for their interaction partners. Gathered interactions were then exported and Cytoscape, Osprey and String algorithms were used to generate network of interacting proteins. PAKs 2-4 and PAK6 were detected with LARC. PAK2 and PAK4 were predicted by algorithms to have a central role in particle uptake. From Western Blotting, endogenous PAKs2-4 and PAK6 were detected in murine macrophages. Immunofluorescent staining was then used to verity presence of these proteins in the forming phagosome and showed localization of PAKs to the phagosome. The same effect was observed with transfection of GFP constructs of PAKs. Upon transfection with dominant negative PAKs reduction in phagocytosis was observed. Thus p21 activated kinase accumulation during phagocytosis was observed and validated that LARC is an accurate and a reliable method of capture of activated supramolecular complex from the cell surface.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr. John Marshall, for his guidance support and motivation without which this would not be possible. He let me learn, on numerous occasions, not only the scientific principals and the importance of sound science, but the life lessons that I will carry with me through the years.

I, most definitely, thank and appreciate all the support from people working side by side with me in the lab: David Vance, Monica Tucholska, Angelique Florentinus and Veronica Barbisan. Without these people days would be filled with gloom and the never ending experiments would have been much worse. Thank you for your advice and support. I want to thank all other graduate students, who cheered each other on; Ryerson University technicians for their patience and supply of equipment; and my supervisory committee, Dr. Kolios and Dr. Killeen, for their valuable guidance.

Family and friends are those that I have to thank the most, for putting up with me through ups and downs of these past couple of years.

Thank you.

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LIST OF SELECTED ABBREVIATIONS

PAK	p21 activated kinase
DGK	diacyl glycerol kinase
DAG	diacyl glycerol
PI3K	phosphotidylinositol 3 kinase
LARC	Live-cell Affinity Receptor Chromatography
GFP	green fluorescent protein
EGFP	enhanced green fluorescent protein
SRBC	sheep red blood cells
IgG	immunoglobulin
iHOP	information hyperlinked over proteins
FcγR	receptor binding constant portion (Fc) of IgG
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
ITAM	immunoreceptor tyrosine based activating motif
AID	auto inhibitory domain
PKC	protein kinase C
PLC	phospholipase C
SYK	spleen tyrosine kinase
PIP3	phosphotydilinositol-3,4,5-trisphosphate
PIP2	phosphotydilinositol-4,5-bisphosphate
NOX	NADPH Oxidase
NOS	nitric oxide synthase
ROS	reactive oxygen species
Arp2/3	actin related protein complex 2/3
WAVE	WASP-family verprolin-homologous protein
WASP	Wiskott–Aldrich syndrome protein
ERK	extracellular regulated kinase
MAPK	mitogen activated protein kinase
WIP	Wiskott–Aldrich syndrome protein interacting protein
LSM	laser scanning microscope
TNF	tumor necrosis factor
HGF	hepatocyte growth factor
MRX	X-linked mental retardation syndrome
HPLC	high performance liquid chromatography
IP3	inositol-1,4,5-triphosphate

INTRODUCTION

Phagocytosis

Receptors expressed on the cell surface recruit proteins to the inner portion of the cell membrane (Corbett-Nelson, Mason et al. 2006). Formation of the active supramolecular complex requires rearrangement of cytoskeletal proteins and recruitment of proteins that are essential for the proper receptor function. Hence, the activated receptor supramolecular complex is comprised of the receptor membrane cytoskeleton and the associated proteins in the activated plasma membrane sub domain (Marshall, Booth et al. 2001).

Phagocytosis is a process internalization of particle such as bacteria, parasites or dying cells of size greater than 1 μm (Garcia-Garcia and Rosales 2002). Fc receptor mediated phagocytosis is triggered by binding of IgG opsonized particles to the surface expressed Fcγ receptors (Greenberg and Grinstein 2002). Molecular interactions with the Fc receptors not only result in phagocytosis, but also other cellular responses including antibody dependent cell mediated cytotoxicity, cytokine production and release of pro inflammatory messengers (Daeron 1997; Sanchez-Mejorada and Rosales 1998), all of which play a role in defence against pathogens.

The professional phagocytes are capable of particle engulfment, they are: neutrophils, monocytes, macrophages, dendritic and mast cells. Although retinal pigmental epithelium cells and Sertoli cells are not termed professional phagocytes they also phagocytose. A large part of the information that is available regarding the signaling events that occur during phagocytosis come from the studies done in murine macrophage cell lines. Signaling differences between macrophages and neutrophils, as expected, have been observed. It was reported that ERK in

FcγR mediated phagocytosis in macrophages is activated but not required for particle uptake (Karimi and Lennartz 1998), while its inhibition in human neutrophils inhibits phagocytosis (Mansfield, Shayman et al. 2000).

There are several Fc receptor isoforms. FcγRI, FcγRIIa and FcγRIIIb are the main receptors of human neutrophils (McKenzie and Schreiber 1998). Differential expression of FcγR isoforms such as presence of FcγRIIIB exclusively in neutrophils which lacks a transmembrane portion and the cytoplasmic tail and is linked to the membrane through glycophosphatidylinositol (GPI); (*Figure 1*); (Ravetch and Bolland 2001) and may result in signalling variation among phagocytic cells. Murine macrophages have FcγRI, FcγRIIa, FcγRIIb and FcγRIV, of which FcγRIIb has been shown to have a negative effect on phagocytosis (*Figure 1*) (Garcia-Garcia and Rosales 2002).

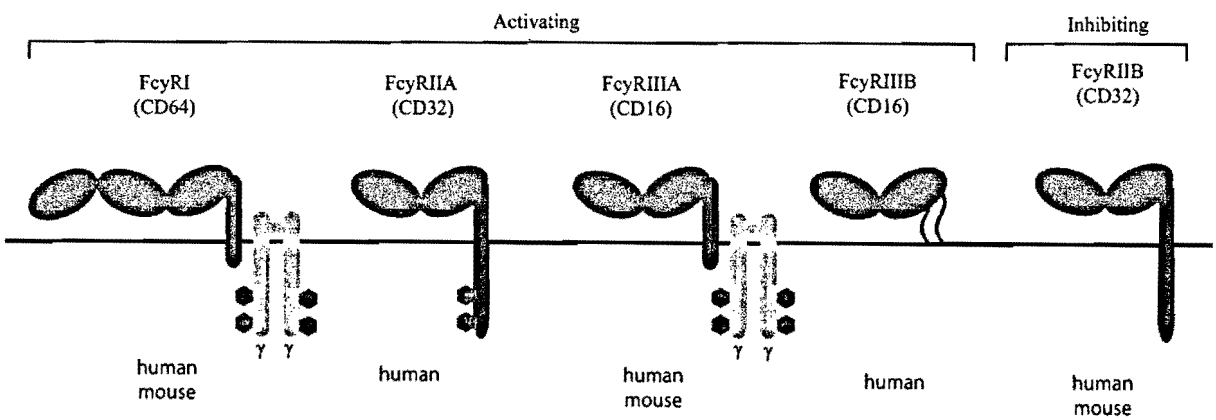


Figure 1. Fcγ receptors. FcγR vary structurally resulting in different affinities for IgGs. Phosphorylation of the intracellular portion of the Fc receptors or ITAMs by Src family kinase upon binding of IgG opsonized particles initiates cascade of signaling events leading to particle uptake. FcγRIIB contains an ITIM phosphorylation of which is inhibitory to phagocytosis.

Binding to Fcγ receptor (FcγR) by Fc portion of IgG initiates intracellular signaling through phosphorylation of FcγRs' immunoreceptor tyrosine-based activating motifs (ITAMs)

(Isakov 1997) by Src family tyrosine kinases (*Figure 2*); (Fitzer-Attas, Lowry et al. 2000). Phosphorylation of ITIMs (immunoreceptor tyrosine-based inhibiting motifs) on FcγRIIB, on the other hand, leads to inhibition of phagocytosis.

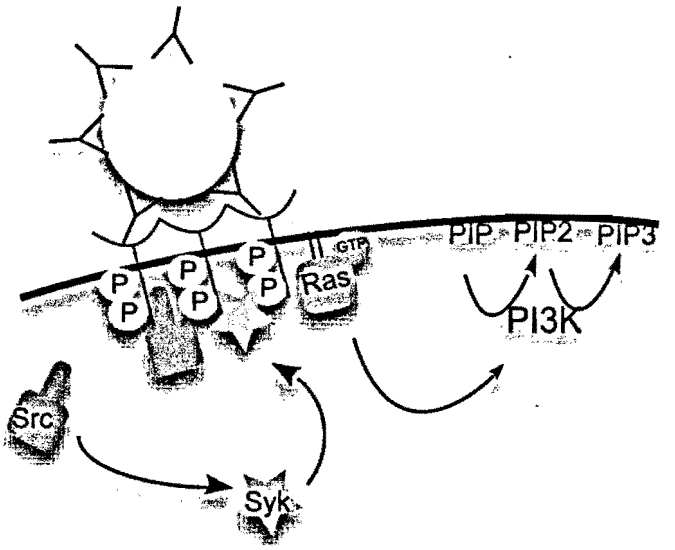


Figure 2. Early signaling events during Fc mediated phagocytosis. Src kinase phosphorylates ITAMs leading to recruitment of more Src to the site of phagocytosis. Activated ITAMs then recruit Syk kinase leading to activation of PI3K.

Phosphorylated ITAMs then recruit more Src family kinases (Kiefer, Brumell et al. 1998), and serve as docking sites for the SH2 domains of Syk (Turner, Schweighoffer et al. 2000). This leads to activation of downstream signaling events resulting in actin polymerization, formation of phagocytic cup and particle internalization. Many signaling proteins are activated and recruited to the site of phagocytosis, including PI3K, PLCs, PLD, PKC, ERK, JNK, small Rho GTPases family proteins, numerous guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), as well as proteins required for actin rearrangement to occur including WASP, Arp2/3 complex, cofilin and myosins (Greenberg and Grinstein 2002).

PI3K is involved in many different signaling events including cell growth and movement. Syk phosphorylation leads to activation of PI3K (Garcia-Garcia and Rosales 2002). PI3K is required for phagocytosis and its activation has been shown to be required for pseudopod extension (Cox, Tseng et al. 1999) and phagosome closure (Araki, Johnson et al. 1996)s. PI3K inhibition leads to arrest in phagocytosis (Cox, Tseng et al. 1999). Type II PI3Ks convert PI(4,5)P into PI(3,4,5)P by phosphorylation of the 3' group on PIP2, which can activate a number of GEFs. Depletion of PI(4,5)P is accompanied by an increase of PI(3,4,5)P in the nascent phagosome membrane, with the concentration of PI(3,4,5)P remaining high until phagosome closure. Lipid mobility was shown to be reduced in the membrane around the particle while lipids on the unstimulated membrane remained freely mobile (Marshall, Booth et al. 2001; Corbett-Nelson, Mason et al. 2006). PIP3 accumulation at the plasma membrane allows regulation of filopodia extension through regulation of Cdc42. Rac1 is also positively regulated by PI(3,4,5)P (Araki, Johnson et al. 1996; Srinivasan, Wang et al. 2003). ERK activation during FcγR mediated particle uptake was demonstrated to be dependent on PI3K (Coxon, Rane et al. 2000). Type I PI3K phosphorylates PIP converting it into PIP2 which serves as a substrate for PLC. Type III PI3K converts PI to PIP and this is important for later stages of phagocytosis and may contribute to activation of NADPH oxidase.

Phosphoinositide specific PLC isoforms catalyze hydrolysis phospholipids, in this process phosphatidylinositol-4,5-bisphosphate is converted into DAG and IP3 (inositol-1,4,5-triphosphate) leading to activation of PKC and an increase in cytosolic calcium concentration, respectively (*Figure 3*); (Wilde and Watson 2001). Ca^{2+} increase is associated with phagocytosis with the highest concentration found in the cytoplasm around the phagocytic cup (Sawyer,

Sullivan et al. 1985). However, at low Ca^{2+} concentration phagocytosis is not disrupted (Di Virgilio, Meyer et al. 1988). Phagocytic cup closure was also shown to be dependent on activation of PKC (Larsen, DiGennaro et al. 2000).

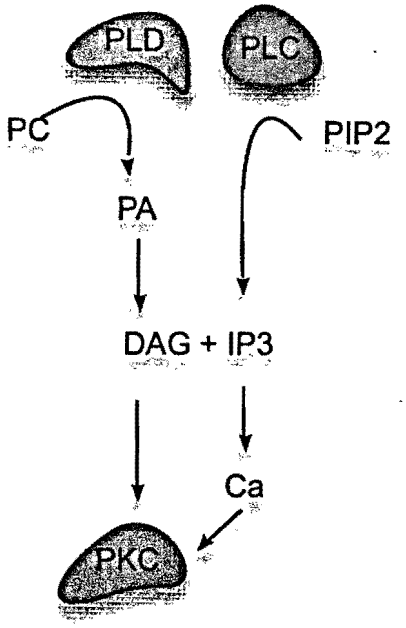


Figure 3. Phospholipids involved in phagocytosis.

PLD catalyses hydrolysis of phosphatidylcholine (PC) into phosphotidic acid (PA) which can activate PLCγ and PLA2 (Lennartz 1999). PI(3,4)P is required for PLD activity. Expression of PLD mutants correlates with a decrease in phagocytosis (Delarue, Taylor et al. 2001). It was also demonstrated that both PLD1 and PLD2 are essential in phagocytosis of IgG opsonized particles in murine macrophages (Corrotte, Chasserot-Golaz et al. 2006). PLD1 is found on endosomes during the formation and maturation of phagosomes while PLD2 is associated with the plasma membrane and is activated upon stimulation of FcγRs leading to production of PA, in turn stimulating PIP2 production which leads to activation of PLD1 on the late endosomes (Corrotte, Chasserot-Golaz et al. 2006) that are then involved in fusion to the phagosome. PLD

can also activate PKC via conversion of PLD-generated PA into DAG by phosphatidic acid phosphatase 1 (Lennartz 1999).

Small Rho GTPases play a prominent role in phagocytosis. Members of the family include proteins such as Rac, Cdc42 and RhoA. Involvement of Cdc42 in phagocytosis is well established. It is mainly involved in modulation of actin dynamics leading to formation of the phagocytic cup. Cdc42 is recruited early in the process around the tips of extending pseudopods (Hoppe and Swanson 2004). It must be inactivated to allow depolymerization of F-actin around the phagosome (Hoppe and Swanson 2004).

Cdc42 is critically involved in initiating the early events in phagocytosis through its ability to activate p21 activated kinases and WASP that triggers the localized polymerization of actin (Robinson and Badwey 2002). Cdc42 was shown to co localize with WASP and actin in the core of the podosome (Linder, Nelson et al. 1999). Actin polymerization is driven by Arp2/3 complex activation through the WAVE complex downstream of Rac (*Figure 4*); (Bosse, Ehinger et al. 2007).

Rac is classified as a member of the Rho GTPases (Bokoch 2005). Rac has been shown to be present during phagocytosis and is involved in variety of cellular functions, including cytoskeletal reorganization, regulation of cell growth and activation of protein kinases (Bokoch 2005). Rac1 and Rac2 are closely related, low molecular weight GTP-binding proteins that have different roles during phagocytosis. Rac 1 is involved in the formation of the phagocytic cup while Rac2 was linked to NADPH oxidase activation (Gu, Jia et al. 2001). Both isoforms have been implicated in regulation of phagocyte NADPH oxidase with Rac2 being the predominant in that function in human neutrophils (Heyworth, Knaus et al. 1993; Bokoch 2005). The amount of

Rac1 and Rac2 that is expressed may play a role in the ability to regulate the Nox complex. Human neutrophils have been shown to express between 1/40 (Heyworth, Bohl et al. 1994) and ¼ (Li, Yamauchi et al. 2002) of Rac1 compared to Rac2, but show equal proportions in murine neutrophils (Li, Yamauchi et al. 2002). Rac1/2 are also differentially expressed during phagocytosis: activation of Rac1 occurs in phagocytic cups and during closure while Rac2 is active during phagosome closure with activity at the lower portion of the phagocytic cup (Hoppe and Swanson 2004).

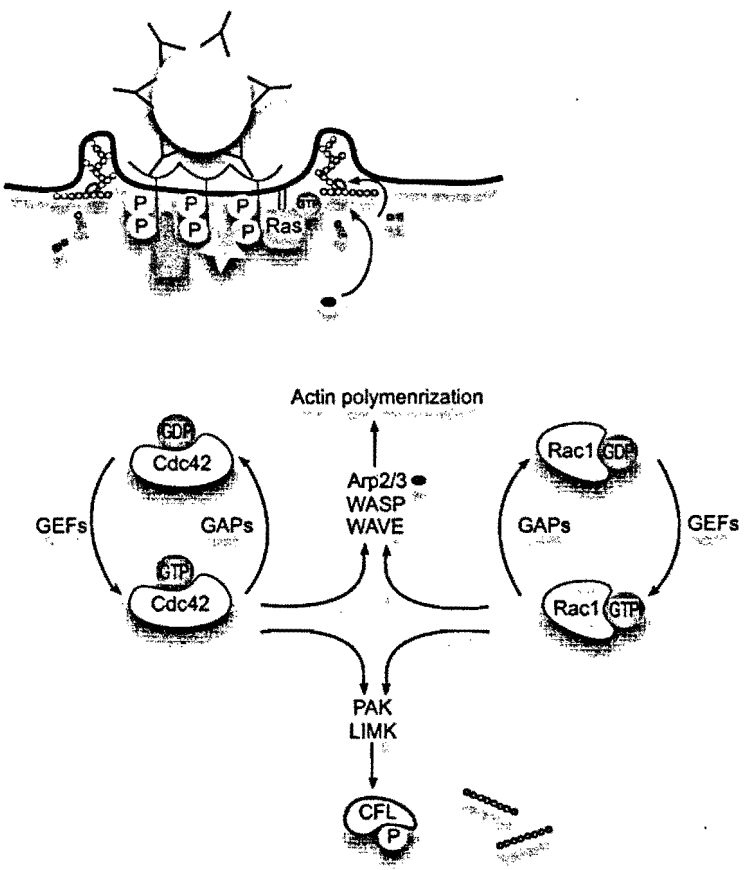


Figure 4. Role of small Rho GTPases during phagocytosis. Cdc42 and Rac1 both play a role in actin dynamics. Rac1/Cdc42 lead to elongation of actin filaments by stimulation of Arp2/3 complex via WAVE and WASP activation. Another mode of action of Rac and Cdc42 is through p21 activated kinases and LIM kinases activation of which arrests function of cofilin, required for severing of actin. Together Rac and Cdc42 act on Arp2/3 complex and cofilin regulating actin polymerization.

DOCK180 (a guanine nucleotide exchange factor) and ELMO (an adaptor protein) function together for optimal Rac activation (Lu and Ravichandran 2006). Also signaling from focal adhesions leads to the local inactivation of Rac1 by inhibiting paxillin (a multi-domain focal adhesion adaptor molecule) via dephosphorylation and blocking of the DOCK180/ELMO signaling (Lu and Ravichandran 2006).

Rho was found to be essential for actin accumulation around the pseudopod extensions and for FcγR-mediated Ca2+ signaling (Hackam, Rotstein et al. 1997). RhoA activity is increased in the presence of active PTP-PEST (protein tyrosine phosphatase 12) (Sastry, Rajfur et al. 2006) which is involved in membrane ruffling and motility and is associated with a decrease in Rac1 (Sastry, Lyons et al. 2002). RhoA activates ROCK, leading to myosin light chain (MLC) phosphorylation, which is dependent on RhoA and ROCK activities (Vicente-Manzanares, Cabrero et al. 2002). It was demonstrated that MAP3K1 binds to p115 RhoGAP and that RhoA can regulate MAP3K1 by binding to its amino terminus (Gallagher, Gutowski et al. 2004).

Cytoskeletal rearrangement is crucial to progression of particle uptake. Actin dynamics are central during phagocytosis with actin localization at the sites of particle attachment and in pseudopod extension during particle engulfment. During actin polymerization, actin monomers are added to the actin's free barbed ends, the number of which has to be increased for efficient polymerization to occur. There are three mechanisms by which free barbed ends can be generated. The first one involves uncapping existing barbed ends by gelsolin related proteins or by a capping protein (Hartwig, Bokoch et al. 1995). The second mechanism involves *de novo* actin filament nucleation by the Arp2/3 complex (Pollard, Blanchoin et al. 2000). The third way

in which barbed ends can be freed is through severing of actin by cofilin (Chan, Bailly et al. 2000).

Cofilin is a final GTPase effector that binds actin. It promotes actin depolymerization by dissociation of actin monomers from the pointed end of actin filaments (Bamburg 1999). Formation of free barbed ends can also be done by cofilin through severing of actin filaments (Chan, Bailly et al. 2000). Availability of free barbed ends can increase actin nucleation of the Arp2/3 complex (DesMarais, Ghosh et al. 2005).

WASP and WAVE family proteins are scaffolds that link upstream signals to the activation of the ARP2/3 complex, leading to actin polymerization. The VCA region of WASP acts as a platform for actin polymerization: CA domains bind to the Arp2/3 complex while V domain binds actin monomers bringing them to the Arp2/3 complex (Takenawa and Suetsugu 2007). The WH1 domain of WASP can bind to and form heterodimers with the WIP family of proteins which help to maintain stability of WASP complex (Ramesh, Anton et al. 1997; Krzewski, Chen et al. 2006). WIP was shown to act as a scaffold that links Nck and CrkL with WASP at the sites of active actin nucleation (Frischknecht, Moreau et al. 1999; Moreau, Frischknecht et al. 2000; Sasahara, Rachid et al. 2002). WASP was shown to bind to Cdc42 (Symons, Derry et al. 1996). Profilin contributes to Cdc42-induced nucleation of actin filaments in high speed supernatant of lysed neutrophils. It enhances nucleation by activating Arp2/3 complex (Yang, Huang et al. 2000). Proline rich domains of WASP and NWASP can bind proteins with SH3 domain and profilin which has a role in supplying actin monomers to WASP (Yang, Huang et al. 2000). WAVE is also capable of binding profilin (Miki, Suetsugu et al. 1998). WAVE promotes Arp2/3 dependent actin polymerization downstream of Rho-GTPase

activation (*Figure 4)*. Initial mechanism of regulation of WAVE induced actin nucleation sites that Rac and Nck cause disassociation of the WAVE complex, releasing active WAVE and leading to actin polymerization (Eden, Rohatgi et al. 2002). WAVE2 relays Rac signaling without binding to it.

The Src family of tyrosine kinases phosphorylate WASP which is enhanced by Cdc42 binding (Torres and Rosen 2003) together contributing to activation of the Arp2/3 complex (Takenawa and Suetsugu 2007).

Cortactin is an Src substrate that drives actin polymerization by activating the Arp2/3 complex and also stabilizes the cortical actin network (Perrin, Amann et al. 2006). Cortactin binds the Arp2/3 and to the actin filaments, which helps activate the Arp2/3 complex and stabilize the newly created branches between the filaments (Weaver, Young et al. 2003). Expressed mutants of cortactin that decreased binding of Arp2/3 complex or dynamin 2 also decreased actin dynamics (Schafer, Weed et al. 2002). Arp2/3 mediated actin polymerization regulates the accessibility of cortactin to dynamin 2 and drive the fission of clathrin-coated pits in an actin polymerization dependent manner (Zhu, Zhou et al. 2005).

Dynamin 2 is a GTPase recruited early during phagocytosis in macrophages. Since PI3K inhibitors blocked dynamin 2 from being recruited to the site of phagocytosis (Gold, Underhill et al. 1999) it should function downstream of PI3K. It is recruited to the forming phagosome through interaction with amphiphysin IIm which is also required for phagocytosis (Gold, Morrissette et al. 2000). Interaction of Dynamin 2 with cortactin was shown to be involved in organization of actin filaments at membranes (Schafer, Weed et al. 2002). Actin rearrangement may also be regulated by dynamin 2 via its interaction with profilin. Dynamin was shown to

modulate localization and function of Rac (Schlunck, Damke et al. 2004) through which it may regulate cytoskeletal events during phagocytosis. It is also associated with endocytic vesicles (Gold, Underhill et al. 1999) which fuse with growing phagosomes.

Myosins are motor proteins that provide the propulsive force for movement from their interaction with actin. ATP hydrolysis by myosins provides energy which is converted into movement of the actin bound myosins. A number of different myosins were shown to be involved in phagocytosis; among them are myosin I (Allen and Aderem 1995), myosin II (Olazabal, Caron et al. 2002) myosin Va (Al-Haddad, Shonn et al. 2001), myosin VII (Titus 1999), myosin X (Cox, Berg et al. 2002). Myosins IC, V and IXb were found around phagosomes in macrophages (Swanson, Johnson et al. 1999). Myosin X was shown to be involved in PI3K dependent signaling (Cox, Berg et al. 2002). The ERK signaling cascade was shown to activate MLCK which then activates myosins (Mansfield, Shayman et al. 2000). Activation of ROCK by Rho inactivates myosin phosphatase which allows phosphorylation of myosin light chain by MLCK (*Figure 5*); (Vicente-Manzanares, Cabrero et al. 2002). The presence of many different myosins around the forming phagosome is an indicator of myosin involvement in the internalization of the forming phagosomes with them either acting as motor and/or structural proteins. Myosins may also play a role in movement of vesicles to the phagocytic cup during focal exocytosis.

Oxidative burst is another process that has been associated with phagocytosis. The first NADPH oxidase (NOX) to be identified was in phagocytic cells and it remains one of the best studied ones (Bokoch and Knaus 2003). NADPH oxidase is a membrane associated enzyme complex composed of cytosolic and membrane associated components (Babior 1999; Bokoch

and Diebold 2002), activation of which leads to generation of reactive oxygen species (ROS); (*Figure 6*); (Babior 1999). Cytosolic subunit p47phox is phosphorylated upon leukocyte activation leading to translocation of p47phox/p67phox to the internal membrane where they interact with flavocytochrome b558 (cyt b), forming an active oxidase (Babior 1999). The combined action of ERK, Akt, and PKC results in translocation of p47phox resulting in the assembly of NOX in human neutrophils (Chen, Powell et al. 2003). NADPH oxidase is also regulated by Rho GTPases with Rac2 predominantly functioning in human neutrophils (Heyworth, Knaus et al. 1993). The GTP bound form of Rac2 activates Nox by binding to p67phox subunit (Diekmann, Abo et al. 1994).

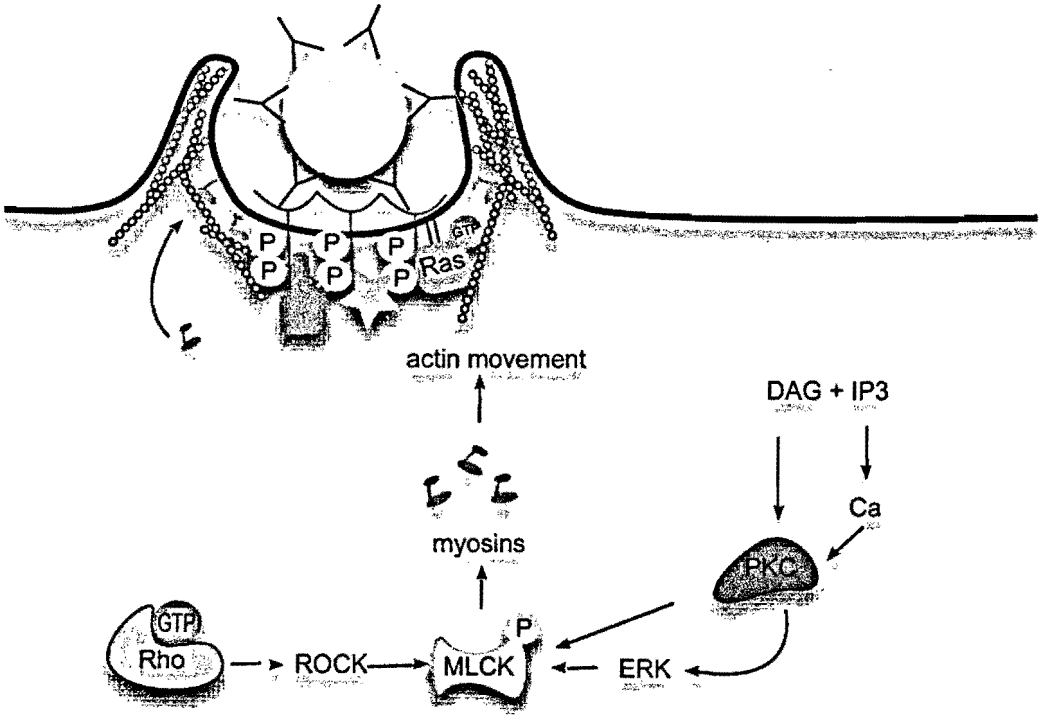


Figure 5. Myosin activation during phagocytosis. Myosins are motor proteins leading to actin movement. Myosins are activated by the phosphorylation of myosin light chain kinase (MLCK). MLCK phosphorylation is a result of either action of PKC, ERK or RhoGTP.

NOX complex and nitric oxide synthase (NOS2) may function together to increase the destruction of pathogens via the formation of reactive species. NOS2A generated NO has been shown to be involved in innate immunity (Diefenbach, Schindler et al. 1999) by being a precursor for cytokine signaling. Rac1 and Rac2 were shown to be interacting partners of NOS2 in activated murine macrophages. Rac2 however may be involved in stimulation of NOS2 activity and may affect its cellular redistribution (Kuncewicz, Balakrishnan et al. 2001). Src is capable of NOS2A tyrosine phosphorylation (Hausel, Latado et al. 2006).

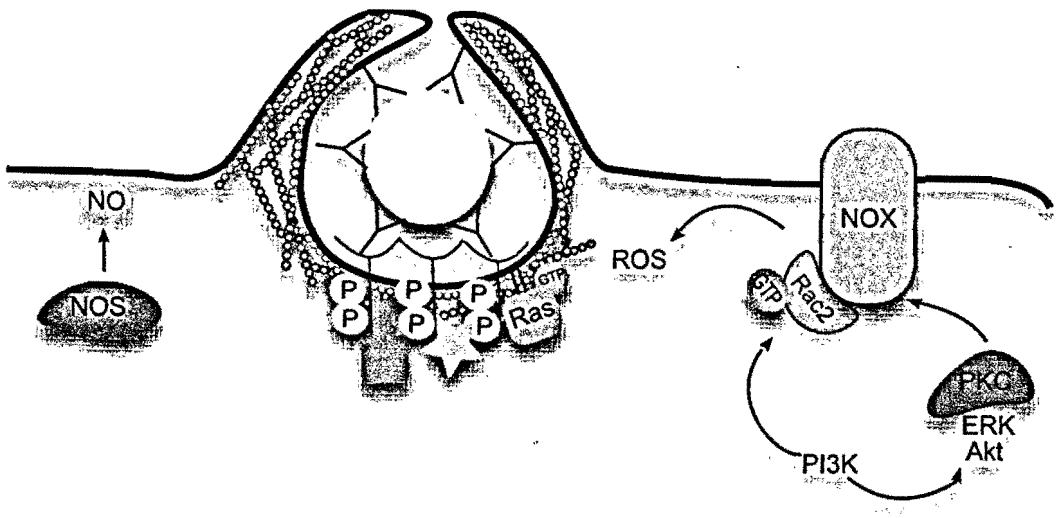


Figure 6. Respiratory burst in phagocytosis. Oxidative burst is one of the processes associated with phagocytosis. NOX complex is activated through combined action of PKC, ERK and Akt. The binding of Rac2 to p67phox subunit of NOX leads to activation of the complex. The active NADPH complex forms reactive oxygen species inhibitory to pathogens being taken. Nitric oxide synthase leads to production of NO, which upon reaction with oxygen can form NO2 or ONOO.

Membrane replenishment during phagocytosis allows macrophages to engulf a large amount of particles from their surface, unlike the enucleated human neutrophils which lack intracellular granules and thus cannot ingest as many particles (Roos, Voetman et al. 1983). Focal exocytosis has been shown to play a role in supplying the ever growing need for membrane

at the site of particle intake. Lack of SNAREs, proteins involved in membrane fusion events, has been shown to reduce phagocytosis (Booth, Trimble et al. 2001). Rab GTPases are associated with vesicle trafficking and are implicated in focal exocytosis. Rab11 deficient cells have reduced phagocytosis (Cox, Lee et al. 2000). Rab7 which regulates traffic to and from endosomes, is important for phagosome maturation in macrophages (Harrison, Bucci et al. 2003). Dynamin 2 was also implicated in movement of vesicles to the forming phagosome (Di, Nelson et al. 2003).

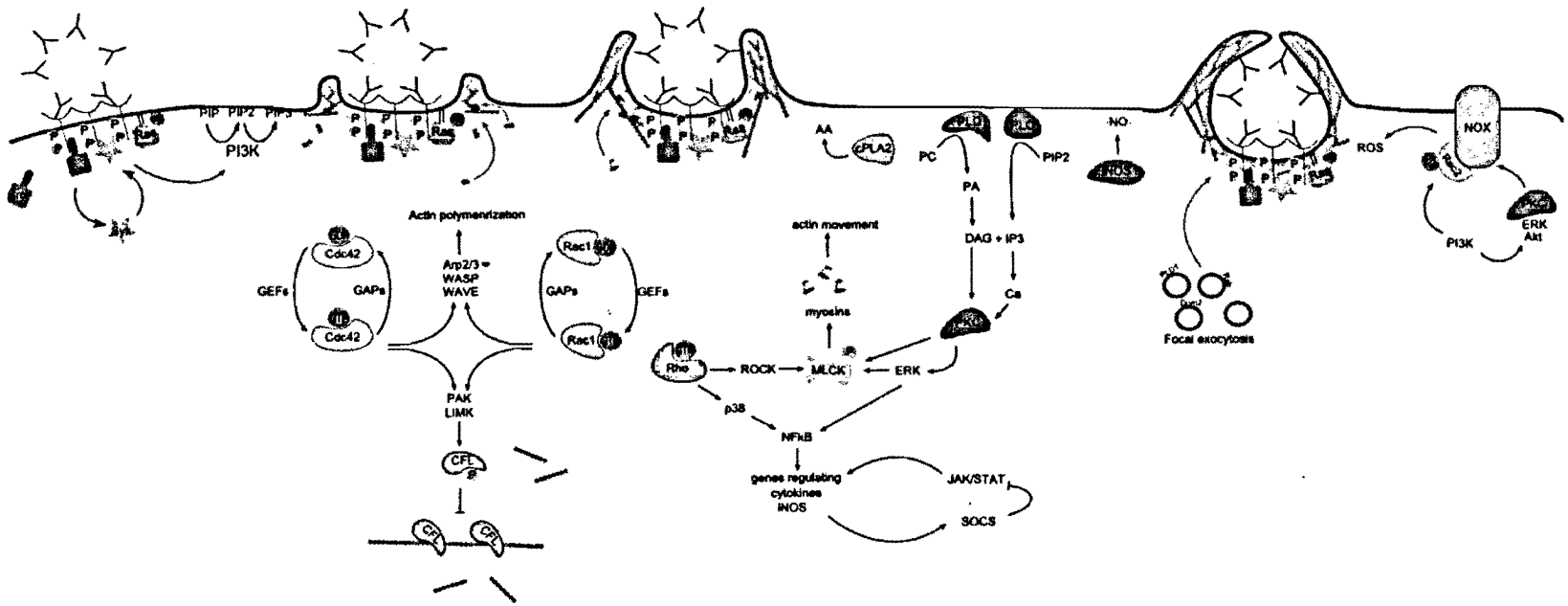

PIP
PIP2
PIP3
PI3K
Actin polymerization
Cdc42
GEFs
GAPs
Cdc42
Arp2/3
WASP
WAVE
Rac1
GAPs
GEFs
Rac1
PAK
LIMK
CFL
CFL
CFL
actin movement
myosins
Rho
ROCK
MLCK
ERK
p38
NFkB
genes regulating
cytokines
iNOS
JAK/STAT
SOCS
AA
cPLA2
PC
PA
PIP2
DAG + IP3
Ca
NO
iNOS
Focal exocytosis
ROS
NOX
PI3K
ERK
Akt

Figure 7. Summary of signaling during FcγR mediated phagocytosis in neutrophils.
Binding of IgG coated microbeads to Fc receptors leads to their phosphorylation through recruitment of Syk and Src family kinases leading to activation of PI3K which is followed by activation of Cdc42 by guanine nucleotide exchange factors specific for it. GTP bound Cdc42 then stimulates activation of the Arp2/3 complex leading to actin nucleation and polymerization. Cdc42 activation also leads to downstream phosphorylation of cofilin which inhibits severing of actin filaments. Rac1 has similar effect on cofilin and Arp2/3 complex however it is activated later on during phagocytosis (Hoppe and Swanson 2004). Internalization of the phagosome can be done through motor action of myosins which can be activated through Rho-ROCK-MLCK action or via PKC mediated phosphorylation of MLCK. NADPH oxidase activity is dependent on regulation of its subunits by Rac2 (Bokoch 2005), PKC, ERK and Akt action (Chen, Powell et al. 2003) that leads to generation of reactive oxygen species. NO is also produced during phagocytosis which is a result of signaling through activation of p38 and ERK which leads to iNOS gene transcription and regulation of pro-inflammatory cytokines.

