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IDENTIFICATION AND CHARACTERIZATION OF WASP AND FKBP-LIKE PROTEIN

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Cover illustration: human fibroblast transfected with WAFL-FLAG (red), actin filaments detected with phalloidin (green), and nucleus stained with DAPI (blue).

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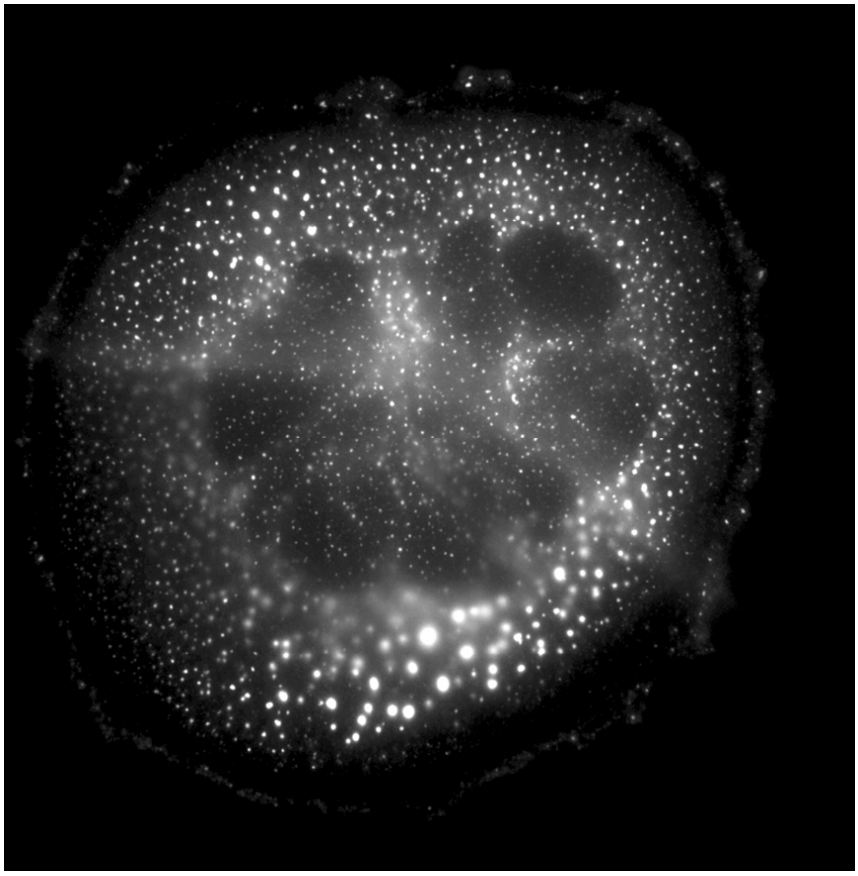

“Somewhere, something incredible is waiting to be known.”

Carl Sagan

ABSTRACT

Ulcerative colitis (UC) is a chronic inflammation of the colon which together with the related Crohn's disease (CD) represent the major forms of Inflammatory Bowel Disease (IBD). IBD is believed to arise in a dysfunctional intestinal barrier which allows the normal gut flora to trigger an unwanted chronic immune response in genetically susceptible individuals. The cause of these illnesses is unknown, and treatment today is solely to alleviate the symptoms.

In order to identify biomarkers that could help us to further our understanding of the pathophysiology of UC, we applied the subtractive suppression hybridization (SSH) method to identify UC-differentially regulated genes in colonic mucosal biopsy specimens (Paper I). The 331 differentially expressed genes were grouped into functional categories and when analyzed revealed a gene expression pattern consistent with UC as an inflammatory disorder with altered epithelial homeostasis. Interestingly, 21 genes were found to be involved in membrane trafficking. Moreover, 37 of the 331 genes were unknown genes with no predicted function. Based on the sequence homology to the WASP-protein (connected to the immunodeficiency disorder Wiskott-Aldrich syndrome) one unknown gene was selected for further investigation. We named the gene WAFL. While the initial characterization of WAFL revealed a broad tissue expression, qPCR analysis indicated that WAFL was upregulated in inflamed tissue of UC compared to inflamed tissue from CD patients (i.e. consistent with the SSH data). To learn about the function of this protein we initiated a detailed investigation. One approach was to obtain the structure of the protein. In Paper II, the expression of regions of WAFL in *E.coli*, and in insect cells via a baculoviral system, is described. These peptides were purified and were found to be folded correctly. They have subsequently been used for functional tests of WAFL.

The major finding in my thesis, Paper III, links WAFL to regulation of early endosomes. WAFL interacts with actin and WASP-interacting protein (WIP), which implies a role in actin-based transport of the endosomes. Moreover, cells depleted of WAFL by RNAi exhibited a disturbed transport of endocytic cargo towards lysosomes. Protein mapping experiments showed that the endosome localization is mediated by WAFL's central coiled-coil domain. This domain of WAFL binds to the integral membrane components monophosphorylated phosphatidylinositols, in particular PtdIns(3)P which is found on early endosomes.