Live-cell Affinity Receptor Chromatography

In depth characterization of FcγR mediated phagocytosis and capture of the fully functional supramolecular receptor complex signaling events was made possible through manual screening of proteins in an MSMS database developed previously using the method of Live-cell Affinity Receptor Chromatography (LARC) (Jankowski, Zhu et al. 2008). LARC is based on the *in situ* isolation of fully functional supramolecular FcγR complex. LARC is an extension of previously established methods that are based on the binding of ligand microbeads to the cell surface of nascent phagosomes (Marshall, Booth et al. 2001). Some phagocytic proteins are thought to remain on the internalized micro bead after phagosome closure (Garin, Diez et al. 2001). Assembly of the receptor complex and its capture from the cell surface is accomplished by micro beads coated with Fc receptor cognate ligand (IgG) followed by careful comparison to control beads is needed to identify proteins that are specifically bound to the Fc receptor.

There are a number of algorithms available that can be used to construct protein interaction networks. Specifically *Information Hyperlinked Over Proteins (iHOP)* (Hoffmann and Valencia 2004) allows navigation of PubMed abstracts and provides easy access to published data as relevant to the gene or protein product queried. Relevant protein relationships can be stored and used in developments of interaction models which can be exported (Hoffmann and Valencia 2005) and visualized through network building software such as Cytoscape (Shannon, Markiel et al. 2003), String (Jensen, Kuhn et al. 2009) or Osprey (Breitkreutz, Stark et al. 2003) which allows visualization, modification and manipulation of complex gene networks. Since my research we focused on p21 activated kinases, these proteins are reviewed in some detail in the following pages.

p21 activated kinases (PAKs)

PAKs are serine/threonine protein kinases with 6 isoforms identified in both human and mouse. They are divided into two groups: PAKs 1, 2, 3 belong to group I and are distinct from the second group (PAKs 4,5,6) in that they are activated by Rho GTPases, specifically Cdc42 and/or Rac1 (Eswaran, Soundararajan et al. 2008). Separation of the two groups is not only based on their regulation but also on the protein structure (*Figure 8*). A highly conserved C-terminal catalytic kinase domain is common to both group I and II PAKs, the regulatory N-terminal domain is the source of structural variation. Group I PAKs contain GTPase (p21) binding domain (PBD) and an auto-inhibitory domain (AID) that overlaps PBD. AID is absent in group II PAKs. AID acts as a pseudo substrate forcing the kinase domain to adopt an autoinhibited inactive conformation. Upon Rho GTPase interaction with PBD, the activating loop is phosphorylated which is otherwise prevented.

The functions of different PAK isoforms were studied through generation of genetic knockout mice. PAK1, 3 and 5 gene knockout mice were viable, while deletions of PAK2 and 4 genes resulted in embryonic lethality (Eswaran, Soundararajan et al. 2008). PAK4 deletion mainly lead to lethality from defects in the fetal heart. Also, neuronal development and axonal outgrowth abnormalities were reported (Qu, Li et al. 2003). Mice that were double knockouts with PAK5 and PAK6 genes had behavioural and locomotory defects, but they were fertile and viable (Nekrasova, Jobes et al. 2008).

Different expression patterns are also observed from isoform to isoform, with ubiquitous expression of PAK2 while PAK1, 3 and 5 are detected in the brain, but not exclusively. PAK4

and PAK6 have high expression in testis and prostate, with PAK4 also being found in the colon (Eswaran, Soundararajan et al. 2008).

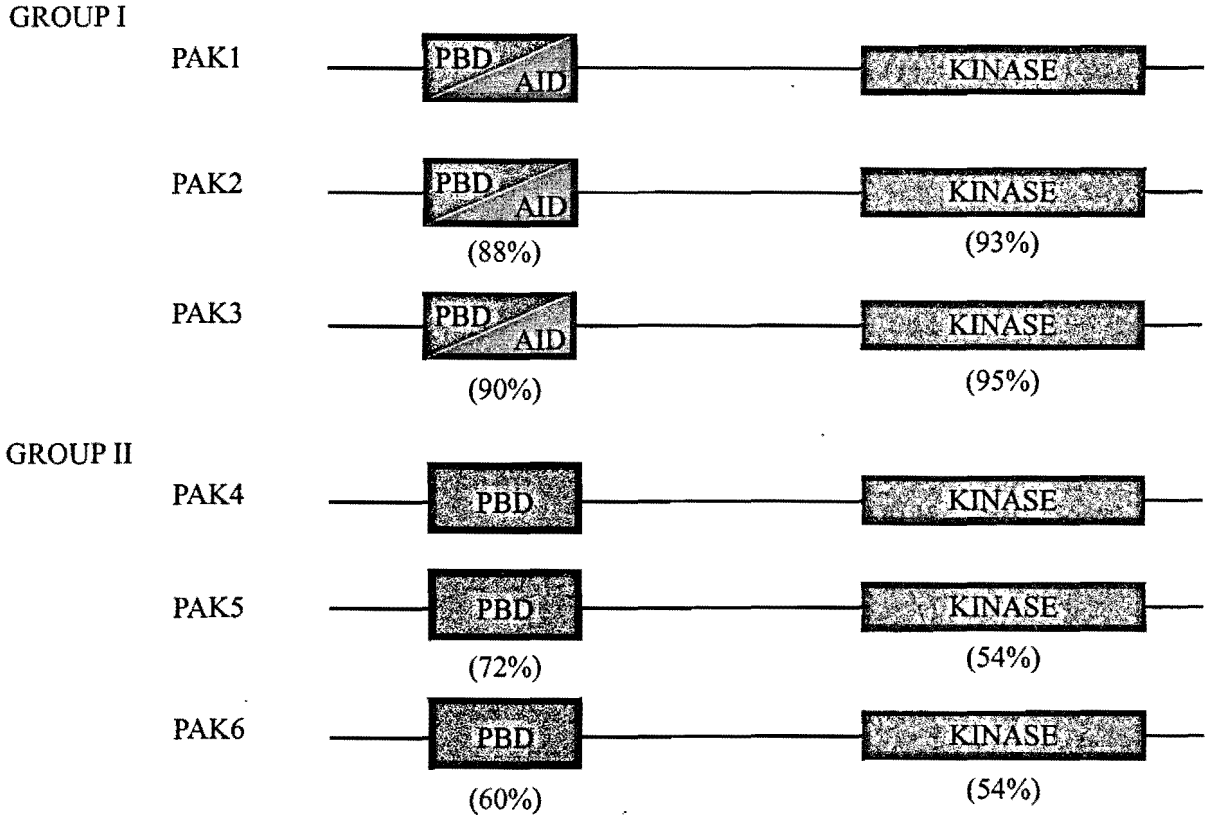


Figure 8. Group I and Group II PAKs structure comparison.
Despite a high degree of variation in the PBD, especially in the Group II PAKs, the kinase domains are highly conserved. Group II PBDs share only 40% similarity to those of Group I PAKs. The degree of similarity is shown in per cent below the domains relative to PAK1 for Group I and PAK4 for Group II. Group I PAKs have an auto inhibitory domain which is lacking in Group II.

PAK2

PAK2 can be activated by small G proteins, Rac1, Cdc42 or through caspase cleavage (Rudel and Bokoch 1997). Caspase cleavage leaves a 34kDa activated Pak2 lacking most of the N-terminal regulatory domain including the AID domain. Caspase 3 cleaves PAK2 adjacent to Asp-212 and this residue is inaccessible in PAK1 and is absent in PAK3 thus this mode of

activation of PAKs is only possible with PAK2. Protease-dependent activation of PAK2 independent of RhoGTPases. This cleaved Pak2 has a function that differs from full length Pak2 which normally stimulates cell survival and cell growth, while the truncated analogue was shown to function in programmed cell death (Jakobi, McCarthy et al. 2003; Koeppel, McCarthy et al. 2004). Transfection of truncated PAK2 promoted apoptosis (Lee, MacDonald et al. 1997). In comparison, kinase dead mutant transfected cells had delayed apoptosis and decreased formation of apoptotic bodies (Rudel and Bokoch 1997).

Phosphorylation plays a role in PAK2 activation by Cdc42 and Rac1. Dephosphorylation is also an important means of PAK2 regulation. Protein phosphatase 1A was shown to act on phospho-Thr-402, required for PAK2 activity, leading to down regulation of its kinase activity (Wang and Wang 2008).

Cdc42 activation of PAK2 via phosphorylation of serine 14 induced c-Abl activity (Roig, Huang et al. 2000). c-Abl is a tyrosine kinase involved in a variety of cellular responses. PAK2 and c-Abl were also shown to colocalize in the cytoplasm of dorsal ruffles and it can also phosphorylate c-Abl interacting proteins (Machuy, Campa et al. 2007). Abl mediated PAK2 tyrosine phosphorylation leads to down regulation of PAK2 activity (Roig, Tuazon et al. 2000) while phosphorylation of Ser 637 and 638 by PAK2 diminishes the ability of c-Abl to interact with its binding partner Abi2. However interaction with Crk is stimulated, where Crk is phosphorylated by c-Abl (Jung, Pendergast et al. 2008).

PAK2 was reported to form a complex with Fyn and Nck (Lamalice, Houle et al. 2006). Furthermore, cotransfection of Pak2 and Syk leads to the activation of JNK (Miah, Sada et al. 2004). Pak2 coexpressed with an inactive form of Cdc42 or kinase-inactive Pak2 interacts to a

lesser extent with Syk, suggesting that Pak2-Syk association is enhanced by Pak2 activation (Miah, Sada et al. 2004). PAK2 was shown to play a role in clathrin-independent endocytosis of IL2R, with cortactin playing a role downstream of PAK2 (Grassart, Dujeancourt et al. 2008).

Enhanced activation of PAK2 by Rac1 and Cdc42 was observed when PAK2 was coexpressed with Src family tyrosine kinases, which led to phosphorylation of Tyr130 in a small fraction of cellular PAK2 (Renkema, Pulkkinen et al. 2002). This phosphorylation may be dependent on the conformational change observed in PAK2 as a result of binding to GTPases (Renkema, Pulkkinen et al. 2002).

One of the major effectors of PAKs are LIM kinases. They are involved in regulation of the actin cytoskeleton dynamics through inactivation of ADF/cofilin. PAK2 and PAK4 were shown to regulate LIMK1 and 2. MRCK and Rho kinase can phosphorylate and thus activate LIMK (Bokoch 2003), providing a parallel signaling mechanism.

Cellular contractions can be in part mediated by PAK2. MCLK phosphorylation by PAK2 on Ser439 and Ser991 inactivates it and thus prevents it from phosphorylation of the myosin II regulatory light chain (Goeckeler, Masaracchia et al. 2000). However, binding of calmodulin, an MLCK activator, prevents Ser991 phosphorylation by PAK2 (Goeckeler, Masaracchia et al. 2000), which could be a more important site for inhibition of catalytic activity by PAKs. PAK2 is capable of R-MLC (regulatory myosin light chain) phosphorylation resulting in increased contractility (Zeng, Lagunoff et al. 2000). Regulation of contractile activity by PAKs is likely a complex interplay between other molecular messengers such as Ca2+/ Calmodulin. Another myosin which maybe regulated by PAK2 is myosin VI, which is an

unconventional myosin in that it translocates towards pointed ends on actin filaments while others move towards barbed ends (Bokoch 2003).

PAK3

This member of the PAK family was shown to be involved in genetic disorders, specifically mutations in the PAK3 gene lead to X-linked non-symptomatic mental retardation syndrome (MRX); where mental retardation is accompanied by normal brain development and few symptoms. PAK3 mutations in MRX affected subjects led to absent or abnormal axonal connections, implicating PAK3 in formation of axonal connections in the human brain (Hofmann, Shepelev et al. 2004).

PAK3 may also be needed for dendritic development and has been implicated in the spine morphogenesis (Kreis, Thevenot et al. 2007) where cytoskeletal rearrangement plays an important role. Actin cytoskeleton in dendritic spines is under the control of RhoGTPases, of which Cdc42 was shown to stimulate the function of PAK3 (Kreis, Thevenot et al. 2007). Dissociation of active RhoGTPases from PAK3 has been reported, after initial activation has occurred (Manser, Leung et al. 1994). Dendritic spines undergo actin based motility (Fischer, Kaech et al. 1998).

It was shown that PAK3 kinase activity induces formation of long, thin dendritic outgrowths, and it was implicated in GIT1/PIX/Rac/PAK complex in regulation of dendritic spine formation through downstream regulation of MLC by PAK proteins (Zhang, Webb et al. 2005). ARHGEF6 was implicated in PAK3 activation in dendritic cells during spine morphogenesis (Node-Langlois, Muller et al. 2006), however it was also shown to be an activator of Cdc42 and Rac1 (Baird, Feng et al. 2005). PAK3 was shown to phosphorylate myosin ID

(Wu, Lee et al. 1996) and myosin VI on Thr406 which significantly enhanced its actin translocating activity (Yoshimura, Homma et al. 2001). MEKK1, a protein that serves a role in stress response signaling, is indirectly activated by PAK3 (Siow, Kalmar et al. 1997). Cells transfected with constitutively active PAK3 or Cdc42 activated PAK3 were shown to activate JNK1 (Bagrodia, Derijard et al. 1995).

There are four PAK3 splice isoforms that have been identified, all of which are expressed in brain, specifically in neurons (Kreis, Rousseau et al. 2008). Alternatively spliced exons b and c are inserted in the regulatory PBD/AID domains of PAK3, resulting in molecular weight differences in expressed proteins: PAK3a, PAK3b, PAK3c and PAK3cb had weights of 65, 68, 69, and 72kDa, respectively (Kreis, Rousseau et al. 2008). The presence of those inserts also alters kinase activity; PAK3b, PAK3c and PAK3cb showed high kinase activity independent of presence of Cdc42, in contrast to that of PAK3a. Also it was shown that presence of insert b reduces binding of Cdc42. The novel PAK3 isoforms also showed altered formation and localization of focal adhesions resulting in major changes in cell morphology (Kreis, Rousseau et al. 2008). Because of the location of those insets, they could bring new regulatory modifications to PAK3 leading to functional diversification during signal transduction, such as redirection of alternatively spliced PAK3s to other GTPase activation. Knock down of PAK3 function in hippocampal splice cultures leads to thin, elongated and immature type spines while constitutively active PAK3 showed no effect (Boda, Jourdain et al. 2008).

alphaPIX was shown to function with PAK3 in spine morphogenesis. Defects result in mental retardation. Constitutively active PAK3 potentially rescues the phenotype resulting from aphaPIX gene destruction (Node-Langlois, Muller et al. 2006). PAK3 is responsive to

thrombonin and other G protein coupled receptors, although JNK activation occurs in their response it is independent of PAK3 (Malcolm, Chambard et al. 2000).

PAK4

PAK4 plays one of the major roles in cytoskeletal reorganization (Eswaran, Soundararajan et al. 2008). It is a known effector of Rac1 (Zhang, Chernoff et al. 1998) and was shown to be central for podosome formation in human macrophages (Gringel, Walz et al. 2006). PAK4 is involved in a protein complex composed of SSH-1L, LIMK, and 14-3-3zeta through which it regulates the actin depolymerization process by regulation of ADF/cofilin. Phosphorylation of SSH-1L by PAK4 exerts a negative effect on it (Soosairajah, Maiti et al. 2005). Pak4 regulates LIMK1 and SSH-1L via phosphorylation which serves as a mechanism of regulation of actin dynamics (Soosairajah, Maiti et al. 2005). Down regulation of PAK4, an inhibitor of neutrite outgrowth, was observed in neuroblastoma cell lines (Nowicki, Kosacka et al. 2007).

PAK4 was shown to be up regulated in prostate cancer (Ahmed, Shea et al. 2008) and recently implicated in cell motility of pancreatic ductal adenocarcinoma (Kimmelman, Hezel et al. 2008). It was reported that variation in expression levels of PAK4 is proportional to LIMK1 mediated phosphorylation of cofilin. This also correlated to changes in cell morphology and a change in cell migration behaviour downstream of hepatocyte growth factor (HGF) (Ahmed, Shea et al. 2008), which is known to increase cancer invasiveness. HGF was previously shown to stimulate PAK4 activity in a PI3K dependent manner which contributed to HGF-induced changes in actin organization (Wells, Abo et al. 2002). Over expression or activation of PAK4

was found to be key step in oncogenic transformation due to its ability to promote cell survival, resulting uncontrolled proliferation (Liu, Xiao et al. 2008).

PAK4 was also shown to be required for full stimulation of pro-survival pathway in response to TNF alpha stimulation (Li and Minden 2005). NFkappaB and ERK pathway activation was reduced in the absence of PAK4 (Li and Minden 2005). Inhibition of apoptotic signals by PAK4 is also consistent with its phosphorylation of a proapoptotic protein BAD on Ser112 and inhibition of caspase activation (Gnesutta, Qu et al. 2001). Inactivation of BAD promotes cell survival through inactivation of a mitochondrial pathway and cytochrome c release (Downward 1999).

Expression of constitutively active or wild type PAK4 showed reduction in apoptosis in response to TNFalpha stimulation and UV radiation (Gnesutta, Qu et al. 2001). Interestingly, and consistent with anti apoptotic properties of PAK4, it is capable of inactivation of caspase 8 in a manner that is not dependent on its kinase activity (Gnesutta and Minden 2003).

PAK4 activates GEF H1 by phosphorylation leading to activation of Rac and inhibition of Rho pathway (Callow, Zozulya et al. 2005). This interaction also stimulates lamelopodia formation and is associated with microtubules thus blocking stress fiber formation (Callow, Zozulya et al. 2005). As a result of PAK4 being targeted by Akt (a survival protein), GEFH1 is also phosphorylated during *Mycobacterium tuberculosis* and *Salmonella typhimurium* infections, this in turn controls cell survival through regulation of actin dynamics as a result of GEFH1 regulation of RhoA, Rac1 and actin (Kuijl, Savage et al. 2007).

PAK4 can antagonize Rho activity through direct interaction with RhoGEF (Barac, Basile et al. 2004). Direct interaction of PAK4 with PDZ-RhoGEF negatively regulates activation of

Rho (Barac, Basile et al. 2004). PAK4 also interacts specifically with the GTP-bound form of Cdc42 and weakly activates the JNK signaling. Downstream activation of NFkappaB and ERK pathways is reduced in the absence of PAK4 (Li and Minden 2005).

PAK6

Limited data is available for PAK6 which belongs to the second group of p21 activated kinases. It was originally identified from its interaction with androgen receptor and was shown to have cytoplasmic localization (Lee, Ramos et al. 2002). Similarly to other members of group II PAKs, PAK6 lacks the AID domain, resulting in alternative regulation of this protein as compared to those belonging to group I PAKs.

Activated Cdc42 has stronger PAK6 binding than GTP-Rac. This binding was observed as a result of interaction with androgen receptors (AR) but does not stimulate kinase function. PAK6 was demonstrated to inhibit AR (Lee, Ramos et al. 2002) for which kinase activity was necessary (Schrantz, da Silva Correia et al. 2004).

Protein phosphatase 1B and IQGAP1 were shown to be PAK6 interacting partners using affinity purification and LC-MS/MS (Kaur, Yuan et al. 2008). PP1B is a member of PP2C family of Ser/Thr protein phosphatases involved in negative regulation of the cellular stress response pathways including p38 and JNK (Takekawa, Maeda et al. 1998), as well as down-regulation of NFkappaB (Prajapati, Verma et al. 2004). PAK6 is activated via phosphorylation by p38 and its upstream regulator MKK6 at Ser-165 and Tyr-566. During this, an auto phosphorylation site Ser-560 remains unaltered thus PAK6 is regulated by both auto phosphorylation and MKK6 (Kaur, Liu et al. 2005).

MATERIALS AND METHODS

Live-cell affinity receptor chromatography (LARC)

RAW264.7 cells were obtained from ATTC and grown in DMEM+5% FBS. To minimize the presence of proteins from the growth media prior to the experiment, cells were washed four times in ice cold, isotonic medium containing 20 mM HEPES, 140 mM NaCl and 1 mM each of $CaCl_2$ and $MgCl_2$. The Fc receptor ligand, IgG, was bound onto polystyrene beads in PBS (Marshall, Booth et al. 2001). The beads were washed followed by binding to the surface of live cells on ice in an experimental medium before washing away unbound beads. 2μm polystyrene beads (Bangs Laboratories, Fishers, IN) coated with human IgG were added to live RAW 264.7 cells in experimental medium on ice for 30 min. Free beads were washed off 3 times in ice cold medium. Incubation on ice prevents IgG receptor complex internalization. The cells were warmed for 0, 2.5, 5, 7.5 and in some cases 10 min at 37°C to activate receptors to their full potential. The experiment was quenched with ice cold experimental buffer; the cells were scrapped, to collect the beads before the receptor complexes could be internalized. At this point the bulk of the beads could be washed from the cells by vigourous mechanical action (*Figure 9*). A French Press was used to disrupt the cells followed by isolation of ligand coated microbeads with a sucrose gradient in a 1 ml sample volume (Beckman, USA); (Burkhardt 1998).

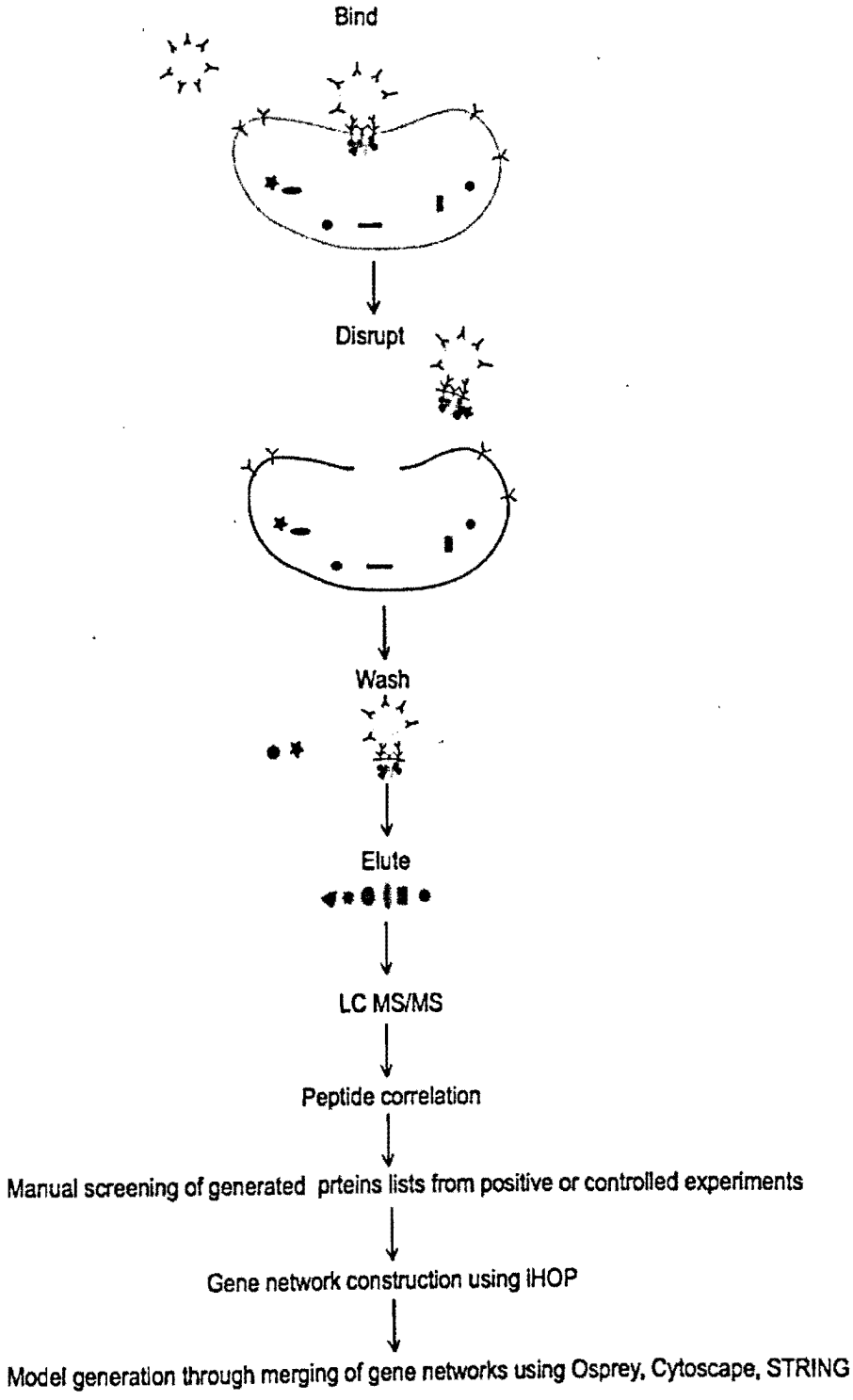
The same beads with ligands were used for traditional immuno affinity chromatography (IAC) of the receptor complex from crude lysates. For the negative control the same beads without the ligands were incubated with crude extracts or growth media. The beads without ligands incubated in crude extracts or only used experimental buffer were isolated through sucrose gradient centrifugation. Proteins from isolated beads were gradually extracted with salts

(Foster, de Hoog et al. 2006) from 0 to 1000mM NaCl in steps of 50 or 100mM. The LARC experiment was performed multiple times and each time compared to affinity ligand capture of the receptor complex or to beads alone incubated with crude extract or growth media.

LC-MS/MS

The Bradford method was used to measure protein content (Bradford 1976). 100 µg of protein were digested in Tris pH 8.8 in 1 M Urea and 5% acetonitrile with 1 µg of trypsin (Calbiochem) for 12h at 37°C. The sample was reduced in 2 mM DTT and then re-digested with trypsin. The tryptic peptides were directly subjected to LC-MS/MS or additionally diluted in 0.1% acetic acid and separated by propyl sulfate chromatography (Link, Eng et al. 1999) using a discontinuous gradient in 50 mM steps to 850 mM and then 1.0 M NaCl creating 15 fractions. All the digested samples were collected by zip tip eluted in 2 µl and diluted to 20 µl and manually loaded via a 20 µl loop of a Rheodyne injector on to the column (Marshall, Jankowski et al. 2004). Samples and controls were resolved separately by C_{18} reversed phase chromatography (Vydac 0.3mm ID, 15 cm column). Samples were analysed over a 90 minute gradient from 5% to 65% acetonitrile with a flow rate of 2µl/min with an Agilent 1100 series capillary pump via a metal needle to form an electro-spray (McCombie, Adams et al. 1992) into a Decca XP-100 ion trap (Thermo Electron Company). MS/MS data files from the LC-ESI-ion trap analysis were searched against a non-redundant library of proteins, cDNAs, EST (McCombie, Adams et al. 1992) and genomic DNA using SEQUEST (Yates 1998). Since digestion was done with trypsin only fully tryptic peptides were accepted.

Figure 9. Live-cell Affinity Receptor Chromatography (LARC).
The method used to generate proteins specific to the Fc Receptor supramolecular complex involved in binding of IgG opsonized particles. Control experiments were carried out under the same experimental conditions with the naked beads.



Screening and Analysis

Data set of proteins putatively associated with phagocytosis was obtained through visual inspection of LC-MS/MS data obtained through LARC of IgG opsonized microbeads (Jankowski, Zhu et al. 2008). To account for non-specific binding, data from control experiments were screened. The presence of three unique peptides and a score of at least 2400 (Chelius, Huhmer et al. 2002) was used as the basis of acceptance of proteins in the construction of protein interaction models of FcγR mediated phagocytosis. These conditions represent 99% probability (Cargile, Bundy et al. 2004) of a protein presence in the pathway.

Proteins that met the criteria above were examined for interacting partners from the same data set using *iHOP* (Hoffmann and Valencia 2004). From the interacting partners interaction models (Hoffmann and Valencia 2005) were created and exported into Cytoscape (Shannon, Markiel et al. 2003), String (Jensen, Kuhn et al. 2009) and Osprey (Breitkreutz, Stark et al. 2003) where they were merged leading to improvements in model visualization through the application of different algorithms.

SDS-PAGE and Western Blotting

RAW264.7 lysates were probed for PAK2, 3, 4 and 6. RAW lysates were prepared by scraping cells grown to 80-90% confluency in 75cm2 flask in AMEM+10%FBS. Approximately 5 ml of 2xSDS-PAGE sample buffer containing around 5mM PMSF, 5mM AEBSF, 5mM EDTA and eukaryotic protease inhibitor cocktail (Sigma-Aldrich) were added to the scrapped cells and immediately denatured for 10min at 100°C. Approximately 50µl of cell lysate along side a molecular weight marker (MWM) were run on a discontinuous polyacrylamide gel with 4% stacking and 7% separating gels (Schagger, Borchart et al. 1987). Proteins from SDS-PAGE

were transfered onto PVDF membrane. The membrane was stained with Coomassie stain without acetic acid, destained with 50% methanol, dried and marked with a pencil to indicate the location of the MWM. The membrane was then soaked in 100% methanol to fully destained and blocked with either 5% skim milk in PBS+0.1% Tween (PBST) or 5%BSA+1% donkey serum in PBS+0.1% Tween for 30min. The membrane was washed 3 times with PBST and then primary anti bodies were added for 30 min, then washed 3 times. The membrane was incubated with 1:10000 dilution of secondary HRP conjugated antibody in PBST for 30 min, then washed extensively with PBST and developed with ECL developing solution (Haan and Behrmann 2007).

PAK2 - blocked with 5%BSA+1%DS in PBST, 1:100 goat anti-PAK2 (sc7117, Santa Cruz Biotechnology) in 5%SM in PBST. PAK3 - blocked with 5%BSA in PBST, 1:500 goat anti-PAK3 (sc1871, Santa Cruz Biotechnology) in 5%SM in PBST. PAK4 - blocked with 5%SM in PBST, 1:1000 rabbit anti-PAK4 (Cell Signaling #3234) in 5%SM in PBST. PAK6 - blocked with 5%SM in PBST, 1:500 rabbit anti-PAK6 (sc32857, Santa Cruz Biotechnology) in 5%SM in PBST. All blots where probed with secondary antibodies at concentrations of 1:10000 in 5%SM in PBST, 705-035-147 Jackson donkey anti goat and 111-035-045 Jackson goat anti rabbit were used.

Immunostaining

Cells were scrapped and grown on 2.5 cm glass cover slips in 6 well plates in 1mL AMEM+10% FBS. Cells were grown for 24-48hrs until ~30% confluency. Sheep red blood cells (SRBC) or 2 μm polystyrene beads were incubate at a room temperature for 30min with rabbit anti-SRBC or human IgG, respectively, then washed 3x with PBS (Marshall, Booth et al.

2001). Cells were incubated with SRBC or polystyrene beads for 5-7min at 37 °C, then washed 3x with ice cold PBS and fixed with 2% formaldehyde in PBS for 30 min, washed 3x with PBS and put into 5% glycine in PBS. Cells were then permeabilized with PBS+0.1% TritonX-100, washed 3 times with PBS and blocked with 5%SM in PBS. Primary and secondary Cy-3 conjugated antibody concentrations were 1:25 and 1:10000, respectively, diluted in PBS. Slides were mounted with Dako mounting media (Dako, Denmark) and allowed to dry overnight in the dark. Confocal laser scanning microscope (LSM) was used to analyze cell staining.

Transfections

Competent DH5α cells were transformed with different plasmid vectors of GFP fused to endogenous proteins. Transformed cells were plated on selective LB media and incubated at 37°C o/n. A single colony was picked and grown in 3mL of selective LB media. 100mL of selective LB media was then inoculated with the 100μL of starter culture and grown in a shaker over night at 37°C. DNA was extracted from 100mL cultures with MaxiPrep Kits (Quaigen).

RAW macrophages were grown on coverslips in 6 well plates in AMEM+10%FBS o/n at 37°C with 5%CO2. Fugene 6 transfection reagent (Roche Diagnostics, USA) was used according to manufacturers directions. Transfected cells were grown o/n at 37°C with 5%CO2. Cells were fed SRBCs opsonized with rabbit anti-SRBC and images were taken with LSM.

Double stain phagocytosis assay

RAW264.7 cells were grown on cover slips in AMEM+10%FBS in 6 well plates for 24-48 hours at 37°C with 5% CO2. Cells were transfected with EGFP-PAK2, EGFP-PAK3, GFP-PAK4, EGFP-PAK6, EGFP-PAK2-T402E, EGFP-PAK3-K297L, EGFP-PAK6-K436A, and empty GFP vector using Fugene 6 transfection reagent and grown overnight.

Polystyrene beads were incubated with human IgG in PBS for 30min at room temperature then washed 3 times with PBS. Cy3-conjugated anti-human IgG antibodies were added and beads were incubated for another 30 min at room temperature followed by 3 washes with PBS. Transfected RAW264.7 macrophages were incubated with beads for 2 hours then washed 3x with PBS, fixed with 2% formaldehyde in PBS for 30 min, washed 3x with PBS and 5% glycine in PBS was added to each well for 30 min. 1:10000 diluted in PBS Cy5-conjugated to anti human IgG was added to each well and allowed to incubate to 30 min. Cells were then washed 3x15 min in PBS and mounted with Dako mounting media and dried overnight. Images were taken with a scanning laser confocal microscope. The number of beads engulfed was counted for all the transfected cells; number of beads engulfed by cells with empty GFP vector, wild type and dominant negative constructs was then compared using ANOVA.

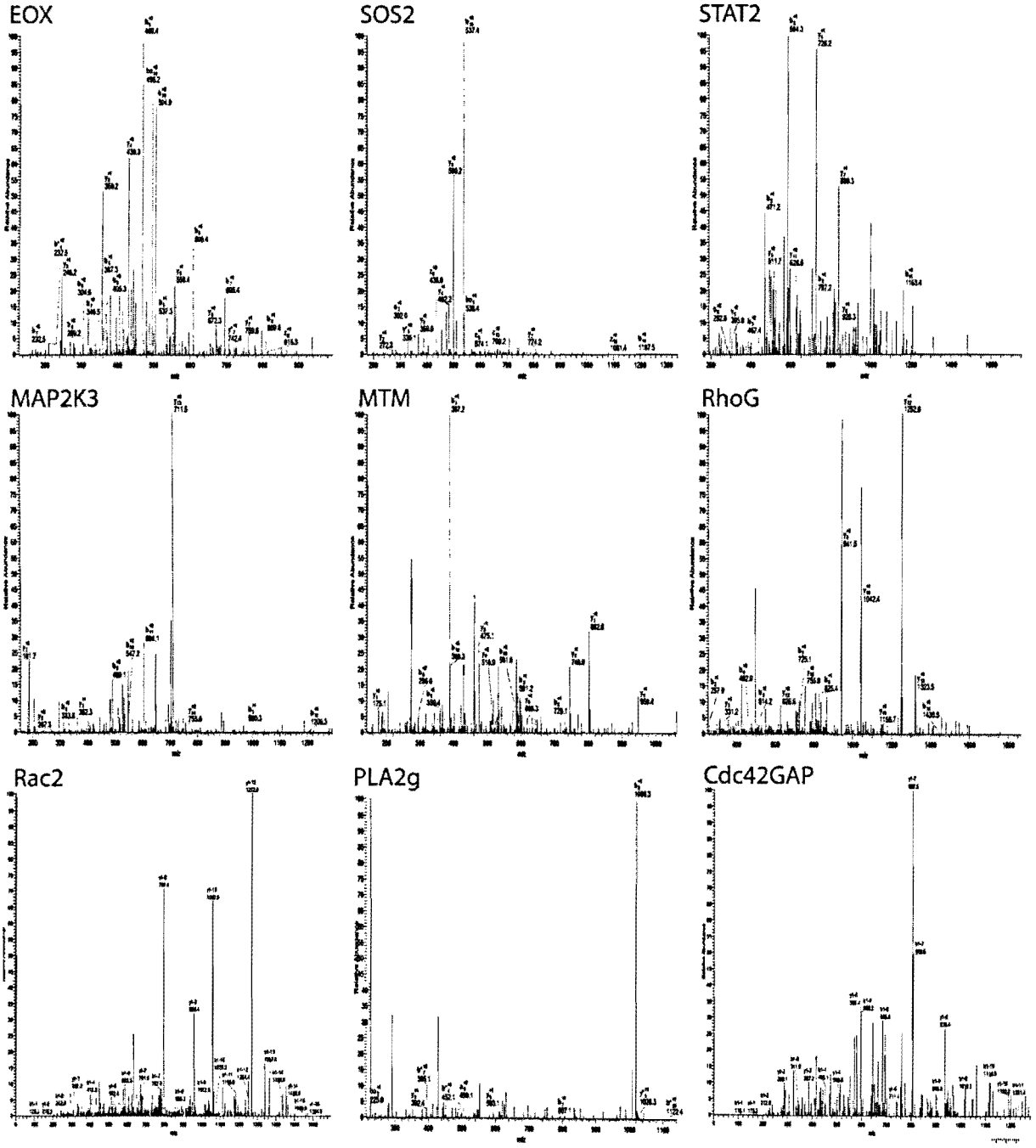
RESULTS

LARC

After manual screening of LC-MS/MS positive and control data, a list of proteins involved in uptake of IgG-opsonized particles was generated. Selected spectra are illustrated in *Figure 10*. Proteins identified through LARC are summarized in *Table 1*. Nearly 900 proteins were identified including channels and receptors. Of those 341 have at least three interactions with other proteins on that list, as determined by the Osprey interaction network builder algorithm. Fc receptors as well as co receptors were detected. Proteins such as serine and tyrosine kinases and phosphatases were detected. Proteins that act on membrane lipids such as PIP5K, PI3K, PLD, PLC, PLA_2, SHIP and their numerous isoforms were detected. Rho family GTPases including Rho, Rac, Cdc42 and PAKs including GEFs and GAPs specific for those proteins were identified. Arp proteins, coronin, myosins, dyneins, profilin, cofilin, filamins, tropomyosin, dynamin are other proteins detected and associated with the cytoskeletal alterations during phagocytosis in neutrophils.

Two classes of PI3K were observed. The p110 subunit which is required for FcγR mediated phagocytosis by macrophages (Leverrier, Okkenhaug et al. 2003) was identified with 9 different peptides with a score of 4369.13. PI3KC2γ had 11 unique peptides with a correlation score of 5739.68. PI3KC2α was also detected and is involved in Ca2+ dependent inactivation of MLCP by ROCK (Yoshioka, Sugimoto et al. 2007) and thus stimulation of myosin based contractile activity.

Figure 10. Fragmentation spectra of selected proteins identified with LARC.
Spectra were obtained from LC-MS/MS using the SEQUEST algorithm.



The presence of the Class I and Class II PI3K can be expected through the importance of PIP2 and PIP3 as second messengers that can lead to activation of PKC, PLD, PLC and other enzymes that act on membrane lipids. PLD2 had 20 different peptides with 4 of them phosphorylated at serine and threonine residues while PLD1 had 9 different peptides correlated with SEQUEST. cPLA2δ had 8 different peptides with nothing found in controls. PKC theta was identified with 8 different peptides.

Rho family GTPases play a dominant role in particle uptake (Greenberg and Grinstein 2002), consistent with this a number of Rho GTPases were detected by LARC, including Cdc42 through 4 peptides and a total correlation score of 136346.39, and Rac2, which is involved in activation of p67phox subunit of NADPH oxidase (Bokoch 2005), was identified with four different peptides and a score of 105143.33. Akt was detected with 3 peptides, a score of 3850.29 with 0 control. One of the functions of Akt is activation of p47phox of phagocyte NADPH oxidase (Chen, Powell et al. 2003) and is dependent on PI3K activity in human neutrophils (Tilton, Andjelkovic et al. 1997).

LARC did not detect some of the proteins thought to be associated with phagocytosis. ARF6 which was shown to have a function in cytoskeletal rearrangements beneath the phagocytic cup (Zhang, Cox et al. 1998) was not detected. However its activator POR1 that functions downstream of Rac (D'Souza-Schorey, Boshans et al. 1997) was detected. LIMK1/2, effectors of Cdc42 and Rac (Greenberg and Grinstein 2002), were detected with scores not significant for acceptance in the protein model. Protein detected with scores below the threshold could be activated away from the receptor or have very low abundance or displaced during the processing.

A large number of proteins was identified including peptides that were correlated to proteins previously known to be involved in particle uptake along with the novel proteins. Identification of different protein isoforms was possible which is important since they may have different functions, cellular distributions or play a role at different stages of the process. Diacylglycerol kinases (DGK) of 4 different classes were identified by LARC, specifically DGKβ1, δ2, ε, ζ and ι. DGK is involved in production of PA from DAG, which together may function as second messengers modulating activity of enzymes such as PIP5K (Jenkins, Fisette et al. 1994) and PKCγ1 (Jones and Carpenter 1993). DGK isoforms contain different domains which alter their function and localization. Class I DGKs (α,β,γ) have a Ca^{2+} binding domain and are activated in Ca2+ presence with DGKβ colocalizing with actin filaments. Class II DGKs (δ, η, κ) contain PH like domains on its N-terminus which may be involved in lipid interactions. Class III DGKε has a preference for arachidonoyl-D (Goto, Hozumi et al. 2008). The only member of Class IV, DGKζ is associated with the nucleus and is transported into the cytoplasm and was found to be expressed in lung macrophages (Katagiri, Ito et al. 2005).

Low abundance regulatory proteins believed to be relevant to Fc receptor (Greenberg, Chang et al. 1994) were identified through LARC with significant scores. False positive identification of proteins was minimized by only accepting proteins with at least three distinct peptides correlated to the protein. Possible incorporation of false positives into the protein model is further reduced by having control experiments.

Reliability of the data from tandem mass spectrometry of the Fc receptor is supported through the presence of peptides having at least three daughter ions in a row from the b or y ion series (DeSouza, Diehl et al. 2005).

The quality of the data is seen from the presence of sharp, discrete and resolved peaks with separation time of less than 10s. Shape and distribution difference in the chormatograms of the elution profiles between positive and controls permits to account for presence of the proteins that are not specifically involved in the Fc receptor complex. Variation in elution conditions including change in salt concentrations led to identification of a greater number of proteins associated with phagocytosis (*Figure 11)*. Further increase in the detection of proteins came from different incubation times, since some proteins are present at different stages of engulfment.

Table 1. LARC generated data set of proteins that may be involved during FcγR mediated phagocytosis in human neutrophils. Proteins that were accepted to specifically associate with the activated supramolecular Fcγ receptor complex had a total cumulative score of at least 2400, a minimum of thee different peptides and low scores in the control experiments. Those proteins that did not meet this criteria were not added to the list. Grey highlighted lines represent the search terms that were used to produce a set of proteins that are listed in that section.

Protein name	Score Positive	Control	Accession #
Cdc42			
CDC42-binding protein kinase alpha isoform A; ser-thr protein kinase r	2919.14	364.54	30089960
Cdc42 From Human, Nmr, 20 Structures 13390 similar to P	136346.39	7371.87	2624582
RAC/CDC42 exchange factor [Homo sapiens]	3725.00	644.28	19311008
truncated Cdc42 interaction protein 4 [Homo sapiens]	5442.82	1126.86	19568094
CDC42 binding protein kinase alpha (DMPK-like) [Homo sapiens]	6090.42	2204.17	29373940
phosphoinositide-3-kinase			
phosphoinositide-3-kinase gamma catalytic subunit [Homo sapiens]	4369.13	611.20	12620871
phosphoinositide-3-kinase, class 2, gamma polypeptide; phosphatidylinos	5739.68	214.76	4758924
phosphoinositide-3-kinase, class 2, beta polypeptide; PI3K-C2beta; pho	6787.56	94.12	15451926
phosphoinositide-3-kinase, regulatory subunit, polypeptide p101 [Homo s	4595.61	959.63	7657433
similar to phosphoinositide-3-kinase, class 2, beta polypeptide; PI3K-	3446.17	387.18	28482204
PC4345 phosphoinositide 3-kinase (EC 2.7.-.-) T96 - human (fragment)	16139.82	231.95	7434348
Transglutaminase			
similar to transglutaminase Z [Homo sapiens] [Mus musculus]	17953.68	583.72	20912421
Phospholipase			
AF263947_1 membrane-associated calcium-independent phospholipase A2 gamma	7384.26	0.00	8896121
cytosolic phospholipase A2 delta [Homo sapiens] 3	3456.06	0.00	29467442
T13162 cytosolic phospholipase A2 gamma - human	3801.96	748.17	7512374
phospholipase C epsilon [Homo sapiens] sapiens] t	6837.82	0.00	11065786
phospholipase C-like 1; phospholipase C, epsilon [Homo sapiens]	4848.60	1202.57	5453912
phospholipase C beta 1 [Homo sapiens] ÿ	7693.70	1139.98	9438229
phospholipase C, beta 2 [Homo sapiens]	4280.63	879.33	4758938
S52099 phospholipase C beta 3 - human	2986.11	263.00	1082692
dJ811H13.1.2 (Phospholipase C, beta 4 (1-Phosphatidylinositol-4,5-Bisph	3250.20	732.01	13374903
phospholipase C, gamma 1 (formerly subtype 148) [Homo sapiens]	2732.22	812.88	4505869
phospholipase D1, phophatidylcholine-specific; phospholipase D1, phosph	3437.14	644.59	4505873
phospholipase C, delta 4; PLC delta4 [Homo sapiens]	2470.40	804.19	14249340
similar to phospholipase A2, group IVB (cytosolic) [Homo sapiens] [Rat	3200.48	0.00	27732107
similar to phospholipase A2, group IVB (cytosolic) [Homo sapiens]	3930.99	387.21	42660438
phospholipase C-like 1; phospholipase C, epsilon [Homo sapiens]	4848.60	1202.57	5453912
phospholipase D2 [Homo sapiens] . Hypothetical prot	9563.47	844.32	2645858
Mitogen activated protein kinase			
M3K1_HUMAN Mitogen-activated protein kinase kinase kinase 1 (MAPK/ERK kinase k	9477.28	0.00	8488988
mitogen-activated protein kinase kinase kinase kinase 1 [Homo sapiens]	2808.92	1108.51	6005810
similar to Mitogen-activated protein kinase kinase kinase 1 (MAPK/ERK	6935.88	434.95	27498231
mitogen-activated protein kinase kinase 3 isoform C; MAP kinase kinase	3568.68	0.00	21618351
mitogen-activated protein kinase kinase kinase 7 interacting protein 1;	3342.85	0.00	5174703
mitogen-activated protein kinase kinase kinase 10; mixed lineage kinas	4246.50	1809.83	21735550
mitogen-activated protein kinase 10 isoform 4; JNK3 alpha protein kina	3086.63	0.00	20986508
mitogen-activated protein kinase kinase kinase 14; serine/threonine pro	7802.33	1340.14	4505397
lysine-deficient protein kinase 2; mitogen-activated protein kinase ki	8780.88	1573.45	32455273
mitogen-activated protein kinase phosphatase x; homolog of mouse dual s	2410.84	298.01	9910432
mitogen-activated protein kinase 4; Erk3-related; protein kinase, mitog	2831.30	0.00	4506089
MK11_HUMAN Mitogen-activated protein kinase 11 (Mitogen-activated protein kina	2579.55	0.00	2499603
Src			
AUTS2 protein [Homo sapiens] similar to human SRC	3560.85	0.00	40555743
adaptor protein with pleckstrin homology and src homology 2 domains [H	4306.12	370.90	10280626
SRC8_HUMAN Src substrate cortactin (Amplaxin) (Oncogene EMS1)	2464.20	649.81	2498954
A45259 desmoyokin - human (fragments) in similar to human SRC	2458.87	0.00	627367
KIAA0921 protein [Homo sapiens] homology and src	8925.66	0.00	20521694
similar to SHC (Src homology 2 domain containing) transforming protein	3866.04	1026.45	20912475

SHC (Src homology 2 domain containing) transforming protein 1; SHC (Sr	2781.38	0.00	32261324
SRC protein [Homo sapiens] s] containing 43 [Homo	2832.71	0.00	30411071
Protein kinase C			
protein kinase CHK2 isoform b; checkpoint-like protein CHK2; serine/th	3814.20	0.00	22209009
protein kinase C, alpha binding protein; protein interacting with C kin	2425.40	796.96	7110697
gi|520587|dbj|BAA01381.1| protein kinase C delta-type [Homo sapiens] 783|dbj|	2766.07	158.58	520587
protein kinase C, eta [Homo sapiens]	3140.33	0.00	28557781
protein kinase C, gamma; Protein kinase C, gamma polypeptide [Homo sap	3515.03	293.99	13384594
bA5N23.1 (protein kinase C theta) [Homo sapiens]	3106.01	386.49	10799537
protein kinase C-like 2 [Homo sapiens]	9286.65	2979.28	5453974
similar to protein kinase C binding protein 1 [Homo sapiens] [Mus musc	7977.79	548.08	25058908
protein kinase C substrate 80K-H; glucosidase II, beta subunit; AGE-bin	3074.92	521.35	4506077
protein kinase C, mu [Homo sapiens]	2863.35	1238.56	4506075
myosin			
myosin IB [Homo sapiens]	4591.91	0.00	37549339
similar to myosin IC [Homo sapiens] beta subunit;	4718.01	0.00	28279972
myosin ID [Homo sapiens]	2792.96	0.00	41150753
AF229172_1 class III myosin [Homo sapiens] r 17, hum	5988.50	246.63	7958618
myosin VA (heavy polypeptide 12, myoxin); Myosin, heavy polypeptide ki	11344.37	1656.39	10835119
MY5B_HUMAN Myosin Vb (Myosin 5B) ma 2; Renal cell carci	2602.13	0.00	39932736
A59255 myosin VIIa, long form - human	33808.58	2574.03	11360310
myosin IXA [Homo sapiens]	13451.05	1274.50	5902012
myosin IXB [Homo sapiens]	13312.00	419.13	4758750
myosin IXB [Homo sapiens] and worm C02C2.6 pred	5511.95	0.00	33356170
AF132021_1 myosin X [Homo sapiens] hosphate 3-kinase	5249.69	0.00	7108753
myosin XV; unconventional myosin-15 [Homo sapiens]	10834.00	797.12	22547229
similar to myosin XVIIIB; myosin 18B [Homo sapiens] [Mus musculus]	3005.66	304.62	28520265
myosin binding protein C, fast type; myosin-binding protein C, fast-typ	8627.83	720.11	4758748
myosin binding protein C gene [Homo sapiens] ÿ	3254.32	1090.32	2058322
myosin, heavy polypeptide 2, skeletal muscle, adult [Homo sapiens]	3651.95	0.00	8923940
myosin, heavy polypeptide 4, skeletal muscle [Homo sapiens]	6560.36	198.39	11024712
myosin, heavy polypeptide 7, cardiac muscle, beta [Homo sapiens]	6980.11	440.25	4557773
myosin, heavy polypeptide 8, skeletal muscle, perinatal [Homo sapiens]	7841.69	1379.04	4505301
myosin, heavy polypeptide 13, skeletal muscle; extraocular muscle myos	19720.02	2740.44	11321579
myosin, heavy polypeptide 14; myosin heavy chain 14; nonmuscle myosin	4571.83	0.00	33563340
myosin heavy chain [Homo sapiens] or 1 isoform b	10045.70	3176.31	20338989
myosin heavy chain (1167 AA) [Homo sapiens]	4346.43	0.00	31144
similar to cardiac alpha-myosin heavy chain [Homo sapiens]	3246.84	560.02	20542063
similar to superfast myosin heavy chain [Homo sapiens]	4232.97	117.71	42658517
myosin heavy chain 6; myosin heavy chain, cardiac muscle alpha isoform	2419.35	209.57	27764861
similar to myosin heavy chain Myr 8b [Rattus norvegicus] [Homo sapiens	5875.50	868.91	27499722
similar to Myosin heavy chain, nonmuscle type B (Cellular myosin heavy	5463.60	1777.57	27482786
MYH2_HUMAN Myosin heavy chain, skeletal muscle, adult 2 (Myosin heavy chain I	5570.70	0.00	13431716
myosin light chain, alkali, nonmuscle [Mus musculus]	29522.28	0.00	33620739
myosin alkali light chain 1 slow a; myosin light chain 1, slow-twitch m	3187.90	1499.63	4505303
myosin light chain kinase isoform 1; myosin light chain kinase [Homo s	4710.25	521.46	16950611
SI:dZ204D19.2 (novel protein similar to human non-muscle myosin, heavy	4209.99	0.00	33468583
tropomyosin 3 [Homo sapiens]	6647.25	2244.38	22748619
tyrosine kinase			
EphA1; eph tyrosine kinase 1 (erythropoietin-producing hepatoma amplifi	8492.48	744.73	4885209
Eph-like receptor tyrosine kinase hEphB1 [Homo sapiens]	2749.84	651.32	4104411
ETS related protein-neurotrophic receptor tyrosine kinase fusion protein	5455.66	783.79	6635287
protein-tyrosine kinase fyn isoform b; proto-oncogene tyrosine-protein	3212.52	1457.16	23510362
A39577 protein-tyrosine kinase (EC 2.7.1.112) JAK1 - human pie	4634.20	707.77	107499
leukocyte tyrosine kinase [Homo sapiens]	3226.57	0.00	4505045
megakaryocyte-associated tyrosine kinase isoform a; Csk-homologous kin	4430.94	335.73	21450846
PTK2 protein tyrosine kinase 2 isoform b; focal adhesion kinase 1 [Hom	6837.22	996.97	27886593
PTK7 protein tyrosine kinase 7 isoform a precursor; colon carcinoma ki	5777.12	429.37	15826840
I78843 receptor protein-tyrosine kinase - human (fragment)	6154.19	597.78	7434436
kinase insert domain receptor (a type III receptor tyrosine kinase); K	7816.57	871.23	11321597
tec protein tyrosine kinase [Homo sapiens]	32334.27	2944.27	4507429
tyrosine kinase precursor [Homo sapiens] s 1] e 1]	3395.79	1320.94	495678
Etk/Bmx cytosolic tyrosine kinase [Homo sapiens] ap	2770.30	794.22	3002963
JN0291 protein-tyrosine kinase (EC 2.7.1.112) (clone lambda-ret-5) - human	2885.01	213.16	88518
macrophage stimulating 1 receptor (c-met-related tyrosine kinase) [Homo	3364.14	506.69	4505265

tyrosine kinase pp60c-src [Homo sapiens] 88) [Homo	4218.80	502.31	3406615
Rho-associated, coiled-coil containing			
Rho-associated, coiled-coil containing protein kinase 1; p160ROCK; p160	3133.58	1348.54	4885583
Rho-associated, coiled-coil containing protein kinase 2 [Homo sapiens]	10647.16	1445.95	4759044
calcium/calmodulin			
calcium/calmodulin-dependent protein kinase I [Homo sapiens]	6645.52	745.99	4502553
Inositol			
A55713 inositol 1,4,5-triphosphate receptor type 1 - human	17013.87	2759.27	1362832
inositol 1,4,5-triphosphate receptor, type 2 [Homo sapiens]	13529.89	4393.12	4504793
IP3T_HUMAN Inositol 1,4,5-trisphosphate receptor type 3 (Type 3 inositol 1,4,	19019.18	5495.45	17366458
bA115P16.2 (inositol 1,4,5-trisphosphate 3-kinase B) [Homo sapiens]	5236.23	0.00	18077667
SH2 containing inositol-5-phosphatase [Homo sapiens]	3783.48	1252.98	1888525
inositol polyphosphate-4-phosphatase, type II, 105kD; inositol polyphos	13187.20	634.58	4504707
inositol polyphosphate-4-phosphatase isoform a; inositol polyphosphate-	6100.50	1350.94	4755140
OCRL_HUMAN Inositol polyphosphate 5-phosphatase OCRL-1 (Lowe's oculocerebrore	5609.63	1660.94	12644378
inositol polyphosphate phosphatase-like 1 [Homo sapiens]	6326.99	566.52	4755142
PIG2_HUMAN 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma 2	6770.42	1141.44	23503086
phosphatidylinositol binding clathrin assembly protein; Clathrin assemb	3486.94	1195.55	6005733
phosphatidylinositol glycan class A isoform 1; Phosphatidylinositol gl	6422.99	119.97	11863130
Similar to phosphatidylinositol glycan, class K [Homo sapiens]	3369.57	0.00	20071719
similar to Homo sapiens (Human). Phosphatidylinositol-glycan-specific ph	3783.17	0.00	28828281
phosphatidylinositol transfer protein, cytoplasmic 1 isoform a; retina	3779.75	855.08	32307140
phosphatidylinositol transfer protein, membrane-associated 2; PYK2 N-t	3792.58	0.00	24308237
A57134 1-phosphatidylinositol 3-kinase (EC 2.7.1.137) gamma isoform - human	3653.08	112.12	2135918
similar to phosphoinositol 3-phosphate-binding protein-2 [Homo sapiens	3225.73	1211.28	27485915
phosphoinositol 3-phosphate-binding protein-3 [Homo sapiens]	4857.58	0.00	7662418
phosphoinositol 3-phosphate-binding protein-3 [Homo sapiens]	7439.88	111.27	37595548
similar to Homo sapiens (Human). Phosphatidylinositol 4-kinase 230 [Dict	14078.66	2880.42	28829504
phosphatidylinositol 4-kinase, catalytic, alpha polypeptide isoform 1;	3485.80	257.79	4505807
phosphatidylinositol 4-kinase, catalytic, alpha polypeptide isoform 2;	6807.50	696.83	17105400
similar to phosphatidylinositol (4,5)bisphosphate 5-phosphatase; match to	2870.84	0.00	4314432
Phosphatidylinositol kinase, primarily involved in telomere length regu	9267.06	439.95	6319383
SYJ2_HUMAN Synaptojanin 2 (Synaptic inositol-1,4,5-trisphosphate 5-phosphatas	6866.99	1140.59	10720297
P11D_HUMAN Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit, d	5155.99	2199.42	3024334
erythrocyte			
erythrocyte membrane protein 4.1N [Homo sapiens] &	10909.41	1687.66	16356663
Similar to erythrocyte membrane protein band 4.1-like 2 [Homo sapiens]	2656.27	1350.90	21961573
erythrocyte membrane protein band 4.1 like 4B; EHM2 gene; FERM-contain	2813.23	0.00	21361812
SPCA_HUMAN Spectrin alpha chain, erythrocyte (Erythroid alpha-spectrin)	10118.02	1171.52	1174412
B Chain B, Human Erythrocyte Catalase 3-Amino-1,2,4-Triazole Complex	12760.18	277.42	7245757
G protein and/or G-protein			
CTOG_HUMAN CH-TOG protein (Colonic and hepatic tumor over-expressed protein)	11472.57	1346.78	3121951
G protein coupled receptor affecting testicular descent [Homo sapiens]	2662.19	939.08	18677729
G protein-binding protein CRFG [Homo sapiens] 4/67	4657.67	103.68	21707262
G protein-coupled receptor 108 [Homo sapiens] ic	7146.14	835.48	37552348
G protein-coupled receptor 123 [Homo sapiens]	5036.48	0.00	29789277
G protein-coupled receptor 125 [Homo sapiens] fa	3015.82	325.53	37540667
G protein-coupled receptor 78 [Homo sapiens]	2925.28	0.00	18201874
G protein-coupled receptor kinase 5 [Homo sapiens]	11399.27	1560.17	4885349
G protein-coupled receptor-associated sorting protein [Homo sapiens]	4258.80	0.00	42662594
G protein-regulated inducer of neurite outgrowth 1 [Homo sapiens]	3501.16	435.96	32698771
guanine nucleotide binding protein (G protein), alpha 14; guanine nucle	2914.55	96.88	4758444
guanine nucleotide binding protein (G protein), alpha activating activ	4567.29	144.91	33695153
leucine-rich repeat-containing G protein-coupled receptor 7 [Homo sapi	21799.75	3754.00	11056008
orphan G protein-coupled receptor GPR44 [Homo sapiens]	12341.37	233.69	4455061
SI:bZ201S.4 (novel protein similar to human G-protein coupled receptor	5182.28	1256.67	26788019
similar to G protein-coupled receptor 101 [Homo sapiens] [Rattus norve	12876.17	3674.28	27710152
Similar to guanine nucleotide binding protein (G protein), alpha activat	4572.47	0.00	29477177
similar to leucine-rich repeat-containing G protein-coupled receptor 7	279490.59	32765.75	27691918
similar to NYD-TSPG protein [Homo sapiens] [Rattus norvegicus]	2456.72	154.56	27730163
similar to putative G-protein coupled receptor [Homo sapiens] [Rattus	2540.90	0.00	27693097
Serine			
AF246705_1 putative serine-rich protein [Homo sapiens]	3677.11	1833.16	7739689
AC006344_3 serine/threonine protein kinase; similar to B-raf proto-oncogen	2787.27	806.98	5441948
AF390028_1 serine/threonine protein kinase kkialre-like 1 [Homo sapiens]	3590.11	1168.23	18087335

CTRO_HUMAN Citron protein (Rho-interacting, serine/threonine kinase 21)	5901.76	1887.74	6225217
Fas-activated serine/threonine kinase isoform 1 [Homo sapiens]	6407.04	794.28	5729822
I38873 serine C-palmitoyltransferase (EC 2.3.1.50) Lcb2 chain - human (fragment)	2719.82	0.00	2136140
JC5314 CDC28/cdc2-like kinase associating arginine-serine cyclophilin - human	3569.05	798.91	7430316
KI11_HUMAN Probable serine/threonine-protein kinase KIAA1811	4920.51	198.61	34395684
KIAA0359 [Homo sapiens] like 3; serine-threonine	3181.97	0.00	40788226
KIAA1982 protein [Homo sapiens] e serine protease	3028.57	0.00	18916886
KIAA2002 protein [Homo sapiens] inine/serine-rich	4569.08	0.00	21693150
male germ cell-associated kinase; serine/threonine protein kinase MAK	3088.09	574.18	11496279
microtubule associated serine/threonine kinase 3 [Homo sapiens]	6280.91	2505.12	37552548
microtubule associated testis specific serine/threonine protein kinase	3727.18	650.43	14149671
misshapen/NIK-related kinase isoform 3; GCK family kinase MINK; serine	3460.95	0.00	27436917
NIMA (never in mitosis gene a)-related kinase 4; Serine/threonine prote	4602.70	0.00	4507277
PCTAIRE protein kinase 1 isoform a; serine/threonine-protein kinase [Ho	2909.69	545.55	5453860
PFT1_HUMAN Serine/threonine protein kinase PFTAIRE-1	2737.85	818.78	22653927
placental protein 11 precursor; 22 serine protease [Homo sapiens]	3093.78	899.29	5174623
polo-like kinase 4; serine/threonine kinase 18; Snk Akin Kinase [Homo	6858.87	481.35	21361433
protein kinase CHK2 isoform b; checkpoint-like protein CHK2; serine/th	3407.35	0.00	22209009
serine/threonine kinase 9 [Homo sapiens] in frabin	4065.81	806.14	23271297
serine/threonine kinase FKSG81; spermiogenesis associated 4 [Homo sapi	3623.64	0.00	14042947
serine/threonine kinase with Dbl- and pleckstrin homology domain [Homo	9121.26	377.92	5902140
serine/threonine protein kinase [Homo sapiens] ru	4592.52	537.07	15131540
serine/threonine protein kinase Kp78 splice variant CTAK75a [Homo sapiens	3450.34	1761.48	5714636
serine/threonine protein kinase; Murine thymoma viral (v-akt) oncogene	3850.29	0.00	4885061
similar to FUS interacting protein (serine-arginine rich) 1 isoform 2;	3263.52	905.66	27732851
similar to phosphoserine aminotransferase isoform 1 [Homo sapiens]	3790.86	0.00	41146632
NIMA (never in mitosis gene a)-related kinase 4; Serine/threonine prote	5016.52	0.00	16160923
similar to Serine/threonine protein kinase PRKX (Protein kinase PKX1)	3492.65	675.97	41150093
similar to Serine/threonine-protein kinase NEK2 (NimA-related protein	4244.78	0.00	27499843
similar to serine-arginine repressor protein (35 kDa) [Homo sapiens] [	3226.16	1219.32	27714289
similar to splicing factor, arginine/serine-rich 2, interacting protei	2539.43	874.15	27708112
similar to tousled-like kinase 1; KIAA0137 gene product; serine threon	3178.20	0.00	25047872
splicing factor, arginine/serine-rich 2, interacting protein; SC35-inte	3926.61	294.26	4759172
splicing factor, arginine/serine-rich 8 isoform 1 [Homo sapiens]	5694.12	2188.22	27664420
Phosphatase			
2ACC_HUMAN Serine/threonine protein phosphatase 2A, 48 kDa regulatory subunit	3457.70	0.00	8134295
A Chain A, Human Vh1-Related Dual-Specificity Phosphatase	3569.76	0.00	1633321
A47114 phosphoprotein phosphatase (EC 3.1.3.16) 2A regulatory chain PR72 - human	3489.12	143.61	539659
A48148 protein-tyrosine-phosphatase (EC 3.1.3.48), receptor type gamma - human	6206.10	583.97	477137
alpha isoform of regulatory subunit B", protein phosphatase 2 isoform	3302.03	0.00	32967586
BC37295_3 [Homo sapiens] , fructose-bisphosphatase;	2975.07	0.00	4567181
C1 domain-containing phosphatase and tensin-like protein isoform 3; pu	5673.35	412.92	38787970
ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin); Phosph	4498.33	1658.32	20070230
F261_HUMAN 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (6PF-2-K/Fru-	5403.48	342.00	2507178
I38127 phosphoprotein phosphatase (EC 3.1.3.16) 1 glycogen-binding regulatory chain	9314.48	1971.42	2135920
Myotubularin [Homo sapiens] ens] ne phosphatase is	5130.20	0.00	21314969
phosphatidic acid phosphatase type 2B; phosphatidic acid phosphohydrol	2484.21	887.54	29171740
Prostatic acid phosphatase precursor [Homo sapiens]	2680.73	0.00	14250150
protein phosphatase 1, regulatory (inhibitor) subunit 12B isoform a; my	5364.87	1714.45	4505319
protein phosphatase 1, regulatory (inhibitor) subunit 13B; apoptosis-s	3764.31	710.37	18699720
protein phosphatase 1, regulatory (inhibitor) subunit 5; Phosphatase 1,	4584.22	0.00	4885559
protein phosphatase 1, regulatory (inhibitor) subunit 9A [Homo sapiens	3695.73	0.00	41147635
protein phosphatase 1, regulatory subunit 10; phosphatase nuclear targ	7216.57	2527.95	25777671
Protein phosphatase 1D [Homo sapiens] mosomal) pro	3493.26	0.00	21707520
protein phosphatase 4, regulatory subunit 1 [Homo sapiens]	4813.57	395.09	4826934
protein tyrosine phosphatase [Homo sapiens] iens] &	27187.00	4064.05	1479976
protein tyrosine phosphatase, non-receptor type 12; protein-tyrosine p	3535.22	0.00	18375652
protein tyrosine phosphatase, non-receptor type 13 isoform 1; protein	11710.04	2205.85	18375646
protein tyrosine phosphatase, non-receptor type 14; cytoskeletal-assoc	4012.97	606.15	34328899
protein tyrosine phosphatase, non-receptor type 21; protein tyrosine ph	2769.08	811.82	5902032
protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), in	4682.52	842.14	42655963
protein tyrosine phosphatase, receptor type, N precursor; islet cell an	2417.95	1177.89	4506321
protein tyrosine phosphatase, receptor type, U isoform 1 precursor; pr	4399.52	1157.17	19743933
protein-tyrosine phosphatase [Homo sapiens] pe 1] n	6763.54	2945.31	633073
PTN3_HUMAN Protein tyrosine phosphatase, non-receptor type 3 (Protein-tyrosine	4936.43	435.03	131530

pyridoxal (pyridoxine, vitamin B6) phosphatase; pyridoxal phosphate ph	4509.16	0.00	10092677
Raichu404X [Homo sapiens] in tyrosine phosphatase	41534.24	0.00	14595132
receptor-type protein tyrosine phosphatase beta, RPTP beta, PTP zeta [huma	3628.47	160.16	300182
receptor-type protein tyrosine phosphatase O isoform b precursor; glome	7359.26	2445.73	4506323
SH2 domain containing phosphatase anchor protein 1 isoform c; SH2 doma	2823.56	0.00	20270265
similar to Dual specificity protein phosphatase 3 (Dual specificity pr	2695.71	187.71	22057039
similar to protein phosphatase 2, regulatory subunit B (B56), alpha is	3827.86	1291.84	20920554
Similar to protein phosphatase 2, regulatory subunit B (B56), epsilon is	2707.31	385.02	29386972
similar to protein tyrosine phosphatase, receptor type, Q isoform 1 pr	4870.00	303.56	41187885
similar to putative protein phosphatase type 2C [Homo sapiens] [Rattus	3709.35	0.00	27691652
Rab			
FYVE-finger-containing Rab5 effector protein rabenosyn-5 [Homo sapiens	3201.76	1091.29	11641241
Rab coupling protein; Rab-interacting recycling protein; Rab effector	2828.04	0.00	20127404
small GTP binding protein Rab7 [Homo sapiens] 7 li	3792.97	997.29	1174149
similar to Rab12 protein [Homo sapiens] ns]	2712.49	843.70	37545057
molecule interacting with Rab13 [Homo sapiens] og	2871.53	423.63	27803369
Rab39 [Homo sapiens] Å	4289.54	0.00	22651417
similar to vascular Rab-GAP/TBC-containing [Homo sapiens] [Mus musculu	3015.05	931.82	28529613
similar to Rab GDP dissociation inhibitor beta (Rab GDI beta) (GDI-2)	3391.69	1002.45	17448269
I37234 Rab geranylgeranyltransferase component A (REP-1) - human	12646.21	0.00	7512350
TBC1 domain family, member 8 (with GRAM domain); vascular Rab-GAP/TBC-c	2815.94	206.10	5902154
Rho			
similar to FERM, RhoGEF, and pleckstrin domain protein 1; chondrocyte-	3200.12	348.66	25030706
FERM, RhoGEF, and pleckstrin domain protein 1; chondrocyte-derived ezri	4323.96	809.21	5031633
AF498974_1 small GTP binding protein RhoG [Homo sapiens]	39072.44	0.00	20379122
F59433 RhoGAP protein [imported] - human	7860.87	3192.33	25535923
hypothetical protein FLJ21817 similar to Rhoip2; likely ortholog of mo	12267.84	0.00	11967979
PTPL1-associated RhoGAP 1 [Homo sapiens] s 1] 54	4244.71	205.63	38016932
similar to PTPL1-associated RhoGAP 1 [Homo sapiens] [Rattus norvegicus	2772.14	1018.79	27696015
IL17Rhom [Homo sapiens] munodeficiency virus 1] X&	2810.95	0.00	37182667
Ras			
CADPS2 [Homo sapiens] ating protein 4 (RasGAP-acti	6200.95	0.00	23573413
GNRP_HUMAN Guanine nucleotide releasing protein (GNRP) (Ras-specific nucleoti	8106.62	1191.23	13124259
kinase suppressor of Ras-2 [Homo sapiens]	3395.00	0.00	34222393
Ras-related GTP-binding protein RAGA [Homo sapiens]	4226.73	0.00	5729999
Ras-GRF2 [Homo sapiens] ocular albinism 1 (Nettles	4384.38	889.36	2522208
ras-related C3 botulinum toxin substrate 2; Ras-related C3 botulinum to	105143.33	26212.47	4506381
Ras-GAP SH3 binding protein [Homo sapiens] I-39 pro	3784.48	0.00	3098601
Raf			
ORF (A-raf) (AA 1-606) [Homo sapiens] 9; TGF beta	4651.90		1340152
SNX6_HUMAN Sorting nexin 6 (TRAF4-associated factor 2)	4140.33	1303.50	10720285
WASP			
IQGAP3 [Homo sapiens]	4770.52	1179.47	30038859
WAS2_HUMAN Wiskott-Aldrich syndrome protein family member 2 (WASP-family prot	3626.13	408.58	13431974
WASPIP protein [Homo sapiens] ein [Homo sapiens] r	4503.33	0.00	12804123
Wiskott-Aldrich syndrome protein; Wiskott-Aldrich syndrome (eczema-thro	2973.34	0.00	4507909
Actin			
similar to axonal-associated cell adhesion molecule [Homo sapiens]	4537.92	470.53	19913548
actin-like 7A; actin-like 7-alpha [Homo sapiens]	6094.00	144.72	5729720
A Chain A, The Yeast Actin Val 159 Asn Mutant Complex With Human Gelsolin Segme	58860.70	11443.71	7546413
alpha-actinin [Homo sapiens] man Apolipoprotein A-I	3548.45	0.00	28723
anillin, actin binding protein (scraps homolog, Drosophila); anillin (D	3666.14	0.00	8923832
actin-related protein 2; ARP2 (actin-related protein 2, yeast) homolog	3689.57	124.58	5031571
actin related protein 2/3 complex subunit 1B; ARP2/3 protein complex su	2796.58	0.00	5031601
ARP3 actin-related protein 3 homolog; ARP3 (actin-related protein 3, ye	3835.10	172.29	5031573
actin-related protein 8; ARP8 (actin-related protein 8, yeast) homolog	17695.33	645.00	27923738
contactin 1 isoform 1 precursor; glycoprotein gP135 [Homo sapiens]	10601.20	919.70	28373117
contactin 6; neural adhesion molecule [Homo sapiens]	8101.76	1553.98	7657361
cortactin binding protein 2; cortactin-binding protein 2; chromosome 7	7537.20	1014.20	16975496
dynactin [Homo sapiens] munodeficiency virus 1] en	3939.97	328.98	1419567
filamin 1 (actin-binding protein-280); filamin 1; filamin A, alpha (act	4639.86	1888.72	4503745
gamma filamin; filamin 2; filamin C, gamma (actin-binding protein-280);	13844.57	4390.39	4557597
actin-binding LIM protein 1 isoform a; LIM actin-binding protein 1; li	5094.96	1878.05	21284383
microfilament and actin filament cross-linker protein isoform c; 620 k	8059.44	2322.67	15011908
microfilament and actin filament cross-linker protein isoform b; 620 k	13966.29	5392.11	33188443

NEB1_HUMAN Neurabin-I (Neural tissue-specific F-actin binding protein I) (Pro	2543.22	0.00	13431727
nebulette; actin-binding Z-disk protein [Homo sapiens]	5207.38	1202.08	5453758
SH3 and multiple ankyrin repeat domains 2 isoform 1; cortactin SH3 dom	4659.51	1999.56	19743794
SRC8_HUMAN Src substrate cortactin (Amplaxin) (Oncogene EMS1)	2464.89	649.81	2498954
SWI/SNF-related matrix-associated actin-dependent regulator of chromat	6144.01	709.43	21237802
SWI/SNF-related matrix-associated actin-dependent regulator of chromat	7250.51	1949.77	21071058
SWI/SNF-related matrix-associated actin-dependent regulator of chromat	5117.65	1051.29	21071044
supervillin isoform 2; membrane-associated F-actin binding protein p20	7592.58	443.34	11496982
Calcium			
voltage-dependent calcium channel gamma-4 subunit; neuronal voltage-gat	4563.00	0.00	7656948
small conductance calcium-activated potassium channel protein 3 isofor	2749.07	834.19	21361129
calcium channel alpha2-delta3 subunit [Homo sapiens]	2524.11	101.23	7024361
ATB4_HUMAN Plasma membrane calcium-transporting ATPase 4 (PMCA4) (Plasma memb	3557.86	617.15	14286105
annexin VI isoform 1; calcium-binding protein p68; annexin VI (p68); ca	8242.25	6070.86	4502109
ATB2_HUMAN Plasma membrane calcium-transporting ATPase 2 (PMCA2) (Plasma memb	11619.73	1784.59	14286115
alpha1A-voltage-sensitive calcium channel [Homo sapiens]	23172.32	3636.01	1763632
alpha 2 delta calcium channel subunit isoform II [Homo sapiens]	2670.78	295.24	2781441
AF127036_1 calcium-activated chloride channel protein 1 [Homo sapiens]	3489.11	1466.15	4585469
similar to calcium activated chloride channel 2 [Homo sapiens] [Rattus	3702.51	657.61	27729605
calcium-activated potassium channel alpha subunit [Homo sapiens]	4976.73	0.00	2570854
calcium-binding transporter [Homo sapiens] DNA-b	7068.12	0.00	33598954
intermediate conductance calcium-activated potassium channel protein 1;	7311.36	0.00	4504859
Calcium-sensing receptor, similar to human metabotropic glutamate rece	8831.08	3275.73	17540172
tumor-associated calcium signal transducer 1 precursor; membrane compon	5964.59	0.00	4505059
voltage-gated calcium channel alpha 1 subunit [Homo sapiens]	4676.48	0.00	3025824
voltage-gated L-type calcium channel alpha-1 subunit [Homo sapiens]	4866.68	785.79	6525019
similar to voltage-gated calcium channel alpha(2)delta-4 subunit [Homo	5742.88	0.00	27499634
calcium channel, voltage-dependent, alpha 1E subunit [Homo sapiens]	4098.10	1336.56	4502529
voltage-dependent calcium channel gamma-3 subunit; neuronal voltage-gat	3454.52	373.02	5729756
calcium channel, voltage-dependent, L type, alpha 1B subunit; calcium c	8200.60	340.64	4502523
CCAH_HUMAN Voltage-dependent T-type calcium channel alpha-1H subunit (Cav3.2)	12693.76	4128.73	23503045
protein disulfide isomerase related protein (calcium-binding protein, i	3979.80	500.69	4758304
low density lipoprotein-related protein 2; megalin; calcium sensor prot	10401.47	2020.15	6806919
calcium-permeable store-operated channel TRPM3c [Homo sapiens]	8276.97	3036.84	28626251
similar to S100 calcium-binding protein A10; S100 calcium-binding prot	7464.90	0.00	41222866
Dedicator			
dedicator of cyto-kinesis 1 [Homo sapiens]	9312.92	4067.34	4503355
Similar to dedicator of cyto-kinesis 1 [Homo sapiens]	3114.32	802.60	27697129
similar to dedicator of cyto-kinesis 1 [Homo sapiens] [Mus musculus]	2631.12	794.92	28482120
dedicator of cytokinesis 4 [Homo sapiens]	11638.88	1006.94	29568109
DOC7_HUMAN Dedicator of cytokinesis protein 7	2727.54	648.85	32469770
Dynein			
dynein, axonemal, heavy polypeptide 8 [Homo sapiens]	18770.83	4396.76	15029526
axonemal dynein heavy chain 7 [Homo sapiens]	6934.50	1823.72	17864092
similar to axonemal dynein heavy chain 7 [Homo sapiens] [Mus musculus]	6385.36	143.20	28479205
similar to axonemal dynein heavy chain 7 [Homo sapiens] [Rattus norveg	27153.38	4712.71	27683429
similar to axonemal dynein heavy chain 7 [Homo sapiens] [Mus musculus]	2523.61	974.47	28478919
dynein, axonemal, heavy polypeptide 9 isoform 2; dynein, axonemal, lig	22493.00	13631.99	13876382
similar to dynein, axonemal, heavy polypeptide 9, isoform 2; dynein, a	2701.62	449.60	20347716
similar to dynein, axonemal, heavy polypeptide 9, isoform 2; dynein, a	6061.73	552.39	28510082
dynein, axonemal, heavy polypeptide 11; dynein, axonemal, heavy chain	5580.88	1704.05	16507235
similar to Dynein heavy chain at 89D CG1842-PA [Homo sapiens]	2774.18	342.09	2772561
DYHC_HUMAN Dynein heavy chain, cytosolic (DYHC) (Cytoplasmic dynein heavy cha	8334.52	2102.12	30581065
dynein, cytoplasmic, heavy polypeptide 2 [Homo sapiens]	4431.71	209.49	42659582
SI:zC220F6.1 (novel protein similar to human dynein heavy chain (DHC))	30517.86	4549.83	29561798
similar to SI:zC220F6.1 (novel protein similar to human dynein heavy c	7783.39	2755.42	38084535
Engulfment			
Cell corpse engulfment protein 5, similar to human major CRK-binding p	30146.15	4533.02	17538408
engulfment and cell motility 3; ced-12 homolog 3 [Homo sapiens]	3243.00	793.47	13376011
SNAP			
NPS1_HUMAN NipSnap1 protein	2908.31	0.00	17380144
guanine nucleotide exchange factor			
CAMP-regulated guanine nucleotide exchange factor II [Homo sapiens]	3982.47	857.82	18645151
guanine nucleotide exchange factor GEF-H1 [Homo sapiens]	3533.61	805.64	19744759
guanine nucleotide exchange factor Lbc [Homo sapiens]	5010.43	979.50	15207794

Rho guanine nucleotide exchange factor 1; 115-kD protein; mouse Lsc ho	5935.73	1920.28	15011972
Rho guanine nucleotide exchange factor 5; oncogene TIM; transforming im	2909.05	0.00	4885633
Rho guanine nucleotide exchange factor (GEF) 11; RhoA-specific guanine	5927.54	1879.42	7662086
Rho guanine nucleotide exchange factor (GEF) 11 isoform 2; RhoA-specif	9559.01	0.00	38026934
Rho guanine nucleotide exchange factor (GEF) 12; leukemia-associated rh	7356.69	1228.23	7662088
similar to Rho-specific guanine nucleotide exchange factor p114 [Homo	9078.45	4181.94	27691570
guanine nucleotide exchange factor p532 [Homo sapiens]	12428.88	4739.64	4557026
PGF2_HUMAN PDZ domain containing guanine nucleotide exchange factor 2 (PDZ-GE	2783.62	341.88	34395686
GTPase			
similar to TBC1 domain family, member 3; Rab GTPase-activating protein	3682.85	0.00	41150825
similar to Rho GTPase activating protein 12 [Homo sapiens]	3360.75	0.00	40548322
Rho GTPase activating protein [Homo sapiens] omo s	2843.64	0.00	34484320
TPA: Ras-related small GTPase [Homo sapiens] tic;	2564.57	253.24	37718739
GTPase-activating protein [Homo sapiens] mo sapien	4722.97	802.98	2389009
retinitis pigmentosa GTPase regulator interacting protein 1; RPGR-inte	6985.75	2693.27	21361839
AF464189_1 WAVE-associated Rac GTPase activating protein [Homo sapiens]	4565.80	745.25	24369934
Rho GTPase activating protein 1; RhoGAP; p50rhoGAP; CDC42 GTPase-activa	7043.31	303.29	4757766
similar to Rab GTPase-activating protein PRC17 [Homo sapiens]	4109.23	558.97	17450832
Rho GTPase activating protein 5; RhoGAP5; p190-B [Homo sapiens]	6198.75	856.32	4502221
SI:zC214P16.5 (novel protein similar to human RAB3 GTPase-activating pr	10038.28	1542.22	27884150
RAS protein activator like 2 isoform 2; Ras GTPase activating protein-	4085.21	1843.02	25121936
Rho-GTPase activating protein 10 [Homo sapiens]	6335.94	2459.70	20977541
retinitis pigmentosa GTPase regulator; retinitis pigmentosa 3 GTPase re	3047.98	1020.01	4506581
rab3 GTPase-activating protein, non-catalytic subunit [Homo sapiens]	2656.37	0.00	19923790
rasGTPase activating protein [Homo sapiens] Homo s	3352.97	891.12	1871475
Ras-GTPase-activating protein SH3-domain-binding protein; GAP binding p	3549.07	0.00	5031703
RAS (RAD and GEM)-like GTP-binding; GTPase GES; REM protein [Homo sapi	2479.16	0.00	20070266
truncated Rho GTPase-activating protein 4 splice form 2 [Homo sapiens]	2793.50	1220.48	29242797
SH3			
3BP1_HUMAN SH3-domain binding protein 1 (3BP-1) n [Homo	3677.87	336.51	20137581
vinexin beta (SH3-containing adaptor molecule-1) [Homo sapiens]	2454.86	0.00	19923335
SH3-domain GRB2-like 2 [Homo sapiens]	2410.38	0.00	4506931
CBLB_HUMAN Signal transduction protein CBL-B (SH3-binding protein CBL-B)	3489.40	576.81	8928017
AF136381_1 c-Cbl-associated protein SH3P12 [Homo sapiens]	5953.17	1651.69	6651089
sorting nexin 9; SH3 and PX domain-containing protein SH3PX1; Wiskott-A	5179.17	1373.07	7706706
Ras-GAP SH3 binding protein [Homo sapiens] I-39 pro	3711.44	0.00	3098601
SH3 multiple domains 3 [Homo sapiens] , H3 lysin	2875.65	412.96	42659727
SH3 and multiple ankyrin repeat domains 1; somatostatin receptor-inter	10140.35	3123.01	11968152
SH3 and multiple ankyrin repeat domains 2 isoform 1; cortactin SH3 dom	4547.03	1999.56	19743794
SHK3_HUMAN SH3 and multiple ankyrin repeat domains protein 3 (Shank3) (Prolin	4321.43	1478.14	22001986
similar to SH3-domain binding protein 1 [Homo sapiens] [Rattus norvegi	3983.16	465.50	27662696
similar to SH3-domain binding protein 4 [Homo sapiens] [Rattus norvegi	2949.51	0.00	27670928
SH3-domain kinase binding protein 1; c-Cbl-interacting protein [Homo s	6073.99	2115.37	13994242
similar to DKFZP586L2024 protein; target of Nesh-SH3 [Homo sapiens] [R	22401.31	621.53	28497066
SH3 protein interacting with Nck, 90 kDa, isoform 2 [Homo sapiens]	4809.22	0.00	20070807
SH2			
SH2A [Homo sapiens] n 569 [Homo sapiens] ency viru	2671.71	626.44	28435534
SH2-B gamma signaling protein [Homo sapiens] e 1] o	2877.53	453.04	8163913
suppressor of cytokine signaling 7; SH2 domain containing SOCS box pro	2899.47	0.00	18254466
SH2 domain containing 3C; novel SH2-containing protein 3; SH2 domain-c	2659.40	355.79	41281821
SH2 domain-containing 3C [Homo sapiens] n - human	4388.33	333.67	21619057
Similar to Kluyveromyces lactis mismatch repair protein Msh2p msh2 SWAL	4756.67	1033.57	23499288
NCK2_HUMAN Cytoplasmic protein NCK2 (NCK adaptor protein 2) (SH2/SH3 adaptor	5480.49	2118.34	20532395
SH2 domain binding protein 1; TPR-containing, SH2-binding phosphoprotei	4028.77	505.38	7661950
kinase			
pyruvate dehydrogenase kinase, isoenzyme 2 [Homo sapiens]	2558.86	0.00	19923736
sphingosine kinase type 1-interacting protein [Homo sapiens]	2814.53	204.90	41191486
similar to rat brain-enriched guanylate kinase-associated protein [Hom	2987.05	431.77	13124765
UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase; N-acylm	2462.33	358.54	4885285
A38282 p58 galactosyltransferase-associated protein kinase - human	2413.30	0.00	107255
glycogen synthase kinase 3 alpha [Homo sapiens]	2897.91	1202.99	11995474
homeodomain-interacting protein kinase 2 [Homo sapiens]	2790.32	501.47	11493928
fucokinase; L-fucose kinase; 1110046B12Rik [Homo sapiens]	2658.52	757.02	21450822
T17287 protein kinase (EC 2.7.1.37) akt3 short splice form - human	2754.93	0.00	7512664
diacylglycerol kinase epsilon; diacylglycerol kinase, epsilon (64kD) [H	2677.62	1278.58	4503313

Similar to Bmp2-inducible kinase [Homo sapiens] X&	2456.59	479.75	23271902
phosphofructokinase, liver; Phosphofructokinase, liver type; human liv	2891.13	656.56	21361070
CDC-like kinase 1 [Homo sapiens] thalamus protein	2913.75	308.54	21618731
ER transmembrane protein kinase receptor in the unfolded protein respo	4157.98	431.64	17567737
creatine kinase, mitochondrial 2 (sarcomeric) [Homo sapiens]	3555.72	937.30	20810521
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 isofor	3128.43	651.60	4503427
A39577 protein-tyrosine kinase (EC 2.7.1.112) JAK1 - human nt	4308.28	707.77	107499
KITH_HUMAN Thymidine kinase, cytosolic	2453.49	752.68	23503074
phosphoglycerate kinase 1 [Homo sapiens]	81424.40	24557.58	4505763
protein kinase PKNbeta [Homo sapiens] (RHAMM) [Ho	2400.25	377.85	6088096
similar to ribosomal protein S6 kinase, 52kDa, polypeptide 1; ribosoma	3040.60	0.00	27679602
AAH08771 Similar to ELKL motif kinase [Homo sapiens]	4761.05	1272.79	14250622
AAK2_HUMAN 5'-AMP-activated protein kinase, catalytic alpha-2 chain (AMPK alp	11183.63	0.00	20178276
aarF domain containing kinase 2; putative ubiquinone biosynthesis prot	12617.69	320.20	32261307
aarF domain containing kinase 5 [Homo sapiens] &	3241.20	0.00	41393593
ABL1_HUMAN Proto-oncogene tyrosine-protein kinase ABL1 (p150) (c-ABL)	6329.75	868.71	125135
AKA3_HUMAN A-kinase anchor protein 3 (Protein kinase A anchoring protein 3) (	4134.86	1543.86	14194457
bA151F5.1.1 (A kinase (PRKA) anchor protein 2) [Homo sapiens]	4126.57	0.00	17384432
A-kinase anchoring protein 7 isoform gamma; A-kinase anchor protein, 18	2760.75	453.60	7706527
A kinase anchor protein 9 isoform 2; yotiao; A-kinase anchoring protei	9238.08	2152.37	22538387
A kinase anchor protein 11 isoform 1; A-kinase anchoring protein 220 [H	3214.07	1236.14	7706457
similar to Neighbor of A-kinase anchoring protein 95 (Homologous to AK	2788.86	1098.55	27480929
similar to protein kinase, putative; protein id: At3g24400.1 [Arabidop	6064.45	0.00	22059483
protein kinase, AMP-activated, beta 2 non-catalytic subunit; AMPK beta	3755.99	0.00	4885561
B54024 protein kinase (EC 2.7.1.37) cdc2-related PITSLRE alpha 2-2 - human	2656.20	0.00	1082284
mitotic checkpoint protein kinase Bub1A [Homo sapiens]	5921.84	833.88	3493533
cell adhesion kinase beta [Homo sapiens] nding immu	12092.80	2355.56	1165219
similar to CamKI-like protein kinase [Homo sapiens] [Rattus norvegicus	2606.89	0.00	27688651
casein kinase 1, alpha 1; down-regulated in lung cancer [Homo sapiens]	4551.06	0.00	19923746
AF049090_1 casein kinase I gamma 3L [Homo sapiens] &	2387.19	409.92	4590042
cyclin-dependent kinase inhibitor [Homo sapiens] &	4092.49	0.00	28629110
cyclin-dependent kinase (CDC2-like) 11; death-preventing kinase [Homo	4726.60	0.00	30387611
similar to cyclin dependent kinase 8 [Danio rerio] [Homo sapiens]	6643.68	0.00	17438621
similar to CDC-like kinase 3; cdc2/CDC28-like protein kinase 3 [Homo s	3605.55	0.00	42655701
cell division cycle 2-like 5 isoform 2; CDC2-related protein kinase 5	3224.46	445.39	14110390
CDC28 protein kinase 1B; CDC2-associated protein CKS1; cell division co	3806.48	0.00	4502857
ceramide kinase isoform a; lipid kinase LK4 [Homo sapiens]	3407.82	628.41	20336726
protein kinase, cGMP-dependent, type I; Protein kinase, cGMP-dependent	2448.19	0.00	10835242
KGPA_HUMAN cGMP-dependent protein kinase 1, alpha isozyme (CGK 1 alpha) (cGKI-	3474.52	546.00	6225588
diacylglycerol kinase alpha [Homo sapiens]	2522.53	715.33	3551828.0
diacylglycerol kinase, beta isoform 1; diacylglycerol kinase, beta (90	5643.17	450.37	22027632
diacylglycerol kinase delta2 [Homo sapiens] prot	4896.33	0.00	22773821
KDGT_HUMAN Diacylglycerol kinase, theta (Diglyceride kinase) (DGK-theta) (DAG	2761.32	683.66	1708624.0
diacylglycerol kinase iota [Homo sapiens] piens] gi	5905.02	254.62	7230557
KDGZ_HUMAN Diacylglycerol kinase, zeta (Diglyceride kinase) (DGK-zeta) (DAG k	6695.02	1288.46	12644407
death-preventing kinase [Homo sapiens] saccharomyc	2947.70	74.31	13540814
deoxyguanosine kinase [Homo sapiens] sapiens]	4420.56	192.46	1480198
A57099 DNA-activated protein kinase, catalytic subunit - human	21100.55	4747.11	1362789
E2K3_HUMAN Eukaryotic translation initiation factor 2-alpha kinase 3 precurso	5289.57	327.22	18203329
EPA3_HUMAN Ephrin type-A receptor 3 precursor (Tyrosine-protein kinase receptor	4169.62	287.29	125387
elongation factor-2 kinase [Homo sapiens] sapiens]	30305.25	6034.51	21618568
putative ethanolamine kinase [Homo sapiens]	4078.89	1401.86	8922650
FES_HUMAN Proto-oncogene tyrosine-protein kinase FES/FPS (C-FES)	7036.10	296.44	400127
FYV1_HUMAN FYVE finger-containing phosphoinositide kinase (1-phosphatidylinosi	7197.30	665.01	9296993
G01743 AMP-activated protein kinase homolog - human (fragment)	6040.16	0.00	7434350
heart alpha-kinase [Homo sapiens]	4782.15	1245.88	16418433
HXK2_HUMAN Hexokinase, type II (HK II) (Muscle form hexokinase)	6115.77	696.43	1708361
Hexokinase 3 [Homo sapiens] uitin activating enzym	2756.92	214.47	20380888
glucokinase regulatory protein; hexokinase 4 [Homo sapiens]	7054.81	466.97	4557621
homeodomain interacting protein kinase 1-like protein; nuclear body as	3763.33	217.41	23397584
dJ8L15.1 (homeodomain-interacting protein kinase 3) [Homo sapiens]	3620.01	788.57	10862835
HPK/GCK-like kinase HGK [Homo sapiens] 7 (ubiquito	6473.95	124.26	4322936
IKK-related kinase epsilon [Homo sapiens]	6676.16	1818.86	42655593
interleukin-1 receptor-associated kinase 3; interleukin-1 receptor-asso	2569.80	391.94	6005792
similar to interleukin-1 receptor-associated kinase 3; interleukin-1 r	3543.84	908.90	27718477

inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase	4486.36	165.28	41352712
similar to Inhibitor of nuclear factor kappa B kinase beta subunit (I-	6186.28	60.68	20538863
JAK3_HUMAN; JANUS KINASE 3; JAK-3; LEUKOCYTE JANUS KINASE; L-JAK [Homo sa	5235.68	0.00	4558771
JC6138 extracellular signal regulated kinase (EC 2.7.1.-) 6 - human	4589.16	0.00	7434299
IkappaBkinase complex-associated protein [Homo sapiens]	10869.64	2045.87	12002688
KIAA0096 gene product is related to a protein kinase. [Homo sapiens]	5100.87	1806.19	20521878
KIAA1275 protein [Homo sapiens] se kinase kinase 1	2839.58	1163.13	6331294
protein kinase, lysine deficient 1; kinase deficient protein [Homo sap	9124.14	0.00	12711660
protein kinase, lysine deficient 4; putative protein kinase WNK4 [Homo	4985.41	907.92	15277312
MAP/ERK kinase kinase 4 isoform a; SSK2/SSK22 MAP kinase kinase kinase,	17728.66	5387.54	5803088
similar to MAP/ERK kinase kinase 5; apoptosis signal regulating kinase	3399.25	538.53	25056550
similar to MAP/ERK kinase kinase 5; apoptosis signal regulating kinase	3230.82	387.78	42662661
membrane-associated guanylate kinase-related 3 [Homo sapiens]	8819.30	560.21	27544925
plaucible mixed-lineage kinase protein [Homo sapiens]	5119.50	1078.62	9927293
AF251442_1 mixed lineage kinase MLK1 [Homo sapiens]	4540.64	0.00	12005724
human Mixed Lineage Kinase homolog (mlk-1) [Caenorhabditis elegans]	2413.05	197.97	25149255
renal tumor antigen; renal cell carcinoma antigen (MOK protein kinase)	5476.87	0.00	7657498
myotonin protein kinase; MtPK [Homo sapiens] rn hexo	2936.83	0.00	633865
myotonic dystrophy protein kinase like protein [Homo sapiens]	2561.81	0.00	41200342
similar to NIMA (never in mitosis gene a)- related kinase 11 [Homo sap	2455.60	0.00	20895109
nucleoside diphosphate kinase type 6 (inhibitor of p53-induced apoptosi	6984.92	88.26	5031951
6-phosphofructo-2-kinase heart isoform [Homo sapiens]	7169.95	1517.80	11933149
p21-activated kinase 4; protein kinase related to S. cerevisiae STE20,	5222.12	780.24	5031975
pantothenate kinase 1 isoform beta; pantothenic acid kinase [Homo sapi	4844.03	1168.26	23510402
TPA: pantothenate kinase 4 putative variant; PANK4p [Homo sapiens]	4692.46	0.00	20043249
A45746 phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) 1 - human	3140.60	133.41	539657
phosphofructokinase, platelet; Phosphofructokinase, platelet type [Hom	5607.77	765.92	11321601
phosphoglycerate kinase 2; phosphoglycerate kinase 1, pseudogene 2 [Ho	7756.26	469.97	20270259
A27816 phosphoglycerate kinase (EC 2.7.2.3) 2 - human	9389.92	359.70	88344
phosphorylase kinase, alpha 1 (muscle); Phosphorylase kinase, muscle, a	6981.61	315.42	4505779
phosphorylase kinase, alpha 2 (liver); Phosphorylase kinase deficiency,	3739.54	516.10	4505781
phosphorylase kinase, beta; Phosphorylase kinase, beta polypeptide [Hom	4735.19	1729.31	4505783
similar to Phosphorylase B kinase gamma catalytic chain, skeletal musc	3848.63	1132.32	37541323
PI 3-kinase enhancer long isoform [Homo sapiens] 5	3877.84	0.00	25989575
polo-like kinase; polo (Drosophia)-like kinase [Homo sapiens]	2698.74	235.45	21359873
S23386 protein kinase (EC 2.7.1.37) cdc2-related PSSALRE - human	4339.04	292.26	420022
pyruvate kinase, liver and RBC; Pyruvate kinase, liver and RBC type [H	2807.93	0.00	10835121
pyruvate kinase, liver and RBC isoform 2; pyruvate kinase, liver and R	4141.17	85.97	32967597
kinase suppressor of Ras-2 [Homo sapiens]	3395.00	0.00	34222393
ribosomal protein S6 kinase, 90kDa, polypeptide 1; ribosomal protein S	5089.42	840.89	20149547
ribosomal protein S6 kinase, 90kDa, polypeptide 4; ribosomal protein ki	3755.97	373.42	4506735
K6A2_HUMAN Ribosomal protein S6 kinase alpha 2 (S6K-alpha 2) (90 kDa ribosomal	4866.13	789.64	6166243
SH3-domain kinase binding protein 1; c-Cbl-interacting protein [Homo s	6539.11	2115.37	13994242
similar to activator of S phase kinase [Homo sapiens] [Rattus norvegic	3424.09	948.18	27714911
sphingosine kinase type 2 isoform [Homo sapiens]	8233.82	0.00	21361699
prostate derived STE20-like kinase PSK [Homo sapiens]	6170.09	1650.86	7706401
tau tubulin kinase 1 [Homo sapiens] F receptor [	3115.58	429.80	37552194
gi|27469428|gb|AAH41876.1| Similar to tau tubulin kinase 1 [Homo sapiens] gi|	2476.95	656.31	27469428
tau tubulin kinase 2; tau-tubulin kinase [Homo sapiens]	14733.88	5805.07	28466991
T45070 protein kinase homolog R31240_1 [imported] - human (fragment)	5041.19	825.49	11360323
Similar to testis-specific kinase 1 [Homo sapiens]	3758.14	0.00	23512307
TVHURS kinase-related protein ros-1 precursor - human A1196	8902.48	1780.88	625223
kinase insert domain receptor (a type III receptor tyrosine kinase); K	5028.62	871.22	11321597
unc-51-like kinase 1 [Homo sapiens]	4639.77	3239.06	4507831
similar to unc-51-like kinase 2 [Homo sapiens] eno	3198.55	388.32	23241685
similar to putative protein kinase WNK3 [Homo sapiens] [Rattus norvegi	2676.53	401.07	27708342
xylulokinase [Homo sapiens] ide exchange factor p5	12508.04	115.65	3298502
NADPH			
NOX1_HUMAN NADPH oxidase homolog 1 (NOX-1) (NOH-1) (NADH/NADPH mitogenic oxida	5463.92	1870.36	8134597
NADPH oxidase-related, C2 domain-containing protein [Homo sapiens]	3441.49	215.33	14249614
NADPH-cytochrome P-450 reductase [Homo sapiens] c	3302.03	858.32	11414998
Oncogene			
v-abl Abelson murine leukemia viral oncogene homolog 2 isoform a; Abels	5151.77	1148.26	6382060
Cas-Br-M (murine) ecotropic retroviral transforming sequence; oncogene	2838.14	269.22	4885117
FGFR1 oncogene partner [Homo sapiens]	3071.14	1081.11	5901954

similar to FGFR1 oncogene partner [Homo sapiens] [Rattus norvegicus]	2796.21	0.00	27730213
similar to GLI-Kruppel family member GLI2 isoform beta; oncogene GLI2;	4549.35	0.00	27675828
jun B proto-oncogene [Homo sapiens]	3406.23	0.00	4504809
MCF.2 cell line derived transforming sequence; Oncogene MCF2 (oncogene	5131.48	519.86	19923310
RAB7, member RAS oncogene family-like 1 [Homo sapiens]	4949.87	0.00	4506375
RAB11B, member RAS oncogene family; RAB11B, member of RAS oncogene fami	10411.91	163.89	4758986
RAB20, member RAS oncogene family; likely ortholog of mouse RAB20, memb	2430.98	972.32	8923401
fGFR1 oncogene partner [Homo sapiens]	2520.35	0.00	33946329
proto-oncogene c-ros-1 protein precursor; transmembrane tyrosine-speci	8458.75	898.59	19924165
S57867 oncogene 1 - human sapiens] à	3394.54	621.51	1362878
translocated promoter region (to activated MET oncogene); Tumor potenti	11982.50	2996.26	4507659
ets variant gene 6; TEL1 oncogene [Homo sapiens]	2467.68	0.00	4503611
similar to ets variant gene 6; TEL1 oncogene [Homo sapiens] [Rattus no	2740.98	0.00	27713431
ubiquitin specific protease, proto-oncogene; Unph [Homo sapiens]	3335.08	55.37	4507853
v-crk sarcoma virus CT10 oncogene homolog isoform a; avian sarcoma vir	2641.09	0.00	41327712
Ribosylation			
ADP-ribosylation factor guanine nucleotide-exchange factor 2; brefeldin	7240.08	655.04	5453573
ADP-ribosylation factor domain protein 1 isoform gamma; ADP-ribosylati	2557.90	0.00	15208643
ARF-GAP, RHO-GAP, ankyrin repeat and plekstrin homology domains-contai	7892.97	1876.04	21264337
LIM domain			
AF330045_1 LIM domain only 7 [Homo sapiens]] it 4	5053.82	895.93	17225574
similar to LIM domain only 7 isoform a [Homo sapiens] [Rattus norvegic	24730.04	1030.73	27704266
Rac			
ras-related C3 botulinum toxin substrate 1 isoform Rac1; rho family, sm	2657.45	0.00	9845511
Random			
paxillin gamma [Homo sapiens] 28 [Homo sapiens] 54	4509.48	1209.88	1912057
cofilin 1 (non-muscle) [Homo sapiens]	14027.98	0.00	5031635
gamma filamin; filamin 2; filamin C, gamma (actin-binding protein-280);	13460.30	4390.39	4557597
filamin 1 (actin-binding protein-280); filamin 1; filamin A, alpha (act	6336.40	1888.72	4503745
Growth Factor			
hepatocyte growth factor-like protein homolog [Homo sapiens]	2953.77	785.73	1141775
fibroblast growth factor 22 precursor [Homo sapiens]	3236.41	0.00	10190672
fibroblast growth factor receptor 3 isoform 1 precursor; hydroxyaryl-pr	2593.47	867.19	4503711
FAT tumor suppressor 2 precursor; multiple epidermal growth factor-lik	12624.67	1879.80	13787217
hepatoma-derived growth factor (high-mobility group protein 1-like); He	2779.46	0.00	4758516
bA288H12.1 (insulin-like growth factor 2 receptor) [Homo sapiens]	14090.38	1055.24	10183620
insulin-like growth factor binding protein, acid labile subunit; INSULI	7418.50	111.55	4826772
nudix-type motif 6 isoform a; antisense basic fibroblast growth factor	3378.29	790.43	20149583
AF172452_1 opioid growth factor receptor [Homo sapiens]	4586.82	405.85	7595305
latent transforming growth factor beta binding protein 1 precursor [Hom	12393.88	320.77	4557731
LTBL_HUMAN Latent transforming growth factor beta binding protein, isoform 1L	3189.79	0.00	20138588
latent transforming growth factor beta binding protein 2 [Homo sapiens]	5781.98	2029.20	4557733
transforming growth factor, beta receptor III (betaglycan, 300kDa); tra	4800.18	613.78	4507471
latent transforming growth factor beta binding protein 4 [Homo sapiens]	2403.36	0.00	4505037
VGF nerve growth factor inducible precursor; neuro-endocrine specific	6692.75	0.00	17136078
vascular endothelial cell growth factor 165 receptor/neuropilin [Homo sap	2517.68	631.99	2978560
Interleukin			
interleukin 18 proprotein; interferon-gamma-inducing factor; interleuki	6773.90	1215.73	4504653
interleukin-1 receptor-associated kinase 3; interleukin-1 receptor-asso	2569.80	391.94	6005792
similar to interleukin-1 receptor-associated kinase 3; interleukin-1 r	3543.84	908.90	27718477
A60386 interleukin-4 receptor precursor - human	6088.80	0.00	280812
interleukin 1 receptor, type I precursor; interleukin 1 receptor alpha,	2595.88	1116.98	4504659
similar to interleukin 20 receptor, alpha; class II cytokine receptor	3609.13	1303.80	27676972
Cadherin			
similar to cadherin 3, type 1, P-cadherin (placental) [Homo sapiens]	5039.01	0.00	27693124
cadherin-5 [Homo sapiens] I protein FLJ38281 [Homo s	5509.06	160.57	29593
CAD9_HUMAN Cadherin-9 precursor	3004.51	0.00	13431362
Cadherin 13, preproprotein [Homo sapiens]	2638.01	613.72	20306944
cadherin 22 precursor; ortholog of rat PB-cadherin [Homo sapiens]	3542.91	0.00	16753221
cadherin related 23 isoform 1 precursor; cadherin-23; otocadherin [Hom	4749.97	245.94	16507962
protocadherin alpha 13 isoform 2 precursor; ortholog of mouse CNR5; KI	2653.39	133.74	14165397
protocadherin beta 1 precursor [Homo sapiens] X&	5778.54	234.23	14195607
protocadherin beta 10 precursor [Homo sapiens]	3697.74	715.30	9256602
protocadherin gamma subfamily B, 3 isoform 2 precursor [Homo sapiens]	4047.42	1240.24	14270496
protocadherin gamma subfamily B, 6 isoform 2 precursor [Homo sapiens]	2425.99	0.00	14270502

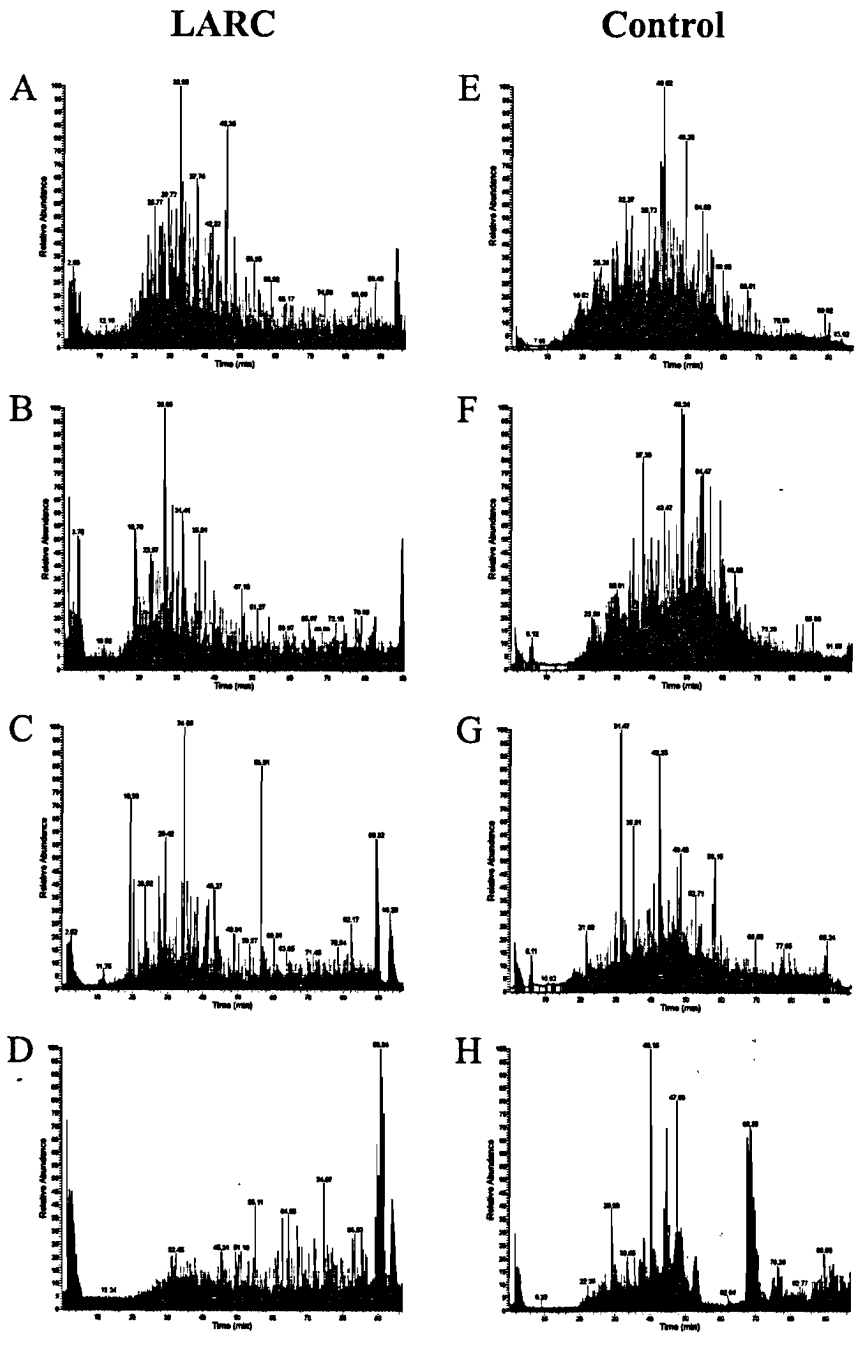
protocadherin LKC [Homo sapiens]]	2790.27	0.00	13537202
protocadherin 11 X-linked isoform a precursor; protocadherin X; protoca	4648.47	1071.63	7657443
protocadherin 12 precursor; vascular endothelial cadherin 2; vascular c	5073.37	265.56	7706113
protocadherin 16 precursor; fibroblast cadherin FIB1; fibroblast cadhe	7610.10	394.86	16933557
protocadherin 17; protocadherin 68 [Homo sapiens]	5048.13	1843.18	14589927
SI:bZ6L08.1 (novel protein similar to human cadherin, EGF LAG seven-pas	5945.04	0.00	26788046
T00041 BH-protocadherin PCDH7 (clone BH-Pcdh-b) - human	4537.09	751.70	7512299
Random 2			
vinculin [Homo sapiens] @à	4688.96	949.33	24657579
Homo sapiens NCK-associated protein 1 [synthetic construct]	2774.28	0.00	30583923
NAP5_HUMAN Nck-associated protein 5 (NAP-5)	4470.63	1207.80	20177972
similar to Nck-associated protein 5 (NAP-5) [Homo sapiens]	2816.58	487.93	41125758
Homo sapiens NCK adaptor protein 1 [synthetic construct]	2559.34	552.88	30584533
AF520705_1 K-dependent Na/Ca exchanger NCKX4 [Homo sapiens]	2778.39	0.00	21702723
SOS2_HUMAN Son of sevenless protein homolog 2 (SOS-2)	5964.18	1574.74	*6175038*
Son of sevenless CG7793-PA	5678.60	932.85	18110536
AF380183_1 SON DNA binding protein isoform E [Homo sapiens]	2432.35	815.78	17046381
SOST [Homo sapiens] apiens] I	2451.21	0.00	37181518
son of sevenless homolog 2; son of sevenless (Drosophilia) homolog 2;	3004.45	351.15	*39930604*
Adaptor			
proapoptotic caspase adaptor protein; caspase-2 binding protein; HSPC19	2446.75	83.73	7706003
adaptor-related protein complex 3, beta 2 subunit; Neuronal adaptin-li	27620.90	822.50	34482047
transcriptional adaptor 3-like isoform b [Homo sapiens]	5505.92	743.82	19743894
Adaptor-related protein complex 4, beta 1 subunit [Homo sapiens]	3373.84	731.86	15559571
PRAM-1 protein; PML-RARA target gene encoding an Adaptor Molecule-1 [H	2891.16	1018.33	40807475
similar to death adaptor molecule RAIDD-2 [Homo sapiens] [Rattus norve	2672.57	405.84	27718087
Neutrophil			
AAH01606 Similar to neutrophil cytosolic factor 2 (65kD, chronic granulom	3317.83	1472.32	12804409
matrix metalloproteinase 8 preproprotein; neutrophil collagenase; PMNL	4398.86	715.31	4505221
Solute			
A40141 mitochondrial solute carrier protein homolog - human	5136.83	1651.66	88158
solute carrier family 2 (facilitated glucose transporter), member 13;	4390.74	412.77	16418395
solute carrier family 3 (activators of dibasic and neutral amino acid	4061.22	562.00	21361344
solute carrier family 4, anion exchanger, member 3; Anion exchanger 3,	7016.77	780.20	4827016
similar to solute carrier family 4, sodium bicarbonate transporter-lik	16808.10	2052.90	27732419
solute carrier family 7 (cationic amino acid transporter, y	3792.41	442.30	4507049
similar to solute carrier family 9 (sodium/hydrogen exchanger), isofor	7808.28	0.00	27721087
similar to solute carrier family 9, member 7; nonselective sodium pota	12527.72	302.89	27477294
similar to solute carrier family 16 (monocarboxylic acid transporters)	3202.74	0.00	28514772
solute carrier family 17 (sodium phosphate), member 4; Na/PO4 cotranspo	3041.23	0.00	4885441
solute carrier family 22 member 5; organic cation transporter 5; organi	5278.03	0.00	4507005
solute carrier family 22 member 11; organic anion transporter 4 [Homo s	5753.69	0.00	8923870
solute carrier family 25 (mitochondrial carrier; adenine nucleotide tra	8606.05	0.00	4502097
solute carrier family 25, member A6; adenine nucleotide translocator 3	6521.11	0.00	27764863
solute carrier family 26, member 8 isoform b; anion transporter/exchan	3341.98	778.28	20336285
similar to solute carrier family 26, member 8, isoform a; anion transp	5741.39	1712.47	27704840
solute carrier family 27 (fatty acid transporter), member 5; very long	2896.93	737.61	13325057
solute carrier family 27 (fatty acid transporter), member 2; very long-	3678.59	0.00	4503653
Cyclin			
annexin A11; annexin XI; autoantigen, 56-kD; calcyclin-associated annex	3422.42	0.00	4557317
cyclin A1 [Homo sapiens] c translation initiation f	2604.40	622.74	2183079
cyclin B2 [Homo sapiens]	3740.59	0.00	4757930
similar to cyclin B3 [Homo sapiens] [Rattus norvegicus]	3314.58	1087.09	27707998
cyclin D2; G1/S-specific cyclin D2 [Homo sapiens]	3396.82	0.00	4502617
cyclin-E binding protein 1 [Homo sapiens]	5219.17	983.10	7705931
similar to cyclin T2 isoform b; cyclin T2a; cyclin T2b; SDS-stable vim	2611.40	706.12	27711766
cyclin F; F-box only protein 1 [Homo sapiens]	3719.14	731.59	4502621
Random 3			
suppressor of cytokine signaling 5 [Homo sapiens]	42983.16	434.28	23273934
Bruton agammaglobulinemia tyrosine kinase [Homo sapiens]	3023.19	502.71	4557377
signal transducer and activator of transcription 2; signal transducer a	22317.73	7272.96	4885615
signal transducer and activator of transcription 4 [Homo sapiens]	15221.41	2027.82	4507255
SI:dZ46I22.3 (novel protein similar to human signal transducer and acti	2838.96	179.00	27817303
LYN protein [Homo sapiens] immunodeficiency virus	2605.06	1425.24	37589566
Insulin			

insulinoma-associated protein IA-6 [Homo sapiens]	2783.40	0.00	13925613
IRR_HUMAN Insulin receptor-related protein precursor (IRR) (IR-related recept	3951.73	844.03	12644000
insulin receptor-related receptor precursor; IR-related receptor [Homo	3511.32	844.03	31657140
insulin receptor substrate like protein [Homo sapiens]	3459.88	1540.26	3135318
insulin receptor substrate 1 [Homo sapiens]	5567.91	1539.58	5031805
insulin receptor substrate 4 [Homo sapiens]	4845.81	890.66	4504733
insulysin; insulinase [Homo sapiens]	10160.06	526.13	4826770
AF440100_1 insulinoma-glucagonoma protein 20 [Homo sapiens]	9009.46	3631.68	17483988
leucyl/cystinyl aminopeptidase; insulin-regulated aminopeptidase; oxyto	2820.82	651.09	5031881
Nitric			
nitric oxide synthase 1 (neuronal) [Homo sapiens]	8542.28	1552.79	10835173
inducible nitric oxide synthase [Homo sapiens] tor	3400.31	749.88	3192917
A Chain A, Human Inducible Nitric Oxide Synthase With Inhibitor	2705.34	0.00	7245982
PDZ			
Similar to PDZ domain protein GIPC2 [Homo sapiens]	2449.71	0.00	23271288
InaD-like protein isoform 3; PDZ domain protein (Drosophila inaD-like)	5255.47	183.29	29029542
AF478468_1 Rap1 guanine nucleotide-exchange factor PDZ-GEF2A [Homo sapien	3290.32	786.23	18874698
multiple PDZ domain protein [Homo sapiens]	4699.73	935.31	4505231
Breakpoint			
synovial sarcoma, X breakpoint 2 interacting protein; afadin- and alpha	2438.95	324.27	7662382
breakpoint cluster region protein 2 [Homo sapiens]	2546.52	579.90	3002953
orofacial clefting chromosomal breakpoint region 1 [Homo sapiens]	3001.26	0.00	24415868
GABA			
gamma-aminobutyric acid (GABA) A receptor, beta 2 isoform 2 [Homo sapie	2494.72	695.30	4503865
gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1;	5016.53	974.10	10181206
Cytochrome			
AF183412_1 cytochrome P450 monooxygenase [Homo sapiens]	2759.13	650.40	9963763
similar to cytochrome P450, subfamily IVF, polypeptide 2; leukotriene	2774.02	0.00	17483447
cytochrome b-245, beta polypeptide (chronic granulomatous disease); gp9	2632.23	0.00	6996021
oxidase (cytochrome c) assembly 1-like [Homo sapiens]	11613.14	0.00	4826880
cytochrome c oxidase assembly protein COX15 [Homo sapiens]	17321.09	468.39	3603251
heme A:farnesyltransferase; cytochrome c oxidase subunit X; cytochrome	3907.91	443.63	17921982
cytochrome P450 [Homo sapiens] angiopoietin 3; an	3969.20	1029.04	18086504
cytochrome P450, family 4, subfamily F, polypeptide 11; cytochrome P45	3356.16	771.19	10863993
cytochrome P450, family 17; steroid 17-alpha-monooxygenase; cytochrome	4011.99	1113.30	4503195
cytochrome P450, family 24 precursor; cytochrome P450, subfamily XXIV (	3942.99	399.35	4503205
CP26_HUMAN Cytochrome P450 26 (Retinoic acid-metabolizing cytochrome) (P450RAI	3790.51	103.05	5921908
similar to family 4 cytochrome P450 [Mus musculus] [Homo sapiens]	10872.69	985.97	20472738
p21			
p21-activated kinase 2; novel serine kinase; hPAK65 [Homo sapiens]	2599.74	0.00	4505599
p21-activated kinase 6; p21-activated protein kinase 6 [Homo sapiens]	2967.64	1542.99	9910476
dJ45P21.3 (butyrophilin, subfamily 3, member A1) [Homo sapiens]	3079.01	0.00	15559188
p21-activated kinase 4; protein kinase related to S. cerevisiae STE20,	5222.12	780.24	5031975
p21-activated kinase 3; bPAK; p21-activated kinase-3; hPAK3; CDKN1A [Ho	4004.01	2010.10	4505601.0
Fc			
AC007842_1 Human Fc gamma BP [AA 1-2843] [Homo sapiens]	3117.69	373.42	5080756
IgG Fc binding protein [AA 4671-5405] [Homo sapiens]	1616.34	507.29	4321127
FcRN protein [Homo sapiens] LIKE protein containing	3029.13	520.27	8101615
unnamed protein product [Homo sapiens] Fc-Fragme	382704.50	10255.65	34527425
Interferon			
interferon-inducible IFI 16 [Homo sapiens] isofor	4044.30	252.23	22531287
IFR2_HUMAN Interferon-related developmental regulator 2 (SKMC15 protein)	2551.86	184.38	7387803
interferon-induced, hepatitis C-associated microtubular aggregat; inte	3375.58	0.00	21361310
Oxidase			
pyridoxine 5'-phosphate oxidase [Homo sapiens]	3151.12	326.69	8922498
PC1219 dihydroorotate oxidase (EC 1.3.3.1) precursor - human	3004.16	881.15	7427731
similar to Amine oxidase [flavin-containing] A (Monoamine oxidase) (MA	4870.25	220.93	41210567
aldehyde oxidase 1 [Homo sapiens]	2448.43	393.76	6598320
coproporphyrinogen oxidase [Homo sapiens]	6029.00	772.63	825648
eosinophil preperoxidase (AA -127 to 575) [Homo sapiens]	125655.52	28069.03	31183
AF117949_1 lysyl oxidase-like protein 2 [Homo sapiens]	6237.46	0.00	4959425
kidney and liver proline oxidase 1 [Homo sapiens]	3336.03	0.00	10864043
Prenylcysteine oxidase 1 [Homo sapiens]	5136.40	421.32	21708072
retina-specific amine oxidase [Homo sapiens] ÿ	3633.81	370.61	3510334
sterol-C4-methyl oxidase-like; C-4 methyl sterol [Homo sapiens]	2595.47	0.00	5803157

thyroid peroxidase [Homo sapiens] ; oxygen-regulate	2875.65	890.23	4680721
Random 4			
GRB2-associated binding protein 2 isoform b; Grb2-associated binder 2 [	2821.04	273.98	6912460
similar to alpha-catenin-like protein [Homo sapiens] [Mus musculus]	2486.38	288.59	28496944
p120 catenin isoform 4AB [Homo sapiens] th thrombos	2813.41	567.64	3152831
AAH04134 Similar to advillin [Homo sapiens]	4754.69	355.51	13278708
similar to plexin D1 [Homo sapiens] [Rattus norvegicus]	2537.92	0.00	27713066
plexin domain containing 1 precursor; tumor endothelial marker 7; 2410	3860.17	0.00	15011862
lectin, mannose-binding, 1 like; complexin III; ERGIC-53-like protein	3500.44	0.00	11141891
MEGF7 [Homo sapiens] ding, 1 like; complexin III;	3131.17	636.65	3449306
plexin B3; plexin 6; plexin-B3 [Homo sapiens]	3500.86	0.00	29336063
alpha-tubulin [Homo sapiens]	3939.38	49.78	32015
beta-tubulin cofactor D [Homo sapiens] sapiens]	2918.43	510.38	41350333
tubulin, gamma complex associated protein 5; gamma-tubulin complex com	2464.33	295.84	24308378
dynamin 2; Dynamin II [Homo sapiens]	2374.32	0.00	4826700
ataxin 1; olivopontocerebellar ataxia 1, autosomal dominant [Homo sapie	4329.75	151.06	4506793
ataxin 2; olivopontocerebellar ataxia 2, autosomal dominant [Homo sapie	9266.12	1518.30	4506795
ataxin 2 related protein isoform C; ataxin-2 domain protein [Homo sapi	4812.04	242.50	27262649
Human ATaXin related ATX-2 (atx-2) [Caenorhabditis elegans]	4254.97	459.82	17552814
ataxin-7 [Homo sapiens] rotein [Homo sapiens] [Mus	5126.26	1118.68	3192950
CXA3_HUMAN Gap junction alpha-3 protein (Connexin 46) (Cx46)	2781.54	0.00	8928078
CXA8_HUMAN Gap junction alpha-8 protein (Connexin 50) (Cx50) (Lens fiber prot	3923.57	0.00	13124697
cell division cycle associated 2 [Homo sapiens]	3546.84	0.00	41148440
cell division cycle 2-like 5 isoform 2; CDC2-related protein kinase 5	3224.46	445.39	14110390
cell division cycle 25C protein isoform b; mitosis inducer CDC25; phos	4245.92	1149.04	12408658
CDC28 protein kinase 1B; CDC2-associated protein CKS1; cell division co	3806.48	0.00	4502857
bA6J24.4 (A novel protein similar to cell division cycle control protei	3156.89	720.71	10696978
similar to neuropilin- and tolloid-like protein 2 precursor [Homo sapi	4369.57	0.00	27658878
toll-like receptor 6 [Homo sapiens] sapiens]	2671.07	1146.04	20143971
Synapto			
chr415 synaptotagmin [Homo sapiens] apiens] [Ratt	2324.31	0.00	13276581
synaptojanin 1; inositol 5'-phosphatase (synaptojanin 1); synaptojanin-	4639.68	1739.86	4507335
SCP1_HUMAN Synaptonemal complex protein 1 (SCP-1 protein)	39518.73	2522.36	3024609
synaptonemal complex protein 1 [Homo sapiens] 90	3797.02	505.32	34878904
synaptonemal complex protein 2 [Homo sapiens]	5157.94	2039.92	7657635
synaptonemal complex lateral element protein [Homo sapiens]	5018.50	1324.82	3256191
synaptotagmin-like 2 isoform a; chromosome 11 synaptotagmin [Homo sapi	3076.96	0.00	15011902
synaptotagmin V; synaptotagmin 5 [Homo sapiens]	3857.90	0.00	4507337
similar to synaptotagmin VI [Homo sapiens] it lo	2457.61	560.21	41058031
synaptotagmin-like 5 [Homo sapiens]	3901.58	849.27	20270305
synaptotagmin 10 [Homo sapiens]	3394.24	452.14	39752671
similar to synaptotagmin 10 [Rattus norvegicus] [Homo sapiens]	3833.62	234.26	20476965
synaptogyrin 2 [Homo sapiens] ng factor-I, splice	10557.74	277.22	2959876
Ran & Raf			
similar to karyopherin beta 3; Ran_GTP binding protein 5; importin bet	3693.09	505.03	16161756
ankyrin 3 isoform 2; ankyrin-3, node of Ranvier; ankyrin-G [Homo sapien	9194.38	1105.18	4502093
unnamed protein product [Homo sapiens] f Ranvier;	5682.91	1079.75	7020450
Yeast Ran Binder #1; suppressor of FUS1; homolog of mouse HTF9a and hum	3746.69	0.00	6320205
REP1_HUMAN RalBP1 associated Eps domain containing protein 1 (RalBP1-interact	3867.76	0.00	34098675
similar to RNA-binding protein Raly [Homo sapiens]	5000.16	1603.40	22050743
Ral-A exchange factor RalGPS2 [Homo sapiens]	2769.77	431.77	8922307
Random 5			
B cell linker protein BLNK-s [Homo sapiens]	3654.70	1389.76	3406751
SNF2 histone linker PHD RING helicase [Homo sapiens]	2838.52	1155.23	27436873
Notch 3 [Homo sapiens] sapiens]	5819.81	0.00	3108187
sno, strawberry notch homolog 1; MOP-3 [Homo sapiens]	6352.13	634.16	33620763
NTC4_HUMAN Neurogenic locus notch homolog protein 4 precursor (Notch 4) (hNot	2830.56	583.48	20139103
A40043 notch protein homolog TAN-1 precursor - human piens] it	4167.39	136.25	107215
delta-notch-like EGF repeat-containing transmembrane [Homo sapiens]	2745.17	152.15	31542543
GDP dissociation inhibitor 1; mental retardation, X-linked 41; mental r	8923.51	0.00	4503971
similar to Rab GDP dissociation inhibitor beta (Rab GDI beta) (GDI-2)	3391.69	1002.45	17448269
TRIO_HUMAN Triple functional domain protein (PTPRF interacting protein)	8433.38	920.73	8928460
rhoA protein [human, blood platelets, Peptide Partial, 9 aa, segment 3 of	17099.01	0.00	257888
Q9BQK8_1 [Segment 1 of 3] Lipin 3 (Lipin 3-like)	2997.64	0.00	23821839
Q9BQK8_2 [Segment 2 of 3] Lipin 3 (Lipin 3-like)	3879.63	0.00	23821840

LAMP1 protein [Homo sapiens] (TRL9)	3269.90	0.00	39644554
N-ethylmaleimide-sensitive factor [Homo sapiens]	4070.10	1529.50	11079228
Integrins			
integrin, beta 7 [Homo sapiens]	2686.42	0.00	4504777
a disintegrin-like and metalloprotease (reprolysin type) with thrombos	2807.05	0.00	21265049
a disintegrin-like and metalloprotease (reprolysin type) with thrombos	4623.40	0.00	19525737
similar to a disintegrin and metalloproteinase domain 23 preproprotein	2667.53	0.00	27684077
a disintegrin and metalloproteinase domain 33 isoform beta preproprote	3671.40	0.00	24041040
a disintegrin-like and metalloprotease with thrombospondin type 1 moti	4291.64	152.37	13569928
a disintegrin-like and metalloprotease (reprolysin type) with thrombos	2546.77	786.15	21265064
a disintegrin and metalloproteinase domain 21 preproprotein [Homo sapi	2577.80	748.89	11497040
a disintegrin and metalloprotease with thrombospondin motifs-2 isoform	3037.00	0.00	7656867
integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD5	2857.14	156.78	4504763
similar to a disintegrin-like and metalloprotease (reprolysin type) wi	2618.71	0.00	25053113
similar to a disintegrin-like and metalloprotease (reprolysin type) wi	3041.19	0.00	28484844
disintegrin-like and metalloprotease with thrombospondin type 1 motif, 5	7269.85	1841.57	7768707
AT14_HUMAN ADAMTS-14 precursor (A disintegrin and metalloproteinase with thro	6598.97	1431.90	29337086
AT20_HUMAN ADAMTS-20 precursor (A disintegrin and metalloproteinase with thro	3779.29	242.74	29611731
a disintegrin and metalloprotease with thrombospondin motifs-7 preprop	4870.17	984.53	10645199
a disintegrin and metalloprotease with thrombospondin motifs-7 preprop	5504.38	1176.97	38683827
a disintegrin and metalloprotease domain 10 [Homo sapiens]	4861.14	485.01	4557251
a disintegrin and metalloproteinase domain 28 isoform 2 preproprotein	6222.42	821.21	11496996
similar to a disintegrin-like and metalloprotease (reprolysin type) wi	7181.65	2316.87	27693936
a disintegrin-like and metalloprotease (reprolysin type) with thrombos	7181.61	142.01	21265061
similar to a disintegrin and metalloprotease with thrombospondin motif	3482.34	1258.04	27721019
similar to a disintegrin and metalloproteinase domain 21 preproprotein	2719.23	0.00	28484011
alpha integrin binding protein 63; Hermansky-Pudlak syndrome 5 [Homo s	6924.47	1195.73	22095345
integrin alpha subunit precursor [Homo sapiens] in p	5366.74	812.55	386831
integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) [Homo sapie	4586.52	135.75	21105795
ITA3_HUMAN Integrin alpha-3 precursor (Galactoprotein B3) (GAPB3) (VLA-3 alph	3656.89	869.86	11467963
integrin alpha 6 [Homo sapiens] [Homo sapiens]	4891.57	456.78	5726563
ITAM_HUMAN Integrin alpha-M precursor (Cell surface glycoprotein MAC-1 alpha s	7012.66	529.03	1708572
A36429 integrin beta-4 chain precursor - human 161568) [Homo	19679.95	2036.50	2119645
AIP1_HUMAN Atrophin-1 interacting protein 1 (Atrophin-1 interacting protein A	7447.76	800.96	37537776
bromodomain adjacent to zinc finger domain, 2A; TTF-I interacting pepti	7337.17	1359.54	7304921
BRCA1 interacting protein C-terminal helicase 1; BRCA1-interacting pro	5826.72	345.37	14042978
phosphorylated CTD interacting factor 1 [Homo sapiens]	3986.17	999.19	18034767
DAZ interacting protein 1; zinc finger DAZ interacting protein 1 [Homo	9092.44	2595.27	7662436
DIP2_HUMAN Disco-interacting protein 2 homolog in-8 [Ho	6787.33	229.21	32700084
huntingtin interacting protein 1 [Homo sapiens] hat	2514.62	1010.97	2072423
huntingtin interacting protein-1-related [Homo sapiens]	2490.68	629.14	29744338

Figure 11. Chromatographs of control and positive LARC runs.
Increasing salt concentrations used for elution of Fc receptor complex from disrupted neutrophil membrane. Panels A-D show LARC chromatographs, panels E-H) show chromatographs of control experiments. A) 0.2M NaCl; B) 0.3M NaCl; C) 0.7M NaCl; D) 1M NaCl; E) 0.2M NaCl control; F) 0.3M NaCl control ; G) 0.7M NaCl control; H) 1M NaCl control.



Models of supramolecular Fcγ receptor complex form human neutrophils

The generated networks represent detailed protein-protein interactions during FcγR mediated phagocytosis in human neutrophils. Probability of false positives is minimized by requiring at least three interacting partners of each protein and that all of the protein interactions in the network come from previously published sources. Incorporation of the interacting proteins into the network was made possible by availability of iHOP (Hoffmann and Valencia 2005).

Three different algorithms were used to build interaction networks - Cytoscape (Shannon, Markiel et al. 2003), Osprey (Breitkreutz, Stark et al. 2003) and STRING (Jensen, Kuhn et al. 2009) *(Figures 7, 8, 9)*. From the generated models list of proteins that are predicted to play a central role in phagocytosis were compared (*Table 2*). From the consensus between the models proteins such as Src, Rac, Cdc42, NOX, PI3Ks and PLCs were found to play a central role in the Fc receptor supramolecular complex. These proteins were previously impllicated in phagocytosis, suggesting accuracy of LARC. Although few proteins identified were found to vary between models there was a close degree of an agreement on critical proteins that were previously established in FcγR mediated phagocytosis (Jankowski, Zhu et al. 2008).

Figure 12. The network of interacting proteins generated from application of Osprey. 341 proteins and 1971 known interactions were detected with at least three interactions in the data set. A global dual spoked ring layout was used, where proteins in the outer ring represent those that are predicted to play a more important role in phagocytosis based on the number of interactions which was obtained through data mining by *iHOP*.

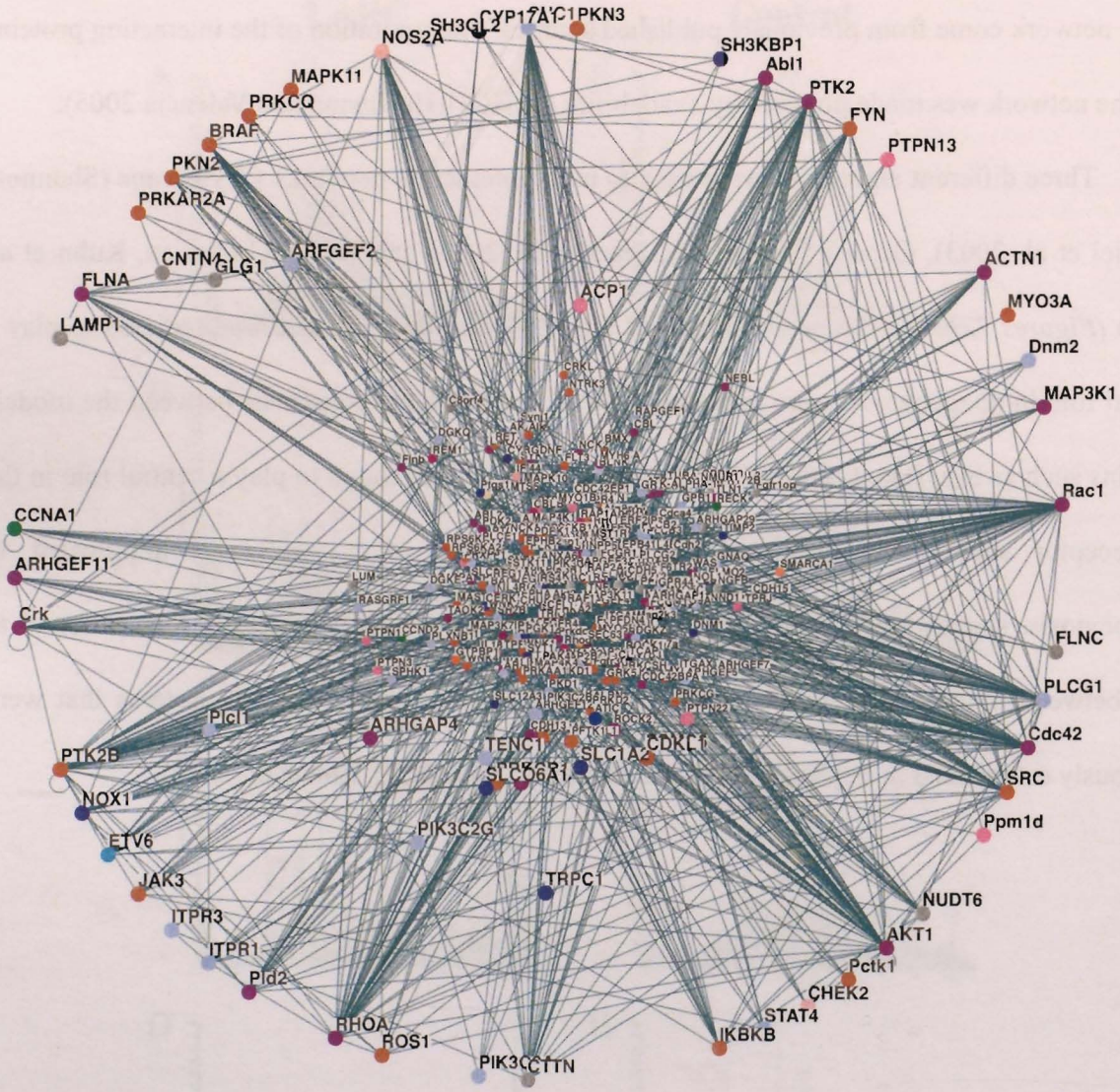


Figure 13. The network of interacting proteins generated with Cytoscape.
Cytoscape is one of the algorithms that is used in construction of protein interaction networks. Interaction data was obtained from iHOP. Spring embedded layout was used for interaction model visualization. Proteins that are clustered in the center are those that are predicted to play a more central role in FcγR mediated particle uptake.

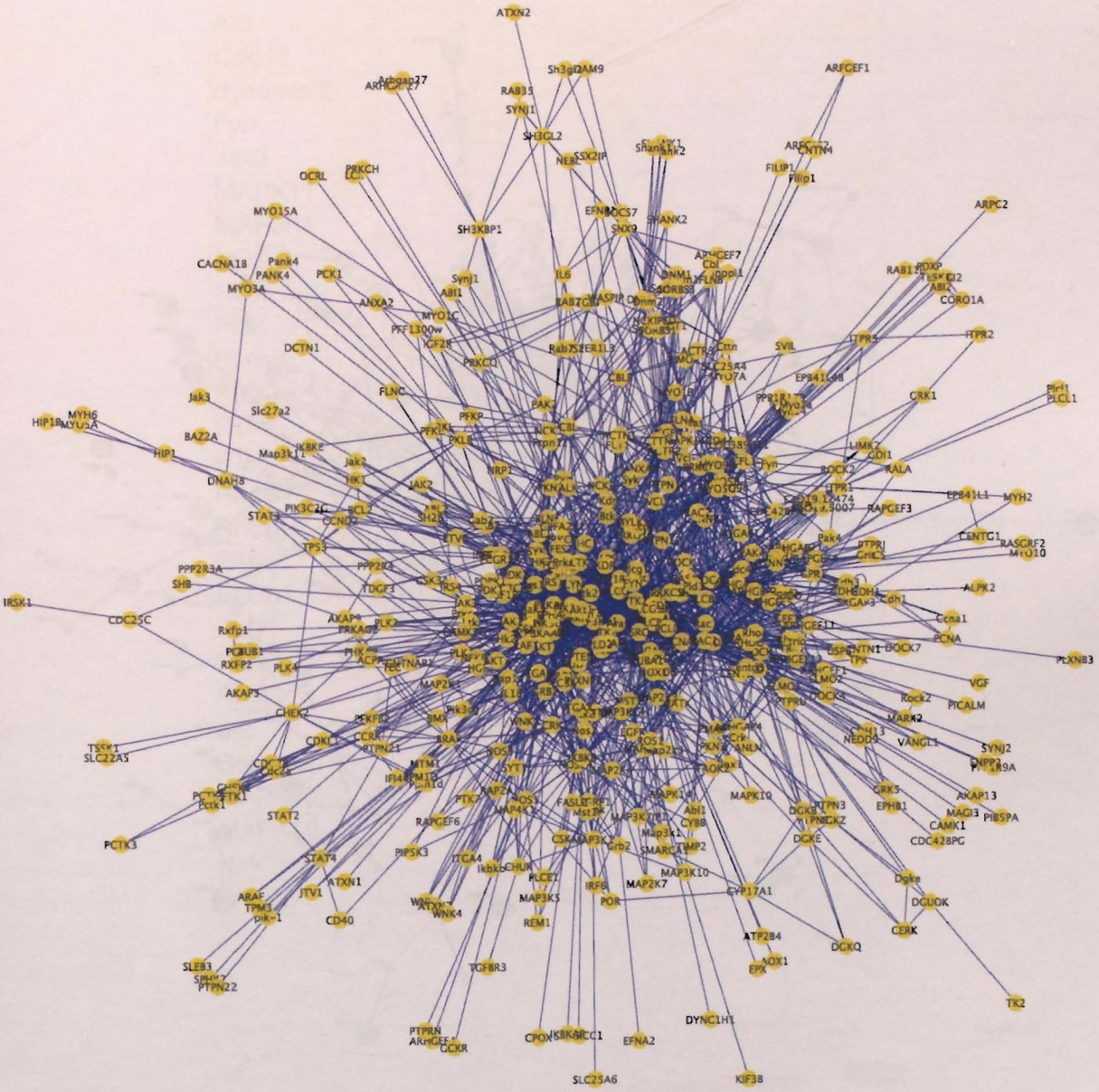


Figure 14. Protein interaction network generated with STRING.

STRING is an online proteins interactions model building algorithm. The list of proteins established to be involved in FcγR driven phagocytosis in human neutrophils was input into the program, generating protein interaction network. The proteins that are found in the center of the model are those that are predicted to have an important function during phagocytosis.

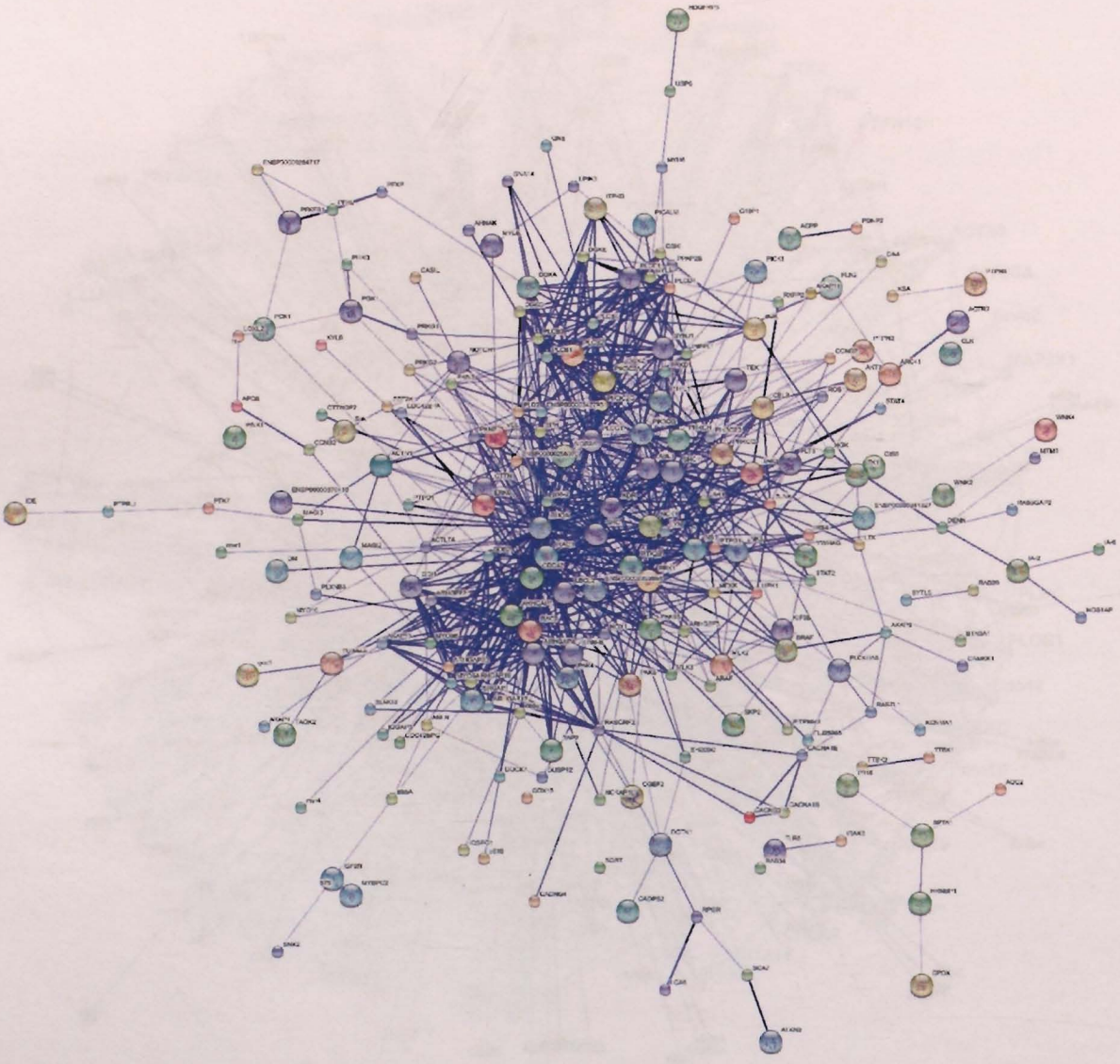
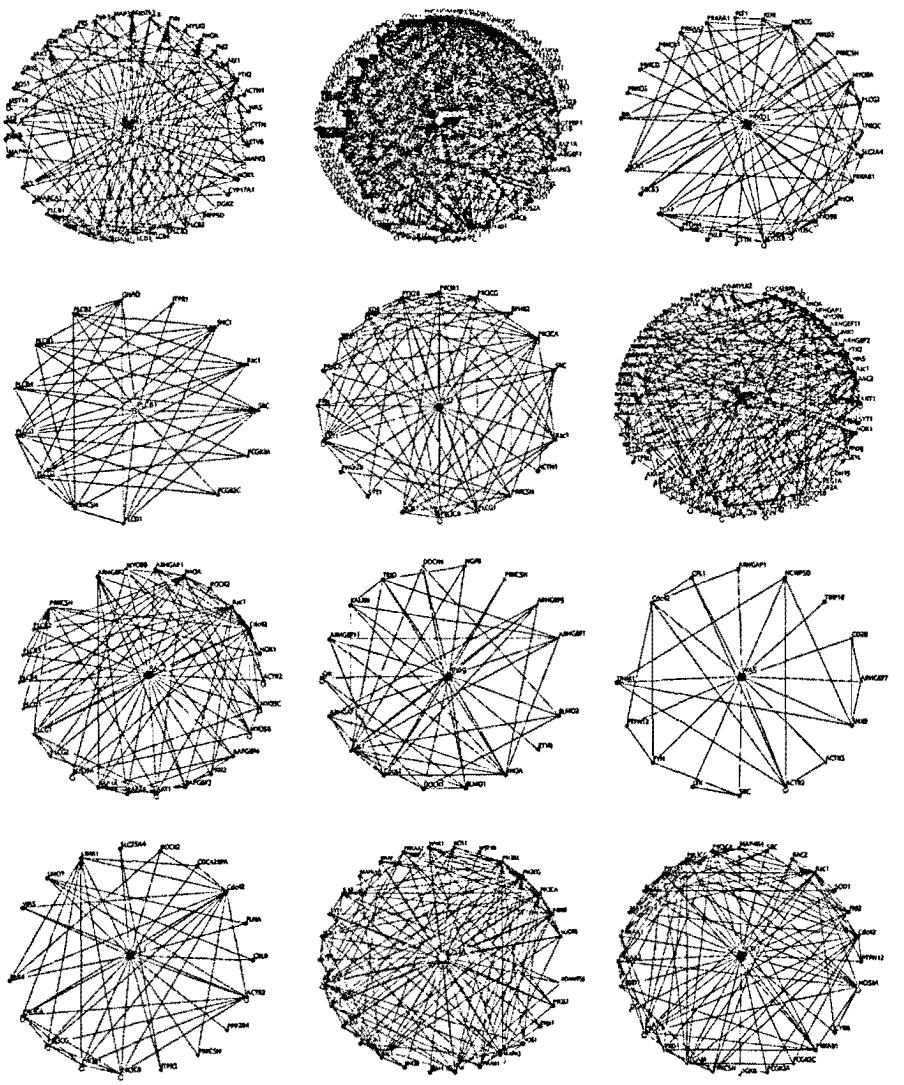


Table 2. List of proteins predicted to play a central role in FcyR mediated phagocytosis in human neutrophils. Osprey, Sting and Cytoscape were used to construct models of interacting proteins from which a list of proteins that were predicted to play a central role was generated.

	Models		
Protein	**Osprey**	**Cytoscape**	**String**
Abl	x		
Akt	x		
Cdc42	x	x	
dynamin 2	x		
Fyn	x	x	x
Jak3	x		
MAPK11	x		
MAP3K1	x		
NOS	x	x	x
NOX	x	x	x
PI3K	x	x	x
PLCy1	x	x	x
PLD2	x	x	
PTK2	x	x	
PTK2B	x	x	x
Rac1	x	x	x
RhoA	x	x	x
PAK4		x	x
PLCb1		x	x
PLD1		x	
Rac2		x	x
RhoG		x	
Src		x	x
Cortactin			x
MAPK12			x
MAPK10			x
Nck			x
PAK2			x

Figure 15. Individual models of some of the proteins known to be involved in phagocytosis.
A, Nodes represent a number of other proteins that the selected protein interacts with while number of edges is the total number of interactions that are relevant to the target protein. All interactions in the models come from the literature which was navigated using iHOP. **B,** Table summarizing proteins in the close up Osprey models with the addition scores, protein accession number, number of nodes and number of edges.

A



B

Protein name	Positive	Control	Accession	Nodes	Edges
SRC protein [Homo sapiens] s] containing 43 [Homo	2832.71	0.00	30411071	41	140
phosphoinositide-3-kinase gamma catalytic subunit [Homo sapiens]	4369.13	611.20	12620871	104	796
protein kinase C, mu [Homo sapiens]	2863.35	1238.56	4506075	27	85
phospholipase C beta 1 [Homo sapiens] y	7693.70	1139.98	9438229	15	51
phospholipase D2 [Homo sapiens]. Hypothetical prot	9563.47	844.32	2645858	20	97
ras-related C3 botulinum toxin substrate 2; Ras-related C3 botulinum to	105143.33	26212.47	4506381	26	104
AF498974 1 small GTP binding protein RhoG [Homo sapiens]	39072.44	0.00	20379122	18	53
Wiskott-Aldrich syndrome protein; Wiskott-Aldrich syndrome [eczema-thro	2973.34	0.00	4507909	16	34
cofilin 1 (non-muscle) [Homo sapiens]	14027.98	0.00	5031635	19	56
inducible nitric oxide synthase [Homo sapiens] tor	3400.31	749.88	3192917	30	148
NOX1_HUMAN NADPH oxidase homolog 1 (NOX-1) (NOH-1) (NADH/NADPH mitogenic oxida	5463.92	1870.36	8134597	21	121

p21 activated kinases

PAKs are likely associated with the Fc receptor supramolecular complex based on both human neutrophil and data gathered from a murine macrophage cell line. PAK2-4 and PAK6 were positively identified with LARC (*Table 3*). The presence of these proteins in both data obtained with human neutrophils and RAW264.7 cells supports that they are involved in Fc receptor mediated particle uptake. PAK2 and PAK4 were predicted to play an important role during phagocytosis via the application of interaction model building algorithms.

Specific peptides identified with LARC and correlated with the proteins are summarized in *Table 4*. These peptides were then searched using BLAST to confirm the protein sequence correlation and the presence of those peptide fragments within the published protein sequences (*Figure 16*). All of the peptides that were identified with LARC were found within the protein sequence returned from BLAST queries. Selected LC-MS/MS spectra of PAK2-4 an PAK6 is illustrated in *Figure 17*.

The presence of these proteins in macrophages, and specifically at the site of particle uptake was confirmed by western blotting, immunostaining and cell transfections with GFP fusion to PAKs.

Table 3. Addition of scores for p21 activated kinases.
Scores of PAKs positively detected by LARC, control scores and accession numbers for corresponding proteins that were identified in both RAW246.7 macrophages and human neutrophils.

Protein name	RAW264.7			Neutrophils		
	LARC	Control	Accession	LARC	Control	Accession
p21-activated kinase 2	2608.54	0.00	28493842	2599.74	0.00	4505599
p21-activated kinase 3	5646.82	604.70	3420949	4004.01	2010.10	4505601
p21-activated kinase 4	6849.56	1729.84	29336032	5222.12	780.24	5031975
p21-activated kinase 6	3903.18	889.45	28487481	2967.64	1542.99	9910476

Table 4. Detection of PAKs in RAW264.7 macrophages. Proteins detected with LARC, analyzed by LC-MS/MS and identified using SEQUEST. Table illustrates all PAK peptide fragments detected using LARC as well as those that were found in control experiments. Peptide sequence, MH+, charge, score, total score and accession numbers are presented

Protein name / File locator	Peptide sequence	MH+	Charge	Score	Score XC	Accession Delta Cn
p21-activated kinase 2 [Mus musculus]						
LARC					2608.54	28493842
	-.QKYLSFTPPEK.-	1338.53	3	737.67	1.61	0.0
	-.MSDNGELEDKPPAPPVR.-	1853.05	2	117.52	1.24	0.0
	-.SVIDPIPAPVGDSNVDSGAK.-	1939.11	2	1518.14	1.73	0.3
	-.AKMTDEEIMEK.-	1325.54	2	235.21	1.05	0.1
CONTROL - 0						
Cdc42/Rac effector kinase; PAK-3 [Mus musculus]						
LARC					5646.82	3420949
	-.SIYTRSVVESIASPAAPNK.-	1991.24	3	202.18	1.07	0.1
	-.LTDFGFCAQITPEQSKR.-	1942.19	3	322.30	0.94	0.0
	-.SIFPGGGDKTNK.-	1221.35	3	279.30	1.12	0.0
	-.QMNLQQQPKK.-	1243.46	3	3133.37	3.01	0.1
	-.SDNILLGMDGSVK.-	1349.54	3	402.94	1.63	0.0
	-.LRSIFPGGGDKTNK.-	1490.69	3	697.42	1.33	0.0
	-.LTDFGFCAQITPEQSKR.-	1942.19	3	237.52	1.43	0.0
	-.PLPMAPEEK.-	1012.21	2	281.80	0.92	0.2
	-.ETVNNQKYMSFTSGDKSAHGYIAAHQSNTK.-	3316.56	2	89.99	0.88	0.1
Control						
					604.70	3420949
	-.CLEMDVDRRGSAK.-	1480.70	2	428.18	1.00	0.1
	-.ELIINEILVMRENK.-	1715.05	2	176.51	1.28	0.0
p21-activated protein kinase 4 [Mus musculus]						
LARC					6849.56	29336032
	-.AGPPASIVPLMR.-	1209.49	2	965.92	1.29	0.0
	-.AALQLVVDPGDPR.-	1351.53	3	251.73	1.28	0.0
	-.AGPPASIVPLMRQHRTR.-	1888.23	3	354.36	1.32	0.0
	-.LLVRDPAQRATAAELLK.-	1866.20	2	83.14	0.95	0.0
	-.VHTGFDQHEQK.-	1326.40	2	430.13	1.33	0.0
	-.GAPSPGVLGPHASEPQLAPPAR.-	2107.36	3	707.21	1.66	0.2
	-.SDSILLTHDGRVK.-	1441.62	2	115.78	0.84	0.0
	-.ATGHSEAGSGSGDR.-	1289.25	2	1075.23	1.38	0.2
	-.DGALTLLLDEFENMSVTRSNSLR.-	2582.87	3	755.24	1.68	0.1
	-.DGPGGPQEASRDK.-	1314.34	2	569.54	1.59	0.1
	-.GAPSPGVLGPHASEPQLAPPAR.-	2107.36	3	235.11	1.65	0.0
	-.VHTGFDQHEQK.-	1326.40	2	786.26	1.55	0.1
	-.DIKSDSILLTHDGRVK.-	1798.04	2	247.39	1.18	0.0
	-.GAPSPGVLGPHASEPQLAPPAR.-	2107.36	3	272.51	1.46	0.0
Control						
					1729.84	29336032
	-.ATGHSEAGSGSGDRR.-	1445.44	2	696.48	1.33	0.1
	-.PKPLIDPACITSIQPGAPKTIVR.-	2416.91	3	798.66	1.59	0.1
	-.ATAAELLKHPFLTK.-	1540.83	2	234.70	1.34	0.0

similar to Serine/threonine-protein kinase PAK 6 (p21-activated kinase

LARC					3903.18	28487481
	-.PNSAFRPPQKDSSSNLVAK.-	2044.26	3	1674.18	2.14	0.2
	-.SMFLSTPATGAASSSKPVPLPQNK.-	2417.77	3	232.05	1.59	0.0
	-.LSVISSNTLR.-	1090.26	2	234.05	0.96	0.0
	-.HSSEEARPQSCLVGSAIGRPGGEGSPSPK.-	2879.12	3	296.045	1.5865	0.0345
	-.SMFLSTPATGAASSSKPVPLPQNK.-	2417.77	3	448.69	1.69	0.1
	-.KRPEISAPQNFQHR.-	1708.91	2	112.91	0.84	0.0
	-.WASERAGQR.-	1061.14	2	172.24	1.11	0.1
	-.SMFLSTPATGAASSSKPVPLPQNK.-	2417.77	3	733.014	2.2594	0.0778
Control						
					889.45	28487481
	-.TAPAAGPLPGRSSPAGSPRTR.-	2005.23	3	426.27	1.0475	0.1602
	-.SMFLSTPATGAASSSKPVPLPQNK.-	2417.77	3	463.18	1.45	0.1

Figure 16. Correlation of peptides identified with LARC with full length FASTA sequence. Peptides highlighted are those that were positively identified through application of LARC. **PAK2**, total of 4 different tryptic peptides were identified and correlated with full length PAK2 sequence. **PAK3**, 9 peptides were identified with MS/MS with 8 of them different. **PAK4**, total of 14 peptides were detected with 6 of them different. **PAK6**, 6 different peptides were identified with LARC out of total of 8 which were correlated to full length PAK6 FASTA sequence. The accession numbers for the FASTA sequences are those as identified by NCBI. Tryptic peptides identified with LARC and SEQUEST algorithm are underlined and bolded, overlapping fragment sequences are in green and those red represent peptides that are next to one another.

PAK2

gi|46559406|ref|NP_796300.1| p21-activated kinase 2 [Mus musculus]

MSDNGELEDKPPAPPVRMSSTIFSTGGKDPLSANHSLKPLPSVPEEKKPRNKIISIFSGTE
KGSKKKEKERPEISPPSDFEHTIHVGFDAVTGEFTGMPEQWARLLQTSNITKLEQKKNPQ
AVLDVLKFYDSNTVK**QKYLSFTPPEK**DGFPSGTPALNTKGSETSAVVTEEDDDDEDAAP
PVIAPRPDHTKSIYTR**SVIDPIPAPVGDSNVDSGAK**SSDKQKKK**AKMTDEEIMEK**LRTIV
SIGDPKKKYTRYEKIGQGASGTVFTATDVALGQEVAIKQINLQKQPKKELIINEILVMKEL
KNPNIVNFLDSYLVGDELFVVMEYLAGGSLTDVVTETCMDEAQIAAVCRECLQALEFLH
ANQVIHRDIKSDNVLLGMEGSVKLTDFGFCAQITPEQSKRSTMVGTPYWMAPEVVTRK
AYGPKVDIWSLGIMAIEMVEGEPPYLNENPLRALYLIATNGTPELQNPEKLSPIFRDFLNR
CLEMDVEKRGSAKELLQHPFLKLAKPLSSLTPLILAAKEAMKSNR

PAK3c

gi|169643246|emb|CAQ16019.1| PAK3c protein [Mus musculus]

MSDSLDNEEKPPAPPLRMNSNNRDSSALNHSSK**PLPMAPEEK**NKKAR**LRSIFPGGGDK**
TNKKKEKERPEISLPSDFEHTIHVGFDAVTGEFTNSPFQTSRPVTVASSQSEGKMGIPEQW
ARLLQTSNITKLEQKKNPQAVLDVLKFYDSK**ETVNNQKYMSFTSGDKSAHGYIAAHQ**
SNTKTASEPPLAPPVSEEEDEEEEEEEDDNEPPPVIAPRPEHTK**SIYTRSVVESIASPAAPN**
KEDIPPSAENANSTTLYRNTDRQRKKSKMTDEEILEKLRSIVSVGDPKKKYTRFEKIGQG
ASGTVYTALDIATGQEVAIK**QMNLQQQPKK**ELIINEILVMRENKNPNIVNYLDSYLVGDE
LWVVMEYLAGGSLTDVVTETCMDEGQIAAVCRECLQALDFLHSNQVIHRDIK**SDNILLG**
MDGSVKLTDFGFCAQITPEQSKRSTMVGTPYWMAPEVVTRKAYGPKVDIWSLGIMAI
EMVEGEPPYLNENPLRALYLIATNGTPELQNPERLSAVFRDFLNRCLEMDVDRRGSAKEL
LQHPFLKLAKPLSSLTPLIIAAKEAIKNSSR

PAK4

gi|29336032|ref|NP_081746.1| p21-activated kinase 4 [Mus musculus]

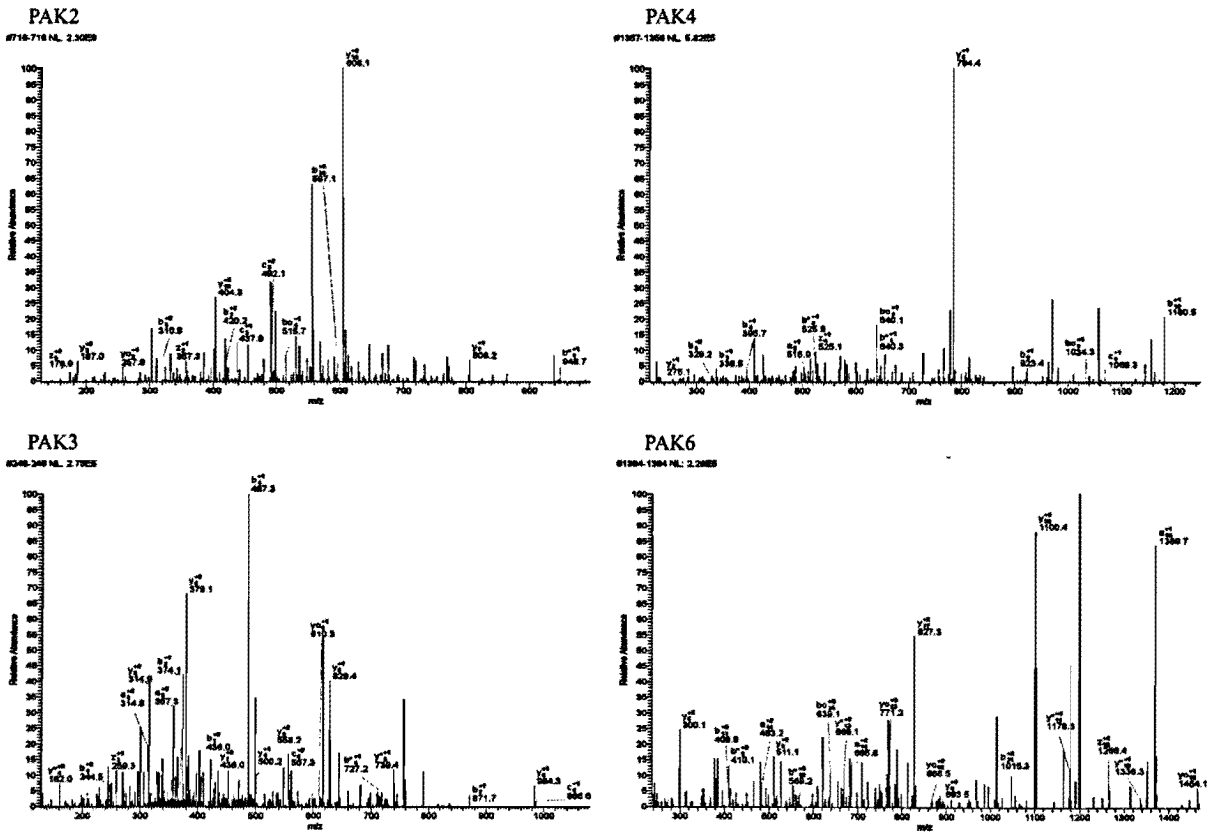
MFGKKKKRVEISAPSNFEHR**VHTGFDQHEQK**FTGLPRQWQSLIEESARRPKPLIDPACIT
SIQPGAPKTIVRGSKGAK**DGALTLLLDEFENMSVTRSNSLR**RESPPPPARAHQENGMLE
ERAAPARMAPDKAGSRARATGHSEAGSGSGDRRRVGPEKRPKSSRDGPGGPQEASRDK

RPLSGPDVSTPQPGSLTSGTKLAAGRPFNTYPRADTDHPPRGAQGEPHTMAPNGPSATGL
AAPQSSSSSRPPTRAR**GAPSPGVLGPHASEPQLAPPAR**ALAAPAVPPAPGPPGPRSPQRE
PQRVSHEQFR**AALQLVVDPGDPR**SYLDNFIKIGEGSTGIVCIATVRSSGKLVAVKKMDLR
KQQRRELLFNEVVIMRDYRHENVVEMYNSYLVGDELWVVMEFLEGGALTDIVTHTRM
NEEQIAAVCLAVLQALAVLHAQGVIHRDIK**SDSILLTHDGRVK**LSDFGFCAQVSKEVPRR
KSLVGTPYWMAPELISRLPYGPEVDIWSLGVMVIEMVDGEPPYFNEPPLKAMKMIRDNL
PPRLKNLHKASPSLKGFLDRLLVRDPAQRATAAELLKHPFLTK**AGPPASIVPLMR**QHRTR

PAK6
gi|187956465|gb|AAI50755.1| P21 (CDKN1A)-activated kinase 6 [Mus musculus]

MFRKKKK**KRPEISAPQNFQHR**VHTSFDPKEGKFVGLPPQWQNILDTLRRPKPVVDPSRI
TRVQLQPMKTVVRGSSVPTEGYISGLLNDIQK**LSVISSNTLR**GRSPTSRRRAQSLGLLGD
DQWAADPDMYLQSPQSEHTDPHGLYLSCNGGTPAGHRQVPWPEPQSPQALPNGMAAK
AQSLGPAEFQGASQRCLQQLGACLQSSPPGTSLPMATGRRGVKVAK**HSSEEARPQSCLV
GSAIGRPGGEGSPSPK**NQESSLKHRLFR**SMFLSTPATGAASSSKPVPLPQNK**PNSAFRP
PQKDSSSNLVAK**AQSLPSEQPMGTFSPLTTSDTSSPQKSLRTVPAAGPLPGRSSPAGSPRTR
HAQISTSNLYLPQDPTVAKGALGGEDTGIVTHEQFKAALRMVVDQGDPRLLLDSYVKIG
EGSTGIVCLAREKHSGRQVAVKMMDLRKQQRRELLFNEVVIMRDYQHLNVVEMYKSY
LVGEELWVLMEFLQGGALTDIISQVRLNEEQIATVCEAVLQALAYLHAQGVIHRDIKSDSI
LLTLDGRVKLSDFGFCAQISKDVPKRKSLVGTPYWMAPEVISRSLYATEVDIWSLGIMVIE
MVDGEPPYFSDSPVQAMKRLRDSAPPKLKNSYKVSPVLRDFLDRMLVREPQERATAQEL
LDHPFLLQTGLPECLVPLIQLYRKQTSTC

Figure 17. Fragmentation spectra of p21 activated kinases identified with LARC.
Spectra were obtained from LC-MS/MS using SEQUEST algorithm. Here selected peptide sequences of murine PAK2-4 and PAK6 are shown. Protein name, score, peptide, charge. **PAK2**, 787.37, QKYSFTPPEK, 3+; **PAK3**, 3133.37, QMNLQQQPKK, 3+; **PAK4**, 786.26, VHTGFDQHEQK, 2+; **PAK5**, 1215.54, SDSILLTSDGR, 3+; **PAK6**, 733.01, SMFLSTPATGAASSSKPVPLPQNK, 3+.



Western blotting of PAKs

The presence of PAK2-4 and PAK6 in RAW264.7 macrophage cell lysates was confirmed using Western blotting (*Figure 18*). A band around 58kDa upon incubation with goat polyclonal antibody against PAK2 corresponds to the presence of PAK2. Goat polyclonal antibody against PAK3 detected a band at 66kDa which corresponds to the presence of PAK3 in cell lysates. PAK4 presence was detected with anti PAK4 rabbit monoclonal antibody which yielded a strong band around 66kDa. PAK6 western blot shows two bands one at the weight of around 80kDa which corresponds to full protein.

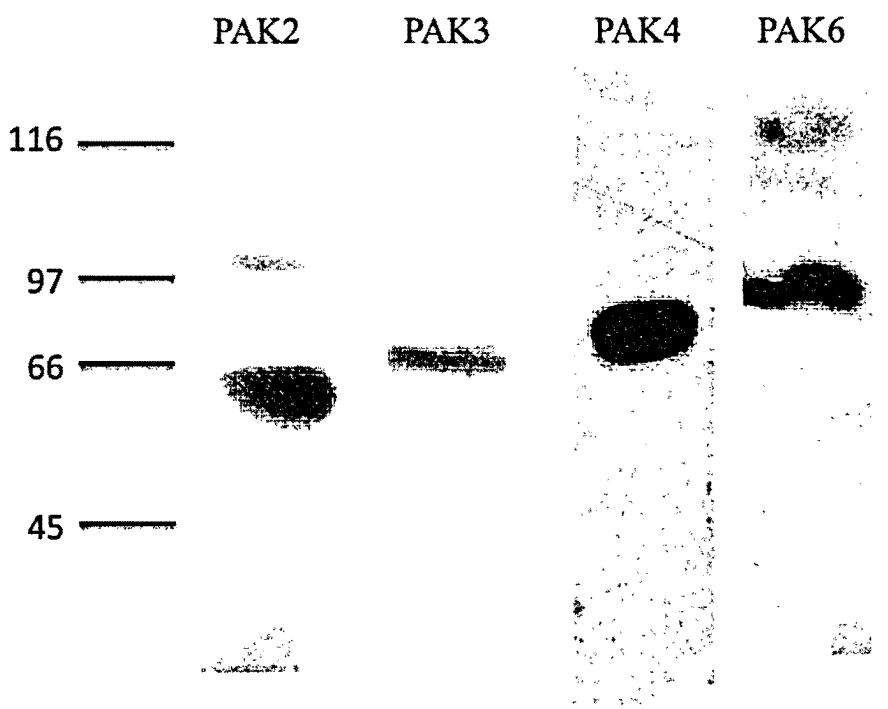


Figure 18. Western blots of p21 activated kinases in RAW264.7 lysates. PAK2-4 and PAK6 are suggested to associate with the activated Fc receptor as determined with application of LARC. Presence of these proteins in RAW lysates was confirmed with western blotting. Cells were boiled in SDS-PAGE sample buffer with addition of EDTA, PMSF, benzamidine and eukaryotic protease inhibitor cocktail. Appropriate primary antibodies were used with HRP conjugated secondary antibodies, detection was done with the use of ECL.

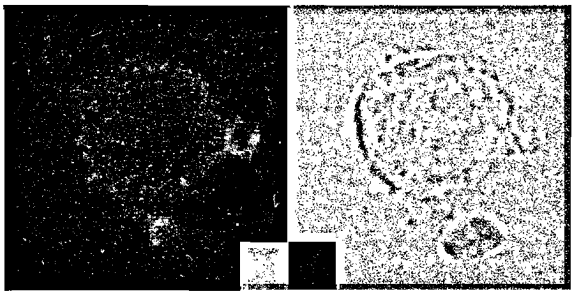
Immunostains of PAK proteins

Localization of PAK2, 3, 4 and 6 to the cell membrane near the site of engulfment of IgG opsonized particles was observed through staining of formaldehyde fixed cells with appropriate primary antibodies and detected with the addition of Cy-3 conjugated secondary antibody and visualized with a laser confocal microscope. PAK2 has higher signal at the formation of the cup which indicates its accumulation during phagocytosis is a result of activation of the Fc receptor. PAK3 also shows some localization to the membrane around the engulfed sheep red blood cell. PAK4 is seen to localize around the beads that are being internalized. Accumulation of PAK6 is seen underneath the formed phagosome as well as around it (*Figure 19*).

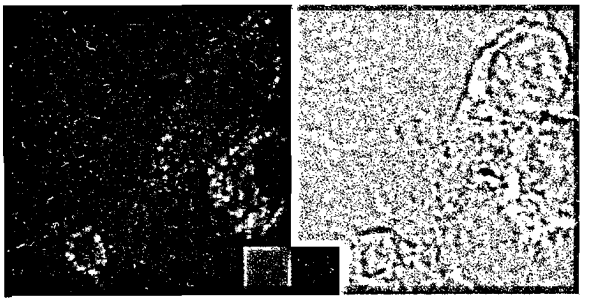
To take into account a possibility of non specific binding of secondary antibody and producing a false signal, cells were stained with the Cy-3 conjugated secondary antibodies alone. Images of the control experiments were taken with the same setting as those when primary antibodies were used. No fluorescence was observed on the beads or SRBC when macrophages were stained with secondary antibodies alone, meaning that signal when primary antibodies are added was only due the specific binding of the antibody to its target protein.

Figure 19. Immunoflorescent stains of RAW264.7 macrophages for PAK2-4 and PAK6.
Cells were grown on glass cover slips in AMEM+10%FBS overnight, opsonised beads or SRBC were incubated with the cells for 7 min, washed, fixed, stained with primary antibodies with 1:25 dilution followed by addition of Cy-3 conjugated secondary antibodies. Panels depict localization of antibodies targeting PAK2-4, 6. Images show the rhodamine (red) channel and the corresponding white light channel on the left. Secondary antibody alone staining are shown as insets.

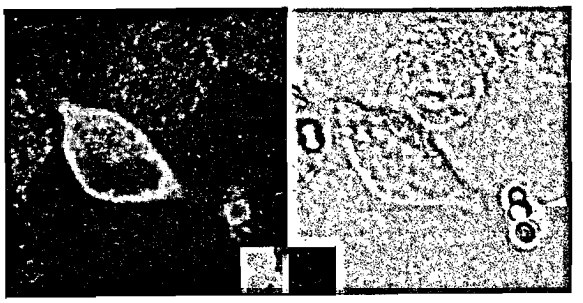
PAK2



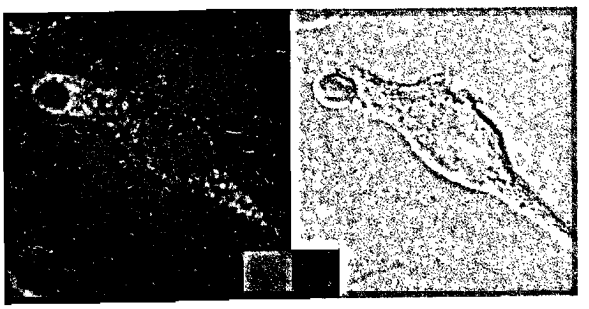
PAK3



PAK4



PAK6



Transfection of wild type GFP-PAK fusion proteins

RAW cells were transfected with GFP constructs of wild type PAK2-4 and PAK6 to confirm their localization at the site of particle uptake. Dominant negative mutants of PAKs were also transfected to see if these proteins have an effect on phagocytosis. ImageJ was used to analyze fluorescent signal intensity across line drawn through the cells.

The presence of a well defined ring around the SRBC in the cell undergoing phagocytosis transfected with wt EGFP-PAK2 is interpreted as a localization of PAK2 to the cup around the engulfed particle. This is supported by the signal intensity of cells transfected with EGFP-PAK2 constructs where the signal is higher on membrane around the engulfed particle as compared to the rest of the cell (*Figure 20A*). The line scan corresponding to the fluorescence intensity distribution throughout the cells shows uniform intensity in the cell body with the signal much higher at the site of phagocytosis (*Figure 20A2*).

Cells transfected with the EGFP-PAK3 construct show some nuclear localization and protein accumulation at the site of phagocytosis (*Figure 20B*). Higher intensity around engulfed SRBC as compared to the rest of the cell can be seen from the signal intensity distribution which was quantified with Image J.

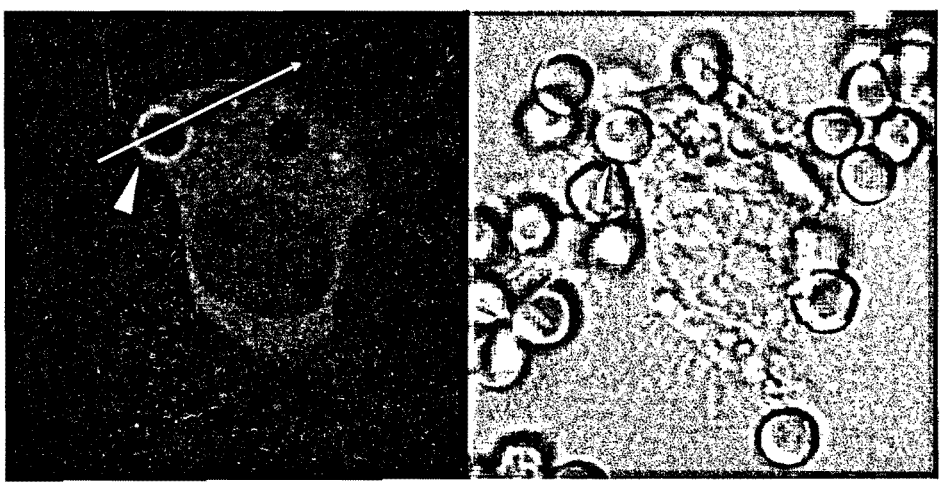
PAK4 linked to GFP transfected cells show no nuclear localization. No significant increase in intensity is observed at the formation of the cup (*Figure 20C*).

A EGFP-PAK6 wild type construct localizes to the site of phagocytosis as seen from the image taken with the LSM and the distribution of signal intensity plot (*Figure 20D*). The fluorescent signal is stronger around the engulfed SRBC as compared to the rest of the cell.

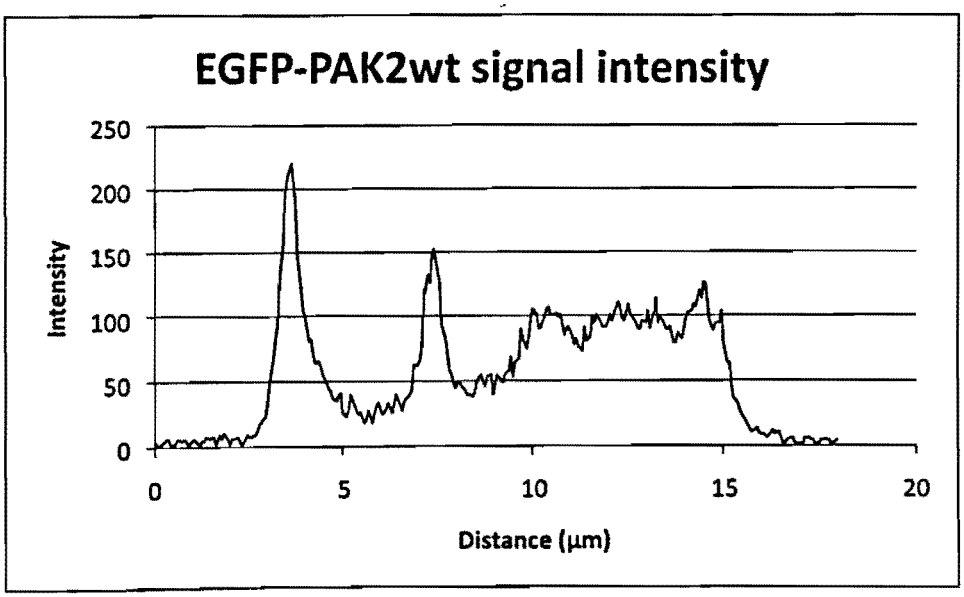
Figure 20. RAW264.7 macrophages transfected with GFP constructs of PAK proteins and quantitative distribution of fluorescent signal.

White arrows are drawn through the cell across which signal intensity was measured with ImageJ and is shown in the graphs below the corresponding image. Blue arrows indicated phagosome formation around SRBC. The image on the left hand side is that taken with the green channel while that on the right side is an image taken with the white channel. **A1**, EGFP-PAK2 transfection; **A2**, EGFP-PAK2 signal intensity measured along the length of the white arrow; **B1**, EGFP-PAK3 transfection; **B2**, EGFP-PAK3 signal intensity measured along the length of the white arrow; **C1**, GFP-PAK4 transfection; **C2**, GFP-PAK3 signal intensity measured along the length of the white arrow; **D1**, EGFP-PAK6 transfection; **D2**, EGFP-PAK6 signal intensity measured along the length of the white arrow.

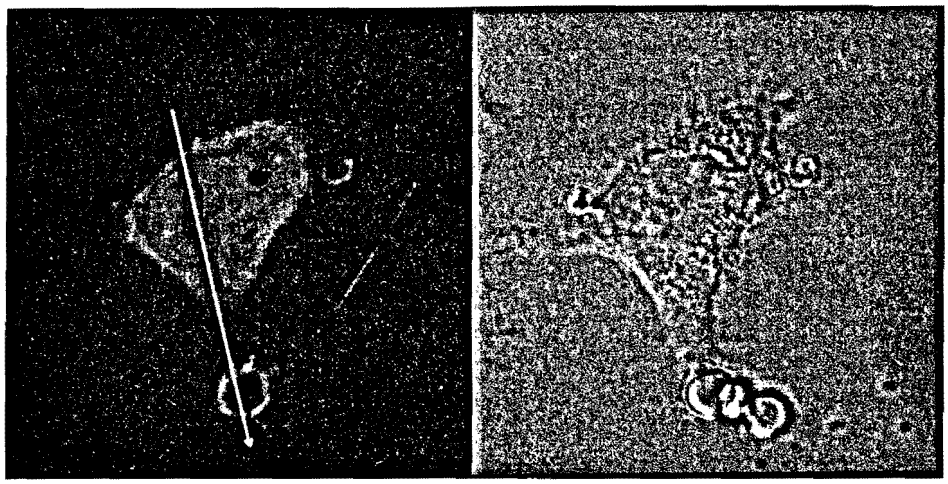
A1.



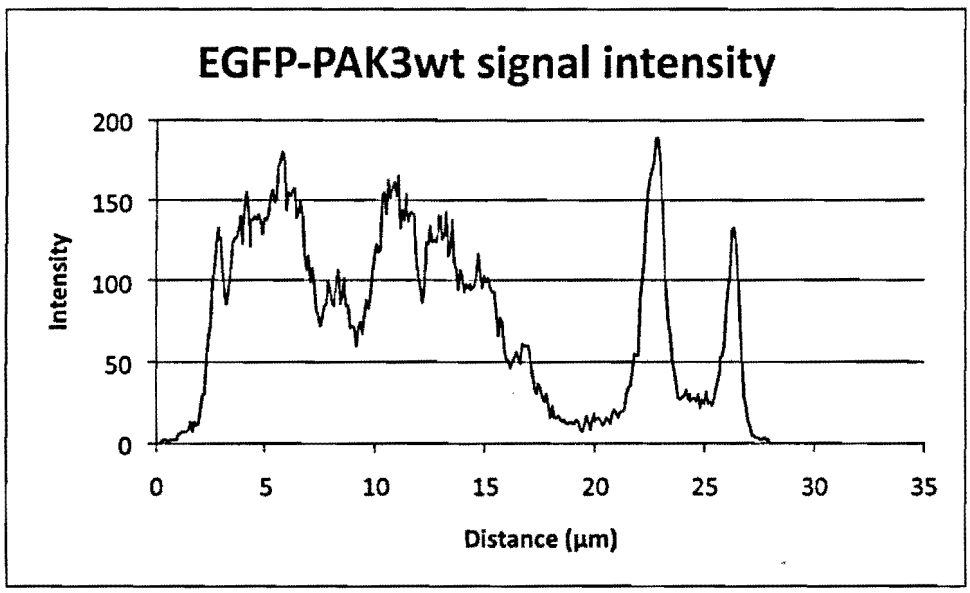
A2.



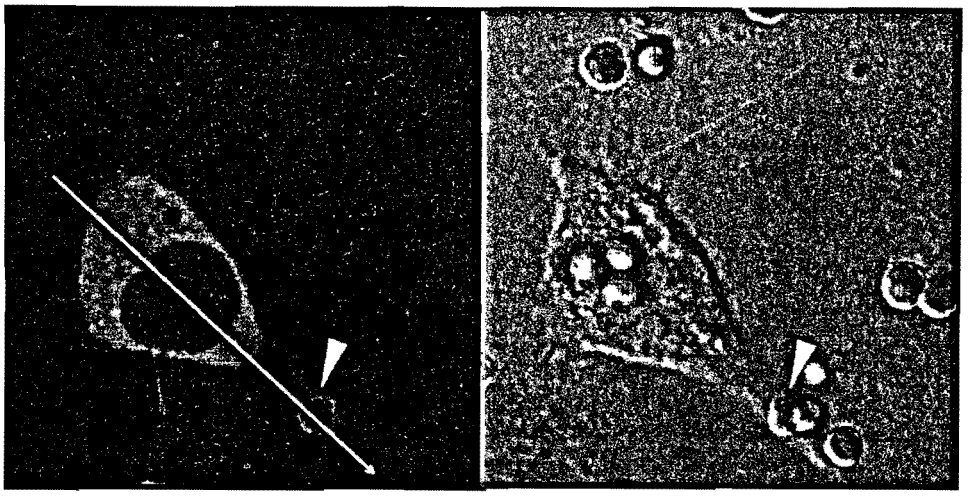
B1.



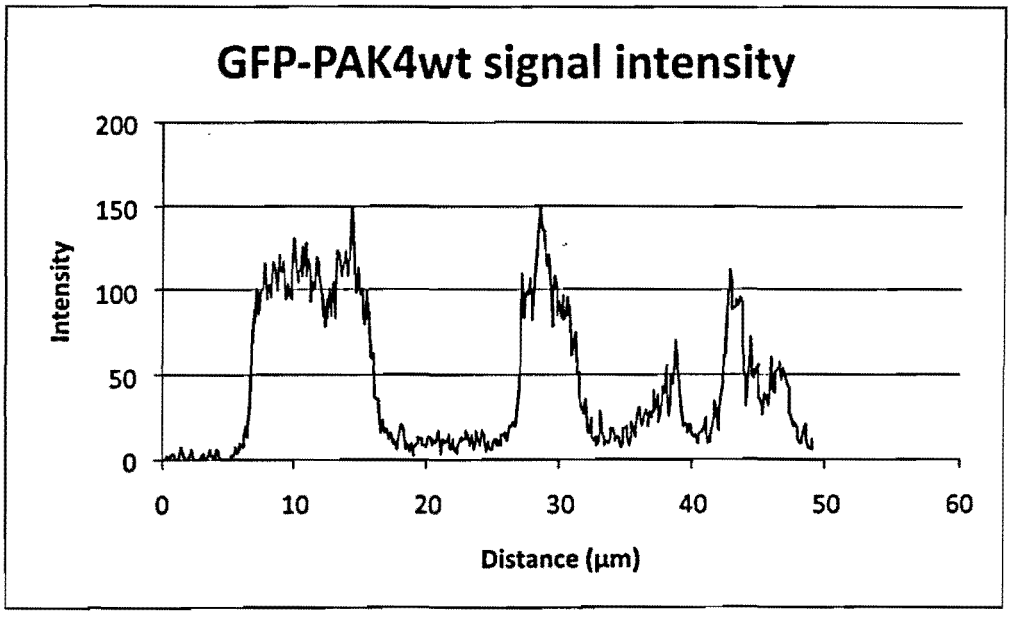
B2.


EGFP-PAK3wt signal intensity
Intensity
200
150
100
50
0
0
5
10
15
20
25
30
35
Distance (µm)

C1.



C2.


GFP-PAK4wt signal intensity
Intensity
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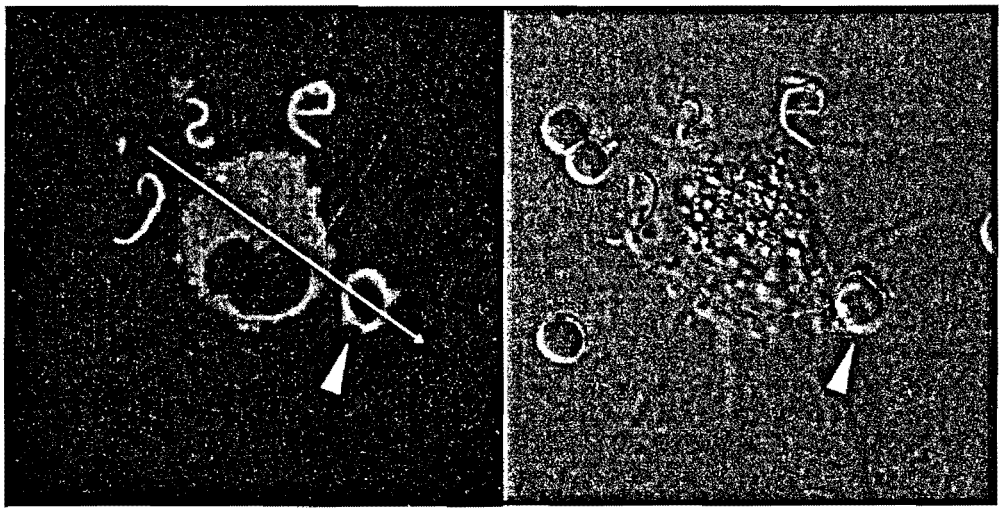
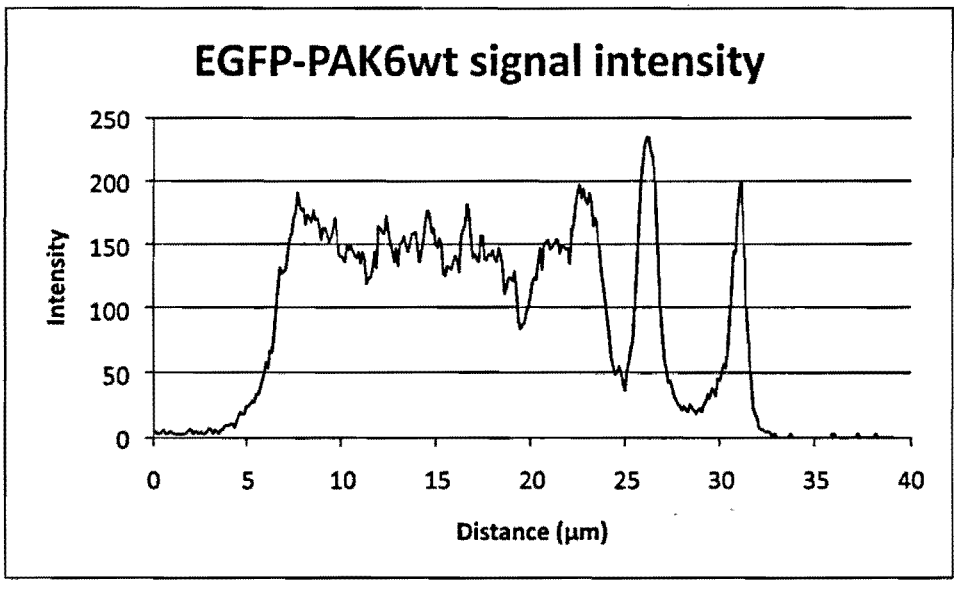
150
100
50
0
0
10
20
30
40
50
60
Distance (µm)

D1.



D2.

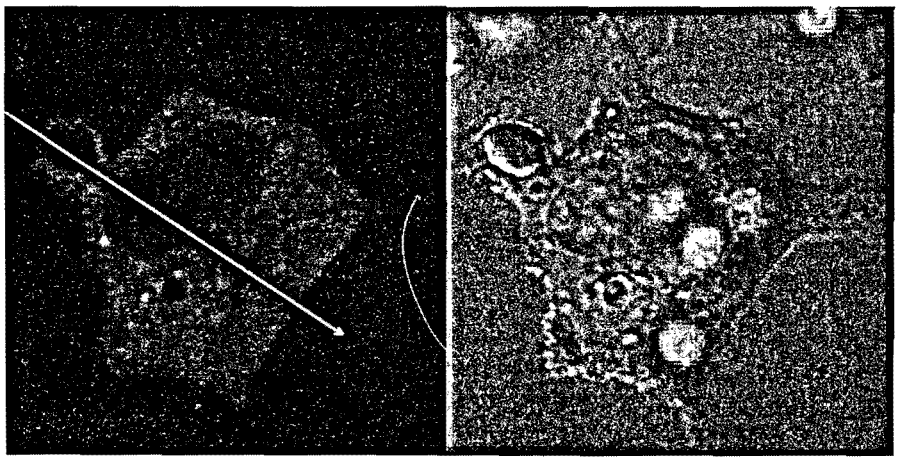

EGFP-PAK6wt signal intensity
Intensity
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
Distance (µm)

Transfection of dominant negative mutants of PAK proteins

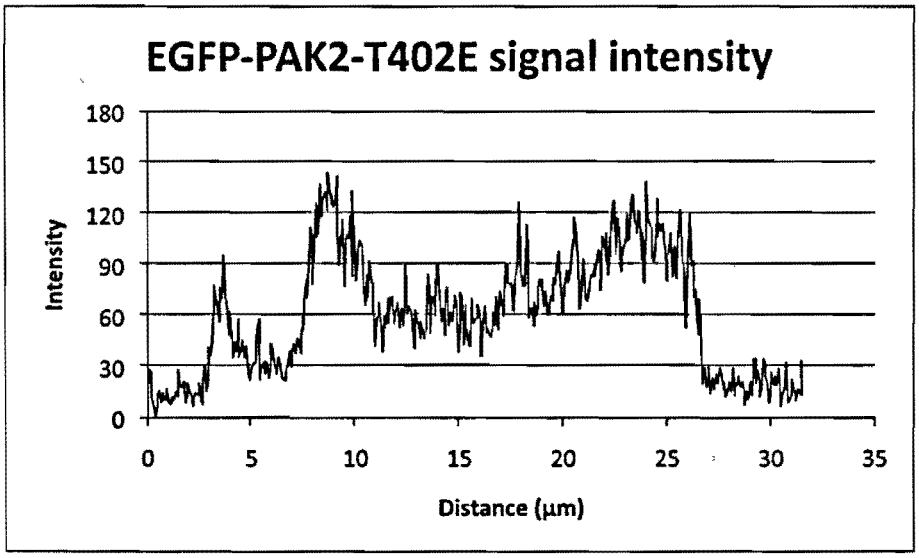
RAW264.7 macrophages were transfected with dominant negative mutants of proteins linked to GFP. A reduction in protein accumulation at the cup was observed for all of the transfections (*Figure 21*). Marked reduction is observed for PAK2 mutant. The PAK2 protein is present at the cup in the same intensity as in the rest of the cell as observed through application of ImageJ software to quantify fluorescent signal. A similar effect is seen with PAK3. The PAK6 mutant seems to accumulate at the site of phagocytosis however the degree of accumulation seems to be lower than that for the wild type EGFP-PAK6.

Figure 21. RAW264.7 macrophages transfected with a dominant negative mutants of PAK proteins that are GFP tagged and their quantitative distribution of fluorescent signal.
White arrows are drawn through the cell across which the signal intensity was measured with ImageJ and is shown in the graphs below the corresponding LSM image. Image on the left hand side is that taken with the green channel while that on the right side is an image taken with the white channel. **A1**, EGFP-PAK2-T402E transfection; **A2**, EGFP-PAK2-T402E signal intensity measured along the length of the white arrow; **B1**, EGFP-PAK3-K297L transfection; **B2**, EGFP-PAK3-K297L signal intensity measured along the length of the white arrow; **C1**, EGFP-PAK-K436A transfection; **C2**, EGFP-PAK-K436A signal intensity measured along the length of the white arrow.

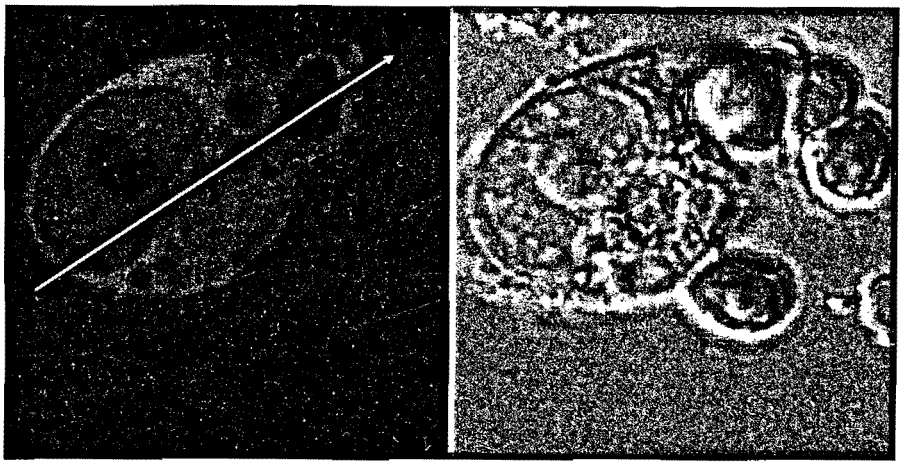
A1. PAK2-T402E



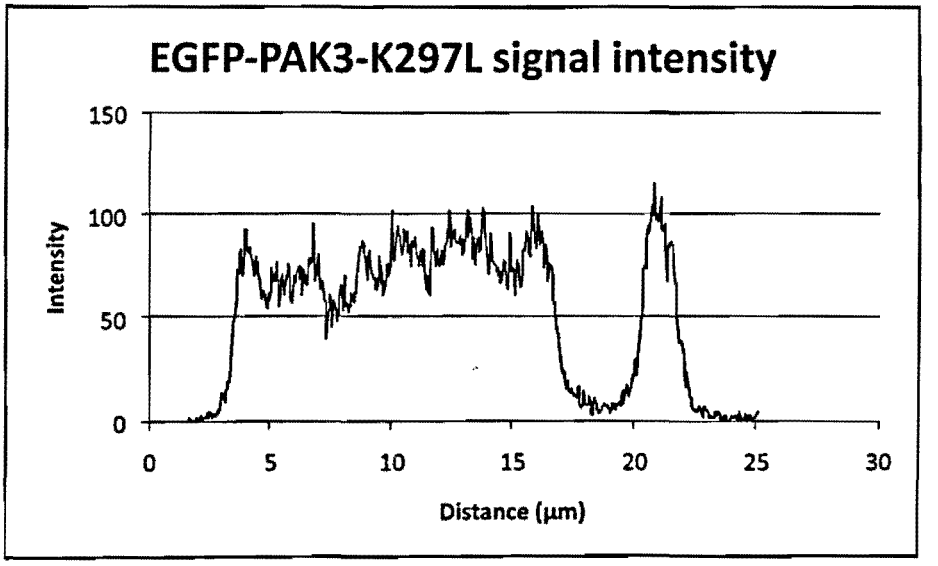
A2.



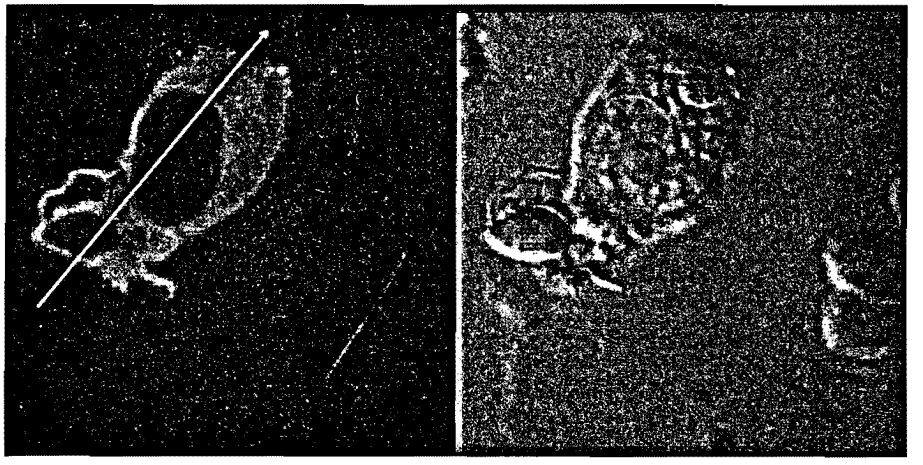
B1. PAK3-K297L



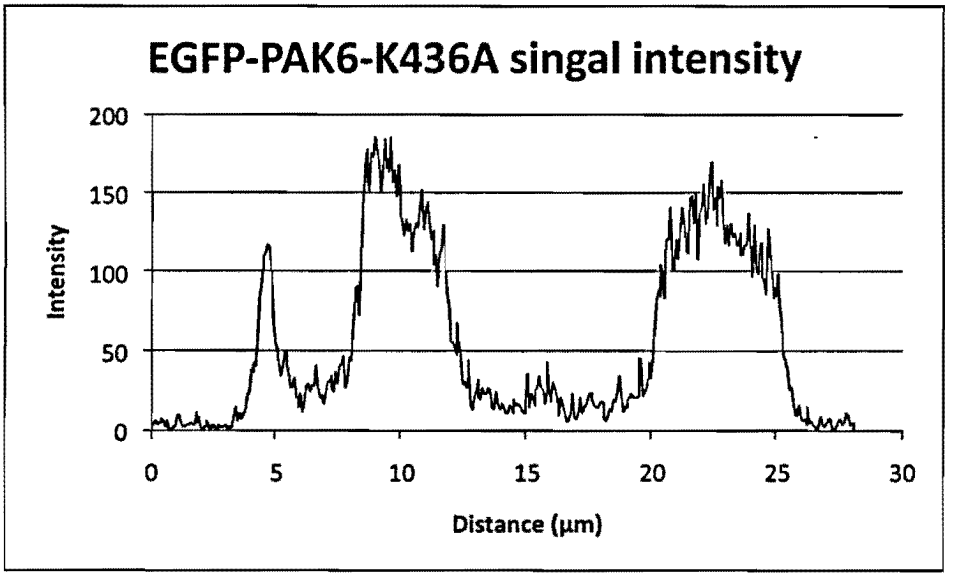
B2.



C1. PAK6-K436A



C2.

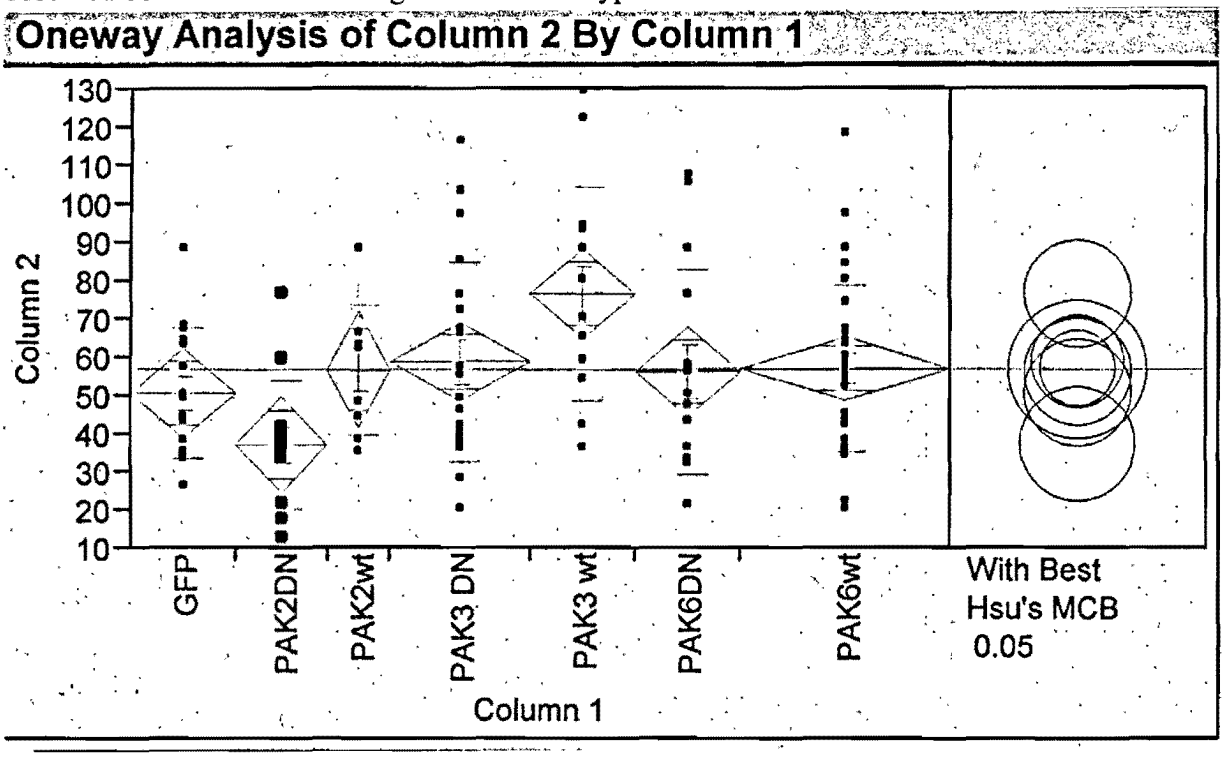


Effect of PAK wild type and dominant negative mutants on phagocytosis

Plasmids containing the GFP control alone, PAK2, PAK3, and PAK6 wild type and the corresponding dominant negative mutants were expressed in RAW macrophages and transefectants identified by the GFP fluorescence from live cells during phagocytosis assays with IgG coated beads. The expression of dominant negative mutants showed a significant reduction in Fc receptor function as measured by IgG coated microbead engulfment and internalization over two hours (ANOVA p=0.039). Analyzing the GFP control alongside both GFP WT and DN mutants for PAK2, PAK3 and PAK6 shown an overall F ration of 3.68 with a probability of p=0.0023. Cells transfected with PAK3 WT engulfed significantly greater number of beads and PAK2 DN internalized significantly less beads than the appropriate GFP tagged controls (*Figure 22*). Hence we observed that PAK proteins play a role in the function of the Fc receptor.

Figure 22. Analysis of microbead uptake by cells transfected with GFP alone, GFP fusion of PAK wt and PAK dn plasmids.

Number of beads engulfed and internalized was counted and compared. Significant difference is observed between dominant negative and wild type PAK transfectants.



DISCUSSION

HPLC

LARC identified numerous proteins from the Fc receptor complex. Many of the identified proteins were previously shown to play a role in phagocytosis. Different subset of proteins was captured from the control beads. From the variation of the LC elution profiles (*Figure 11)* it appears that the chromatographs of the non-specific proteins are different from those that are involved with the activated supramolecular Fcγ receptor complex. Thus the use of ligand receptor microbeads apparently captured a complex group of proteins from the surface of live cells that showed different chromatographic characteristics compared to non-specific binding to the similar control beads. From this we conclude that the Fc receptor supramolecular complex has likely been captured from the surface of the live human neutrophils.

LS-MS/MS

Control experiments allowed for elimination of the false positive identification. Proteins present in the Fc complex LC-MS/MS data with significant scores are supported through previous biochemical data found in literature. Comparison to controls leads us to believe that the Fc receptor was captured. Close to 900 proteins that have a positive score of minimum of 2400 and are either absent from the controls or have non-significant scores as compared to the Fc LC-MS/MS application. The list of specific proteins included integral membrane proteins, the cytoskeletal and many associated proteins. The list of proteins associated with the ligand coated microbeads contained specific members of the Fc receptor family and many proteins that are known to associate with the membranes and cytoskeleton in contrast to the control beads. Hence a large number of proteins were observed to be specifically associated with the ligand coated

microbeads at the site of the activated receptor complex. From the LC-ESI-MS/MS data we conclude that the Fc receptor complex was differentially captured from the surface of live cells by its cognate ligand IgG affixed to microbeads. Stringent criteria for protein acceptance into positive LARC experiments such as the presence of the score of at least 2400 and presence of at least three different tryptic peptides, compared to control experiments for non specific binding, minimized the possibility of false positive acceptance of proteins into the protein interaction models.

Agreement with Literature

Proteins that have been established to associate with the Fc receptor pathway by previous genetic and biochemical experiments were specifically observed on the ligand coated microbeads. Members of the Fc receptor superfamily itself, Src, Syk, PI3K, PLC, PLD, NOX, Rac, Cdc42, actins and myosins have been shown to play a role in phagocytosis (Greenberg and Grinstein 2002) and were detected with LARC. Hence it appears that the use of LARC has successfully captured many proteins that are known to be tightly associated with Fc receptor function. However, there were hundreds of new proteins identified by LARC that might be associated with the Fcγ receptor singaling. Hence, further protein interaction modeling might assist in the depiction of the interactions between these various proteins and the level of their association with the Fc receptor supramolecular complex.

Protein-Protein Interaction Models

The model of Fc receptor complex that arises from protein interaction networks is in agreement with the literature. IHOP was found to efficiently assist with the examination of the genetic and biochemical literature to reveal the interactions of the proteins detected from LARC

or their homologues in a variety of heterologous cells and conditions. After filtering for proteins from the Fc ligand specific data that showed at least three previous interactions with the primary data set, the basis for a model of protein-protein interactions was revealed. We used three different protein-protein interaction algorithms to calculate the central or nodal proteins that might exert the greatest influence on the regulation of the entire receptor supramolecular complex. All three programs revealed that members of the Src, PI3K, Cdc42, NOX complex, PLC, PLD, Rac1, RhoA, MAPK, dynamin-2, NOS2 and others are apparently central interactors in the Fc receptor complex in agreement with previous experiments. However hundreds of new proteins and isoforms not previously known to associate with the activated Fc receptor were detected and from this we conclude that LARC has the capacity to capture most previously established interactors and many novel members of a receptor pathway.

p21 Activated Kinases (PAK)

The Fc receptor is somewhat unique in its capacity to drive actin assembly and thus to form the pseudopods that may reach out from the cell to capture IgG coated particles of greater than 1 micron in size. These particles include bacteria that are recognized by the adaptive immune system. PAK kinases are thought to be regulated by the Rho GTPase family to drive actin formation (Eswaran, Soundararajan et al. 2008). P21 activated kinases were found to be associated with the activated Fc receptor from the LC-MS/MS data. PAK2, PAK3, PAK4 and PAK6 were predicted to play a role in FcyR mediated phagocytosis in both human neutrophils and murine macrophage cell lines (*Tables 3, 4*). From the protein interaction models that were constructed with Cytoscape, Osprey and String, PAK2 and PAK4 were predicted to play a more important role during phagocytosis (*Table 2*). Because of the agreement between neutrophils and

macrophage data means that it is likely that PAKs play an important role in Fc mediated phagocytosis and that LARC shows reproducibility between the receptor complexes captured from live human primary cells or cultured murine macrophages.

Western Blots

Protein expression of selected proteins detected with LARC was confirmed with Western Blots. PAK2, PAK3, PAK4 and PAK6 were detected with Western Blotting in RAW264.7 macrophages (*Figure 18*). The bands that were observed on Western Blots correspond to the correct preited molecular weights of these proteins. The Western blot results indicate that the proteins significantly detected by LC-ESI-MS/MS (Chelius, Huhmer et al. 2002) can be unambiguously confirmed by entirely independent biochemical methods. Detection of these proteins in an isolated receptor complex would likely support detection of these proteins with the LC-MS/MS at the activated Fcγ receptor complex.

Laser Confocal Microscopy

Putative Fc receptor associated proteins can be further suported by immunostaining and the use of GFP fused protein expression. Based on the presence of PAK2-4 and PAK6 in the murine macrophage cell line as confirmed with Western blotting, PAKs were immunostained to see whether they associate with the Fc receptor. PAK2, PAK3, PAK4 and PAK6 were found to be present in the phagocytic membrane around the IgG opsonised polystyrene microbeads or sheep red blood cells (*Figure 22*). Furthermore, PAK2 and PAK6 show accumulation at the site of phagocytosis, as believed from the higher fluorescent signal present at the cup formation as compared to the rest of the cell body. Some signal increase is observed around the engulfed particle when formaldehyde fixed cells were stained with antibodies specific for PAK3 and

PAK4. The presence of PAKs on the forming phagosome, as observed from LSM images of the immunofluorescent staining, potentially supports the idea that the protein capture and identification with LARC is a suitable method of detection of proteins at the activated Fcγ receptor. The differential staining is consistent with selective enrichment of some signal factors at the site of the active receptor.

To further support validity of LARC, cells were transfected with GFP constructs of PAK proteins expressed in macrophages (*Figure 20*). Similar data was gathered with the live cell imaging of the transfected cells as that with the immunostaining of fixed cells. EGFP-PAK2 and EGFP-PAK6 are seen to accumulate around the engulfed red blood cells during phagocytosis. This is further supported from the fluorescent intensity plots, constructed with ImageJ, which show a higher signal intensity around the engulfed particle. Higher intensity around the IgG opsonised particle may indicate involvement of these proteins in the Fc receptor function. EGFP-PAK3 shows a slightly higher signal intensity, which could potentially localize to the activated Fcγ receptor. The PAK4-GFP construct is seen to be present in the membrane around the engulfed particle. Accumulation of PAKs during phagocytosis of IgG opsonized particles is evidence of their association with the activated Fcγ receptor, whether directly or indirectly. Hence we conclude that LARC is able to capture proteins recruited to the plasma membrane sub domain surrounding the activated Fc receptor complex as a result of Fc receptor interaction with the ligand coated microscopic particles.

Role of PAK in Receptor Function

The experiments with GFP labeled controls, wild type PAK proteins and their corresponding dominant negative mutants have shown that the specific isoforms of PAK that

were shown to be tightly associated with Fc from both human primary cells and murine macrophages play a role in receptor function. The general enhancement of receptor function by expression of all three wild type kinases compared to the general trend of inhibition from their corresponding mutants shows that while PAKs are required for Fc function there is redundancy derived from the multiple isoforms. The significant enhancement of receptor function observed from the expression of the PAK3 and marked loss receptor function in the presence of the PAK2 DN indicates that individual PAK proteins have a significant effect on the function of the Fc supramolecular complex

FUTURE OBJECTIVES

Additional independent experiments explaining dominant negative PAK-GFP constructs are required in order to obtain stronger statistical correlation about the functional importance of p21 activated kinases during phagocytosis. Repetition of these experiments with different GFP mutants will be an asset since a particular mutation may not fully impair protein function under different circumstances. Silencing RNA experiments would be an asset to this project and would provide valuable data regarding the effect of p21 activated kinase knockouts on phagocytosis. Experiments with application of pharmaceutical agents blocking PAKs would further yield an insight into their function (Eswaran, Soundararajan et al. 2008). All of the mentioned experiments would further improve the quality of already existing data and would prove validity of LARC without any ambiguity.

CONCLUSION

Chromatographic, mass spectrometric, Western blot, calculations from the literature, immunocytological, GFP fusion and mutatant expression data all show that LARC captured the activated Fc receptor complex from the surface of live primary human cells in agreement with previous results from murine macrophages. Data gathered here indicate that PAK2, PAK3, PAK4 and PAK6 are recruited to and may play a functionally significant role in Fc receptor mediated phagocytosis. Moreover, the detection and presence of Fc specific proteins and subsequent calculations support a general case for the capture and identification of proteins associated with an activated receptor complex through application of Live-cell Affinity Receptor Chromatography.

REFERENCES

Ahmed, T., K. Shea, et al. (2008). "A PAK4-LIMK1 pathway drives prostate cancer cell migration downstream of HGF." Cell Signal **20**(7): 1320-8.

Al-Haddad, A., M. A. Shonn, et al. (2001). "Myosin Va bound to phagosomes binds to F-actin and delays microtubule-dependent motility." Mol Biol Cell **12**(9): 2742-55.

Allen, L. H. and A. Aderem (1995). "A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages." J Exp Med **182**(3): 829-40.

Araki, N., M. T. Johnson, et al. (1996). "A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages." J Cell Biol **135**(5): 1249-60.

Babior, B. M. (1999). "NADPH oxidase: an update." Blood **93**(5): 1464-76.

Bagrodia, S., B. Derijard, et al. (1995). "Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation." J Biol Chem **270**(47): 27995-8.

Baird, D., Q. Feng, et al. (2005). "The Cool-2/alpha-Pix protein mediates a Cdc42-Rac signaling cascade." Curr Biol **15**(1): 1-10.

Bamburg, J. R. (1999). "Proteins of the ADF/cofilin family: essential regulators of actin dynamics." Annu Rev Cell Dev Biol **15**: 185-230.

Barac, A., J. Basile, et al. (2004). "Direct interaction of p21-activated kinase 4 with PDZ-RhoGEF, a G protein-linked Rho guanine exchange factor." J Biol Chem **279**(7): 6182-9.

Boda, B., L. Jourdain, et al. (2008). "Distinct, but compensatory roles of PAK1 and PAK3 in spine morphogenesis." Hippocampus **18**(9): 857-61.

Bokoch, G. M. (2003). "Biology of the p21-activated kinases." Annu Rev Biochem **72**: 743-81.

Bokoch, G. M. (2005). "Regulation of innate immunity by Rho GTPases." Trends Cell Biol **15**(3): 163-71.

Bokoch, G. M. and B. A. Diebold (2002). "Current molecular models for NADPH oxidase regulation by Rac GTPase." Blood **100**(8): 2692-6.

Bokoch, G. M. and U. G. Knaus (2003). "NADPH oxidases: not just for leukocytes anymore!" Trends Biochem Sci **28**(9): 502-8.

Booth, J. W., W. S. Trimble, et al. (2001). "Membrane dynamics in phagocytosis." Semin Immunol **13**(6): 357-64.

Bosse, T., J. Ehinger, et al. (2007). "Cdc42 and phosphoinositide 3-kinase drive Rac-mediated actin polymerization downstream of c-Met in distinct and common pathways." Mol Cell Biol **27**(19): 6615-28.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Breitkreutz, B. J., C. Stark, et al. (2003). "Osprey: a network visualization system." Genome Biol **4**(3): R22.

Burkhardt, J. K. (1998). "Purification of phagosomes and assays for microtubule binding." Methods Enzymol **298**: 373-81.

Callow, M. G., S. Zozulya, et al. (2005). "PAK4 mediates morphological changes through the regulation of GEF-H1." J Cell Sci **118**(Pt 9): 1861-72.

Cargile, B. J., J. L. Bundy, et al. (2004). "Potential for false positive identifications from large databases through tandem mass spectrometry." J Proteome Res 3(5): 1082-5.
Chan, A. Y., M. Bailly, et al. (2000). "Role of cofilin in epidermal growth factor-stimulated actin polymerization and lamellipod protrusion." J Cell Biol **148**(3): 531-42.
Chelius, D., A. F. Huhmer, et al. (2002). "Analysis of the adenovirus type 5 proteome by liquid chromatography and tandem mass spectrometry methods." J Proteome Res **1**(6): 501-13.
Chen, Q., D. W. Powell, et al. (2003). "Akt phosphorylates p47phox and mediates respiratory burst activity in human neutrophils." J Immunol **170**(10): 5302-8.
Corbett-Nelson, E. F., D. Mason, et al. (2006). "Signaling-dependent immobilization of acylated proteins in the inner monolayer of the plasma membrane." J Cell Biol **174**(2): 255-65.
Corrotte, M., S. Chasserot-Golaz, et al. (2006). "Dynamics and function of phospholipase D and phosphatidic acid during phagocytosis." Traffic **7**(3): 365-77.
Cox, D., J. S. Berg, et al. (2002). "Myosin X is a downstream effector of PI(3)K during phagocytosis." Nat Cell Biol **4**(7): 469-77.
Cox, D., D. J. Lee, et al. (2000). "A Rab11-containing rapidly recycling compartment in macrophages that promotes phagocytosis." Proc Natl Acad Sci U S A **97**(2): 680-5.
Cox, D., C. C. Tseng, et al. (1999). "A requirement for phosphatidylinositol 3-kinase in pseudopod extension." J Biol Chem **274**(3): 1240-7.
D'Souza-Schorey, C., R. L. Boshans, et al. (1997). "A role for POR1, a Rac1-interacting protein, in ARF6-mediated cytoskeletal rearrangements." Embo J **16**(17): 5445-54.
Daeron, M. (1997). "Fc receptor biology." Annu Rev Immunol **15**: 203-34.
Delarue, F. L., B. S. Taylor, et al. (2001). "Ras and RhoA suppress whereas RhoB enhances cytokine-induced transcription of nitric oxide synthase-2 in human normal liver AKN-1 cells and lung cancer A-549 cells." Oncogene **20**(45): 6531-7.
DesMarais, V., M. Ghosh, et al. (2005). "Cofilin takes the lead." J Cell Sci **118**(Pt 1): 19-26.
DeSouza, L., G. Diehl, et al. (2005). "Proteomic analysis of the proliferative and secretory phases of the human endometrium: protein identification and differential protein expression." Proteomics **5**(1): 270-81.
Di, A., D. J. Nelson, et al. (2003). "Dynamin regulates focal exocytosis in phagocytosing macrophages." Mol Biol Cell **14**(5): 2016-28.
Di Virgilio, F., B. C. Meyer, et al. (1988). "Fc receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca2+ levels." J Cell Biol **106**(3): 657-66.
Diefenbach, A., H. Schindler, et al. (1999). "Requirement for type 2 NO synthase for IL-12 signaling in innate immunity." Science **284**(5416): 951-5.
Diekmann, D., A. Abo, et al. (1994). "Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity." Science **265**(5171): 531-3.
Downward, J. (1999). "How BAD phosphorylation is good for survival." Nat Cell Biol **1**(2): E33-5.
Eden, S., R. Rohatgi, et al. (2002). "Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck." Nature **418**(6899): 790-3.
Eswaran, J., M. Soundararajan, et al. (2008). "UnPAKing the class differences among p21-activated kinases." Trends Biochem Sci **33**(8): 394-403.

Fischer, M., S. Kaech, et al. (1998). "Rapid actin-based plasticity in dendritic spines." Neuron **20**(5): 847-54.

Fitzer-Attas, C. J., M. Lowry, et al. (2000). "Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn." J Exp Med **191**(4): 669-82.

Foster, L. J., C. L. de Hoog, et al. (2006). "A mammalian organelle map by protein correlation profiling." Cell **125**(1): 187-99.

Frischknecht, F., V. Moreau, et al. (1999). "Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling." Nature **401**(6756): 926-9.

Gallagher, E. D., S. Gutowski, et al. (2004). "RhoA binds to the amino terminus of MEKK1 and regulates its kinase activity." J Biol Chem **279**(3): 1872-7.

Garcia-Garcia, E. and C. Rosales (2002). "Signal transduction during Fc receptor-mediated phagocytosis." J Leukoc Biol **72**(6): 1092-108.

Garin, J., R. Diez, et al. (2001). "The phagosome proteome: insight into phagosome functions." J Cell Biol **152**(1): 165-80.

Gnesutta, N. and A. Minden (2003). "Death receptor-induced activation of initiator caspase 8 is antagonized by serine/threonine kinase PAK4." Mol Cell Biol **23**(21): 7838-48.

Gnesutta, N., J. Qu, et al. (2001). "The serine/threonine kinase PAK4 prevents caspase activation and protects cells from apoptosis." J Biol Chem **276**(17): 14414-9.

Goeckeler, Z. M., R. A. Masaracchia, et al. (2000). "Phosphorylation of myosin light chain kinase by p21-activated kinase PAK2." J Biol Chem **275**(24): 18366-74.

Gold, E. S., N. S. Morrissette, et al. (2000). "Amphiphysin IIm, a novel amphiphysin II isoform, is required for macrophage phagocytosis." Immunity **12**(3): 285-92.

Gold, E. S., D. M. Underhill, et al. (1999). "Dynamin 2 is required for phagocytosis in macrophages." J Exp Med **190**(12): 1849-56.

Goto, K., Y. Hozumi, et al. (2008). "Lipid messenger, diacylglycerol, and its regulator, diacylglycerol kinase, in cells, organs, and animals: history and perspective." Tohoku J Exp Med **214**(3): 199-212.

Grassart, A., A. Dujeancourt, et al. (2008). "Clathrin-independent endocytosis used by the IL-2 receptor is regulated by Rac1, Pak1 and Pak2." EMBO Rep **9**(4): 356-62.

Greenberg, S., P. Chang, et al. (1994). "Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages." J Biol Chem **269**(5): 3897-902.

Greenberg, S. and S. Grinstein (2002). "Phagocytosis and innate immunity." Curr Opin Immunol **14**(1): 136-45.

Gringel, A., D. Walz, et al. (2006). "PAK4 and alphaPIX determine podosome size and number in macrophages through localized actin regulation." J Cell Physiol **209**(2): 568-79.

Gu, Y., B. Jia, et al. (2001). "Biochemical and biological characterization of a human Rac2 GTPase mutant associated with phagocytic immunodeficiency." J Biol Chem **276**(19): 15929-38.

Haan, C. and I. Behrmann (2007). "A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background." J Immunol Methods **318**(1-2): 11-9.

Hackam, D. J., O. D. Rotstein, et al. (1997). "Rho is required for the initiation of calcium signaling and phagocytosis by Fcgamma receptors in macrophages." J Exp Med **186**(6): 955-66.

Harrison, R. E., C. Bucci, et al. (2003). "Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP." Mol Cell Biol **23**(18): 6494-506.

Hartwig, J. H., G. M. Bokoch, et al. (1995). "Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets." Cell **82**(4): 643-53.

Hausel, P., H. Latado, et al. (2006). "Src-mediated phosphorylation regulates subcellular distribution and activity of human inducible nitric oxide synthase." Oncogene **25**(2): 198-206.

Heyworth, P. G., B. P. Bohl, et al. (1994). "Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558." J Biol Chem **269**(49): 30749-52.

Heyworth, P. G., U. G. Knaus, et al. (1993). "Requirement for posttranslational processing of Rac GTP-binding proteins for activation of human neutrophil NADPH oxidase." Mol Biol Cell **4**(3): 261-9.

Hoffmann, R. and A. Valencia (2004). "A gene network for navigating the literature." Nat Genet **36**(7): 664.

Hoffmann, R. and A. Valencia (2005). "Implementing the iHOP concept for navigation of biomedical literature." Bioinformatics **21 Suppl 2**: ii252-8.

Hofmann, C., M. Shepelev, et al. (2004). "The genetics of Pak." J Cell Sci **117**(Pt 19): 4343-54.

Hoppe, A. D. and J. A. Swanson (2004). "Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis." Mol Biol Cell **15**(8): 3509-19.

Isakov, N. (1997). "Immunoreceptor tyrosine-based activation motif (ITAM), a unique module linking antigen and Fc receptors to their signaling cascades." J Leukoc Biol **61**(1): 6-16.

Jakobi, R., C. C. McCarthy, et al. (2003). "Caspase-activated PAK-2 is regulated by subcellular targeting and proteasomal degradation." J Biol Chem **278**(40): 38675-85.

Jankowski, A., P. Zhu, et al. (2008). "Capture of an activated receptor complex from the surface of live cells by affinity receptor chromatography." Anal Biochem **380**(2): 235-48.

Jenkins, G. H., P. L. Fisette, et al. (1994). "Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid." J Biol Chem **269**(15): 11547-54.

Jensen, L. J., M. Kuhn, et al. (2009). "STRING 8--a global view on proteins and their functional interactions in 630 organisms." Nucleic Acids Res **37**(Database issue): D412-6.

Jones, G. A. and G. Carpenter (1993). "The regulation of phospholipase C-gamma 1 by phosphatidic acid. Assessment of kinetic parameters." J Biol Chem **268**(28): 20845-50.

Jung, J. H., A. M. Pendergast, et al. (2008). "Phosphorylation of c-Abl by protein kinase Pak2 regulates differential binding of ABI2 and CRK." Biochemistry **47**(3): 1094-104.

Karimi, K. and M. R. Lennartz (1998). "Mitogen-activated protein kinase is activated during IgG-mediated phagocytosis, but is not required for target ingestion." Inflammation **22**(1): 67-82.

Katagiri, Y., T. Ito, et al. (2005). "Expression and localization of diacylglycerol kinase isozymes and enzymatic features in rat lung." Am J Physiol Lung Cell Mol Physiol **288**(6): L1171-8.

Kaur, R., X. Liu, et al. (2005). "Activation of p21-activated kinase 6 by MAP kinase kinase 6 and p38 MAP kinase." J Biol Chem **280**(5): 3323-30.

Kaur, R., X. Yuan, et al. (2008). "Increased PAK6 expression in prostate cancer and identification of PAK6 associated proteins." Prostate **68**(14): 1510-6.

Kiefer, F., J. Brumell, et al. (1998). "The Syk protein tyrosine kinase is essential for Fcgamma receptor signaling in macrophages and neutrophils." Mol Cell Biol **18**(7): 4209-20.

Kimmelman, A. C., A. F. Hezel, et al. (2008). "Genomic alterations link Rho family of GTPases to the highly invasive phenotype of pancreas cancer." Proc Natl Acad Sci U S A **105**(49): 19372-7.

Koeppel, M. A., C. C. McCarthy, et al. (2004). "Identification and characterization of PS-GAP as a novel regulator of caspase-activated PAK-2." J Biol Chem **279**(51): 53653-64.

Kreis, P., V. Rousseau, et al. (2008). "The four mammalian splice variants encoded by the p21-activated kinase 3 gene have different biological properties." J Neurochem **106**(3): 1184-97.

Kreis, P., E. Thevenot, et al. (2007). "The p21-activated kinase 3 implicated in mental retardation regulates spine morphogenesis through a Cdc42-dependent pathway." J Biol Chem **282**(29): 21497-506.

Krzewski, K., X. Chen, et al. (2006). "Formation of a WIP-, WASp-, actin-, and myosin IIA-containing multiprotein complex in activated NK cells and its alteration by KIR inhibitory signaling." J Cell Biol **173**(1): 121-32.

Kuijl, C., N. D. Savage, et al. (2007). "Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1." Nature **450**(7170): 725-30.

Kuncewicz, T., P. Balakrishnan, et al. (2001). "Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages." Am J Physiol Renal Physiol **281**(2): F326-36.

Lamalice, L., F. Houle, et al. (2006). "Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK2/p38 activation and endothelial cell migration in response to VEGF." J Biol Chem **281**(45): 34009-20.

Larsen, E. C., J. A. DiGennaro, et al. (2000). "Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells." J Immunol **165**(5): 2809-17.

Lee, N., H. MacDonald, et al. (1997). "Activation of hPAK65 by caspase cleavage induces some of the morphological and biochemical changes of apoptosis." Proc Natl Acad Sci U S A **94**(25): 13642-7.

Lee, S. R., S. M. Ramos, et al. (2002). "AR and ER interaction with a p21-activated kinase (PAK6)." Mol Endocrinol **16**(1): 85-99.

Lennartz, M. R. (1999). "Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis." Int J Biochem Cell Biol **31**(3-4): 415-30.

Leverrier, Y., K. Okkenhaug, et al. (2003). "Class I phosphoinositide 3-kinase p110beta is required for apoptotic cell and Fcgamma receptor-mediated phagocytosis by macrophages." J Biol Chem **278**(40): 38437-42.

Li, S., A. Yamauchi, et al. (2002). "Chemoattractant-stimulated Rac activation in wild-type and Rac2-deficient murine neutrophils: preferential activation of Rac2 and Rac2 gene dosage effect on neutrophil functions." J Immunol **169**(9): 5043-51.

Li, X. and A. Minden (2005). "PAK4 functions in tumor necrosis factor (TNF) alpha-induced survival pathways by facilitating TRADD binding to the TNF receptor." J Biol Chem **280**(50): 41192-200.

Linder, S., D. Nelson, et al. (1999). "Wiskott-Aldrich syndrome protein regulates podosomes in primary human macrophages." Proc Natl Acad Sci U S A **96**(17): 9648-53.

Link, A. J., J. Eng, et al. (1999). "Direct analysis of protein complexes using mass spectrometry." Nat Biotechnol **17**(7): 676-82.

Liu, Y., H. Xiao, et al. (2008). "The pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice." Mol Cancer Res **6**(7): 1215-24.

Lu, M. and K. S. Ravichandran (2006). "Dock180-ELMO cooperation in Rac activation." Methods Enzymol **406**: 388-402.

Machuy, N., F. Campa, et al. (2007). "c-Abl-binding protein interacts with p21-activated kinase 2 (PAK-2) to regulate PDGF-induced membrane ruffles." J Mol Biol **370**(4): 620-32.

Malcolm, K. C., J. C. Chambard, et al. (2000). "Independent activation of endogenous p21-activated protein kinase-3 (PAK3) and JNK by thrombin in CCL39 fibroblasts." J Cell Physiol **185**(2): 235-43.

Manser, E., T. Leung, et al. (1994). "A brain serine/threonine protein kinase activated by Cdc42 and Rac1." Nature **367**(6458): 40-6.

Mansfield, P. J., J. A. Shayman, et al. (2000). "Regulation of polymorphonuclear leukocyte phagocytosis by myosin light chain kinase after activation of mitogen-activated protein kinase." Blood **95**(7): 2407-12.

Marshall, J., A. Jankowski, et al. (2004). "Human serum proteins preseparated by electrophoresis or chromatography followed by tandem mass spectrometry." J Proteome Res **3**(3): 364-82.

Marshall, J. G., J. W. Booth, et al. (2001). "Restricted accumulation of phosphatidylinositol 3-kinase products in a plasmalemmal subdomain during Fc gamma receptor-mediated phagocytosis." J Cell Biol **153**(7): 1369-80.

McCombie, W. R., M. D. Adams, et al. (1992). "Caenorhabditis elegans expressed sequence tags identify gene families and potential disease gene homologues." Nat Genet **1**(2): 124-31.

McKenzie, S. E. and A. D. Schreiber (1998). "Fc gamma receptors in phagocytes." Curr Opin Hematol **5**(1): 16-21.

Miah, S. M., K. Sada, et al. (2004). "Activation of Syk protein tyrosine kinase in response to osmotic stress requires interaction with p21-activated protein kinase Pak2/gamma-PAK." Mol Cell Biol **24**(1): 71-83.

Miki, H., S. Suetsugu, et al. (1998). "WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac." Embo J **17**(23): 6932-41.

Moreau, V., F. Frischknecht, et al. (2000). "A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization." Nat Cell Biol **2**(7): 441-8.

Nekrasova, T., M. L. Jobes, et al. (2008). "Targeted disruption of the Pak5 and Pak6 genes in mice leads to deficits in learning and locomotion." Dev Biol **322**(1): 95-108.

Node-Langlois, R., D. Muller, et al. (2006). "Sequential implication of the mental retardation proteins ARHGEF6 and PAK3 in spine morphogenesis." J Cell Sci **119**(Pt 23): 4986-93.

Nowicki, M., J. Kosacka, et al. (2007). "The myelin-associated glycoprotein inhibitor BENZ induces outgrowth and survival of rat dorsal root ganglion cell cultures." J Neurosci Res **85**(14): 3053-63.

Olazabal, I. M., E. Caron, et al. (2002). "Rho-kinase and myosin-II control phagocytic cup formation during CR, but not FcgammaR, phagocytosis." Curr Biol **12**(16): 1413-18.

Perrin, B. J., K. J. Amann, et al. (2006). "Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration." Mol Biol Cell **17**(1): 239-50.

Pollard, T. D., L. Blanchoin, et al. (2000). "Molecular mechanisms controlling actin filament dynamics in nonmuscle cells." Annu Rev Biophys Biomol Struct **29**: 545-76.

Prajapati, S., U. Verma, et al. (2004). "Protein phosphatase 2Cbeta association with the IkappaB kinase complex is involved in regulating NF-kappaB activity." J Biol Chem **279**(3): 1739-46.

Qu, J., X. Li, et al. (2003). "PAK4 kinase is essential for embryonic viability and for proper neuronal development." Mol Cell Biol **23**(20): 7122-33.

Ramesh, N., I. M. Anton, et al. (1997). "WIP, a protein associated with wiskott-aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells." Proc Natl Acad Sci U S A **94**(26): 14671-6.

Ravetch, J. V. and S. Bolland (2001). "IgG Fc receptors." Annu Rev Immunol **19**: 275-90.

Renkema, G. H., K. Pulkkinen, et al. (2002). "Cdc42/Rac1-mediated activation primes PAK2 for superactivation by tyrosine phosphorylation." Mol Cell Biol **22**(19): 6719-25.

Robinson, J. M. and J. A. Badwey (2002). "Rapid association of cytoskeletal remodeling proteins with the developing phagosomes of human neutrophils." Histochem Cell Biol **118**(2): 117-25.

Roig, J., Z. Huang, et al. (2000). "p21-activated protein kinase gamma-PAK is translocated and activated in response to hyperosmolarity. Implication of Cdc42 and phosphoinositide 3-kinase in a two-step mechanism for gamma-PAK activation." J Biol Chem **275**(22): 16933-40.

Roig, J., P. T. Tuazon, et al. (2000). "Functional interaction between c-Abl and the p21-activated protein kinase gamma-PAK." Proc Natl Acad Sci U S A **97**(26): 14346-51.

Roos, D., A. A. Voetman, et al. (1983). "Functional activity of enucleated human polymorphonuclear leukocytes." J Cell Biol **97**(2): 368-77.

Rudel, T. and G. M. Bokoch (1997). "Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2." Science **276**(5318): 1571-4.

Sanchez-Mejorada, G. and C. Rosales (1998). "Signal transduction by immunoglobulin Fc receptors." J Leukoc Biol **63**(5): 521-33.

Sasahara, Y., R. Rachid, et al. (2002). "Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation." Mol Cell **10**(6): 1269-81.

Sastry, S. K., P. D. Lyons, et al. (2002). "PTP-PEST controls motility through regulation of Rac1." J Cell Sci **115**(Pt 22): 4305-16.
Sastry, S. K., Z. Rajfur, et al. (2006). "PTP-PEST couples membrane protrusion and tail retraction via VAV2 and p190RhoGAP." J Biol Chem **281**(17): 11627-36.
Sawyer, D. W., J. A. Sullivan, et al. (1985). "Intracellular free calcium localization in neutrophils during phagocytosis." Science **230**(4726): 663-6.
Schafer, D. A., S. A. Weed, et al. (2002). "Dynamin2 and cortactin regulate actin assembly and filament organization." Curr Biol **12**(21): 1852-7.
Schagger, H., U. Borchart, et al. (1987). "Isolation and amino acid sequence of the 'Rieske' iron sulfur protein of beef heart ubiquinol:cytochrome c reductase." FEBS Lett **219**(1): 161-8.
Schlunck, G., H. Damke, et al. (2004). "Modulation of Rac localization and function by dynamin." Mol Biol Cell **15**(1): 256-67.
Schrantz, N., J. da Silva Correia, et al. (2004). "Mechanism of p21-activated kinase 6-mediated inhibition of androgen receptor signaling." J Biol Chem **279**(3): 1922-31.
Shannon, P., A. Markiel, et al. (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." Genome Res **13**(11): 2498-504.
Siow, Y. L., G. B. Kalmar, et al. (1997). "Identification of two essential phosphorylated threonine residues in the catalytic domain of Mekk1. Indirect activation by Pak3 and protein kinase C." J Biol Chem **272**(12): 7586-94.
Soosairajah, J., S. Maiti, et al. (2005). "Interplay between components of a novel LIM kinase-slingshot phosphatase complex regulates cofilin." Embo J **24**(3): 473-86.
Srinivasan, S., F. Wang, et al. (2003). "Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis." J Cell Biol **160**(3): 375-85.
Swanson, J. A., M. T. Johnson, et al. (1999). "A contractile activity that closes phagosomes in macrophages." J Cell Sci **112 (Pt 3)**: 307-16.
Symons, M., J. M. Derry, et al. (1996). "Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization." Cell **84**(5): 723-34.
Takekawa, M., T. Maeda, et al. (1998). "Protein phosphatase 2Calpha inhibits the human stress-responsive p38 and JNK MAPK pathways." EMBO J **17**(16): 4744-52.
Takenawa, T. and S. Suetsugu (2007). "The WASP-WAVE protein network: connecting the membrane to the cytoskeleton." Nat Rev Mol Cell Biol **8**(1): 37-48.
Tilton, B., M. Andjelkovic, et al. (1997). "G-Protein-coupled receptors and Fcgamma-receptors mediate activation of Akt/protein kinase B in human phagocytes." J Biol Chem **272**(44): 28096-101.
Titus, M. A. (1999). "A class VII unconventional myosin is required for phagocytosis." Curr Biol **9**(22): 1297-303.
Torres, E. and M. K. Rosen (2003). "Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP." Mol Cell **11**(5): 1215-27.
Turner, M., E. Schweighoffer, et al. (2000). "Tyrosine kinase SYK: essential functions for immunoreceptor signalling." Immunol Today **21**(3): 148-54.
Vicente-Manzanares, M., J. R. Cabrero, et al. (2002). "A role for the Rho-p160 Rho coiled-coil kinase axis in the chemokine stromal cell-derived factor-1alpha-induced lymphocyte actomyosin and microtubular organization and chemotaxis." J Immunol **168**(1): 400-10.

Wang, J. and Z. Wang (2008). "Negative regulation of caspase 3-cleaved PAK2 activity by protein phosphatase 1." Sci China C Life Sci **51**(1): 1-11.

Weaver, A. M., M. E. Young, et al. (2003). "Integration of signals to the Arp2/3 complex." Curr Opin Cell Biol **15**(1): 23-30.

Wells, C. M., A. Abo, et al. (2002). "PAK4 is activated via PI3K in HGF-stimulated epithelial cells." J Cell Sci **115**(Pt 20): 3947-56.

Wilde, J. I. and S. P. Watson (2001). "Regulation of phospholipase C gamma isoforms in haematopoietic cells: why one, not the other?" Cell Signal **13**(10): 691-701.

Wu, C., S. F. Lee, et al. (1996). "Activation of myosin-I by members of the Ste20p protein kinase family." J Biol Chem **271**(50): 31787-90.

Yang, C., M. Huang, et al. (2000). "Profilin enhances Cdc42-induced nucleation of actin polymerization." J Cell Biol **150**(5): 1001-12.

Yates, J. R., 3rd (1998). "Database searching using mass spectrometry data." Electrophoresis **19**(6): 893-900.

Yoshimura, M., K. Homma, et al. (2001). "Dual regulation of mammalian myosin VI motor function." J Biol Chem **276**(43): 39600-7.

Yoshioka, K., N. Sugimoto, et al. (2007). "Essential role for class II phosphoinositide 3-kinase alpha-isoform in Ca2+-induced, Rho- and Rho kinase-dependent regulation of myosin phosphatase and contraction in isolated vascular smooth muscle cells." Mol Pharmacol **71**(3): 912-20.

Zeng, Q., D. Lagunoff, et al. (2000). "Endothelial cell retraction is induced by PAK2 monophosphorylation of myosin II." J Cell Sci **113 (Pt 3)**: 471-82.

Zhang, B., J. Chernoff, et al. (1998). "Interaction of Rac1 with GTPase-activating proteins and putative effectors. A comparison with Cdc42 and RhoA." J Biol Chem **273**(15): 8776-82.

Zhang, H., D. J. Webb, et al. (2005). "A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC." J Neurosci **25**(13): 3379-88.

Zhang, Q., D. Cox, et al. (1998). "A requirement for ARF6 in Fcgamma receptor-mediated phagocytosis in macrophages." J Biol Chem **273**(32): 19977-81.

Zhu, J., K. Zhou, et al. (2005). "Regulation of cortactin/dynamin interaction by actin polymerization during the fission of clathrin-coated pits." J Cell Sci **118**(Pt 4): 807-17.

APPENDIX A

Oneway ANOVA of PAK wild type and donimant negative mutants

Oneway Anova

Summary of Fit

Rsquare	0.167329
Adj Rsquare	0.12191
Root Mean Square Error	22.7861
Mean of Response	56.61538
Observations (or Sum Wgts)	117

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Column 1	6	11477.017	1912.84	3.6842	0.0023*
Error	110	57112.676	519.21		
C. Total	116	68589.692			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
GFP	15	50.6000	5.8833	38.941	62.259
PAK2DN	13	37.1538	6.3197	24.630	49.678
PAK2wt	9	56.6667	7.5954	41.614	71.719
PAK3 DN	20	58.7500	5.0951	48.653	68.847
PAK3 wt	15	76.4667	5.8833	64.807	88.126
PAK6DN	15	56.0667	5.8833	44.407	67.726
PAK6wt	30	56.9667	4.1602	48.722	65.211

Std Error uses a pooled estimate of error variance

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
GFP	15	50.6000	16.9908	4.3870	41.191	60.009
PAK2DN	13	37.1538	16.6876	4.6283	27.070	47.238
PAK2wt	9	56.6667	16.8745	5.6248	43.696	69.638
PAK3 DN	20	58.7500	26.0725	5.8300	46.548	70.952
PAK3 wt	15	76.4667	27.8282	7.1852	61.056	91.877
PAK6DN	15	56.0667	26.7568	6.9086	41.249	70.884
PAK6wt	30	56.9667	21.7120	3.9641	48.859	65.074

Means Comparisons

Comparisons with the best using Hsu's MCB

d	Alpha
2.31560	0.05
2.34506	
2.37540	

Means Comparisons

Comparisons with the best using Hsu's MCB

d
2.24646
2.31560
2.31560
2.29838

Level	vs. Max p-Value	vs. Min p-Value
PAK3 wt	.	<.0001*
PAK3 DN	0.0656	0.0268*
PAK6wt	0.0234*	0.0294*
PAK2wt	0.0883	0.1038
PAK6DN	0.0432*	0.0760
GFP	0.0082*	0.2329
PAK2DN	<.0001*	.

Mean[i]-Mean[j]-LSD

	PAK3 wt	PAK3 DN	PAK6wt	PAK2wt	PAK6DN	GFP	PAK2DN
PAK3 wt	-19.2665	-0.53479	2.383835	-1.78278	1.133491	6.600158	19.46776
PAK3 DN	-35.7388	-16.8976	-13.8415	-18.4629	-15.3388	-9.87217	2.938325
PAK6wt	-36.1853	-17.2086	-13.9753	-19.1545	-15.7853	-10.3186	2.423094
PAK2wt	-42.047	-23.5313	-20.8711	-24.1303	-21.647	-16.1804	-3.19677
PAK6DN	-39.6665	-20.9348	-18.0162	-22.1828	-19.2665	-13.7998	-0.93224
GFP	-45.1332	-26.4015	-23.4828	-27.6494	-24.7332	-19.2665	-6.39891
PAK2DN	-59.3066	-40.633	-37.7853	-41.7095	-38.9066	-33.44	-20.5416

If a column has any positive values, the mean is significantly less than the max.

Mean[i]-Mean[j]+LSD

	PAK3 wt	PAK3 DN	PAK6wt	PAK2wt	PAK6DN	GFP	PAK2DN
PAK3 wt	19.26651	35.96812	36.61617	41.38278	39.66651	45.13318	59.15789
PAK3 DN	0.305502	16.89756	17.40818	22.62953	20.7055	26.17217	40.25398
PAK6wt	-2.81471	13.64196	13.97529	19.75445	17.58529	23.05195	37.20255
PAK2wt	2.447048	19.36467	20.27107	24.13028	22.84705	28.31371	42.22241
PAK6DN	-1.13349	15.56812	16.21617	20.98278	19.26651	24.73318	38.75789
GFP	-6.60016	10.10145	10.7495	15.51611	13.79984	19.26651	33.29122
PAK2DN	-19.319	-2.55934	-1.84032	2.683809	1.080981	6.547647	20.54159

If a column has any negative values, the mean is significantly greater than the min.