Interestingly, WAFL appears also on the phagosome of invasive bacteria such as *Salmonella, Yersinia,* and *Shigella* indicating that WAFL may be involved in the phagocytosis process. The observation that induced expression is observed in macrophages support this possibility.

In conclusion, we have identified and characterized a novel gene, WAFL, from UC bioptic material. This interesting gene is upregulated in inflamed tissue of UC patients and has potential functions in the correct processing of phagosomes and early endosomes.

LIST OF PUBLICATIONS

I. **Viklund IM**, Kuznetsov NV, Löfberg R, Daperno M, Sostegni R, Astegiano M, Rizzetto M, von Stein O, D'Amato M, von Stein P, Pettersson S.

Identification of a new WASP and FKBP-like (WAFL) protein in inflammatory bowel disease: a potential marker gene for ulcerative colitis

Int J Colorectal Dis. 2008 Oct;23(10):921-30.

II. Kopec J, Ågren D, **Viklund IM**, Pettersson S, Schneider G.

Expression and purification of domains of the human WASP- and FKBP-like protein WAFL, a novel member of the WASP family involved in early endocytosis

Under revision for Protein Expres Purif

III. WAFL, a new protein involved in regulation of early endocytic transport at the intersection of actin and microtubule dynamics

Viklund IM, Aspenström P, Meas-Yedid V, Zhang B, Kopec J, Ågren D, Schneider G, D'Amato M, Olivo-Marin JC, Sansonetti P, Tran Van Nhieu G, S Pettersson.

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CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
Arp	Actin-related protein
ATP	adenosine triphosphate
CD	Crohn's disease
CME	clathrin-mediated endocytosis
CR16	corticosteroid and regional expression-16
CRIB	Cdc42 and Rac interactive binding
EEA1	early endosomal antigen 1
EGFP	enhanced green fluorescent protein
ESCRT	endosomal sorting complex required for transport
EVH1	Ena-VASP homology-1
FACS	fluorescence-activated cell sorting
FKBP	FK506-binding protein
GAP	GTPase activating protein
GEF	GDP/GTP-exchange factor
GBD	GTPase binding domain
GDP	guanosine diphosphate
GTP	guanosine triphosphate
IBD	inflammatory bowel disease
IP	immunoprecipitation
MVB	multivesicular body
PtdIns/PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol phosphate
PPIase	peptidyl-prolyl isomerase
RT-PCR	reverse transcription polymerase chain reaction
SCAR	suppressor of cAMP receptor
SCV	Salmonella-containing vacuole
SHD	SCAR homology domain
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SSH	subtractive suppression hybridization
TGN	trans-Golgi network
UC	ulcerative colitis
VCA	verprolin central acidic
WAFL	WASP and FKBP-like
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin homologous
WH1	WASP-homology domain 1
WH2	WASP-homology domain 2
WIP	WASP-interacting protein
WIRE	WIP-related
XLT	X-linked thrombocytopenia

1 INTRODUCTION

"An organism is an integrated system of interdependent structures and functions. An organism is constituted of cells, and a cell consists of molecules which must work in harmony."
André Lwoff

Medicine can be studied on many different levels: genetic, protein, cell, tissue, organism, or population. In the clinical setting, medical investigations will start with a disease, with symptoms from a patient. A clinical diagnosis will start to emerge as other patients appear with the same symptoms. Usually a diagnosis is a relief to the patient, but it does not necessarily explain what the problem is. However, as a clinical diagnosis, a disease, is established, the search for a cure or a relief of symptoms commences. If a treatment for similar ailments exists this will be employed, and could often prove to be helpful. For some diseases, there is no need for deeper investigations into causes, if the treatment is effective and curative. This is however seldom the case, usually temporary relief of symptoms can be offered, but to find a cure one also needs to find what is causing the disease.

On the other side of the spectrum of medical research is the study of the functioning organism. How can we ever describe a flaw in the system, when we do not know how it works when healthy? The human genome project has uncovered the human genes, the blueprint of our existence. Genes code for proteins which are the workers of life. They carry out the tasks that are needed to keep the body at health, and they do it in the context of the cell. The cell is a perfect machine in itself. It is self-repairing and usually renewable. In simpler forms of life the cell *is* the organism. In complex beings like ourselves the cells are cooperating and specialize into tissues which seemingly work as units within the body. But let us not forget that the cell is still the basic unit of life. If dysfunction arises here, it will cause disease for the whole organism.

Ulcerative colitis is a diagnosis which is based on certain clinical findings such as colonic inflammation and bloody diarrhea. It is grouped together with Crohn's disease as inflammatory bowel disease. The two diagnoses are often difficult to differentiate and there are no clear molecular markers for either one. Treatment is aimed at attenuating the inflammation and when this is unsuccessful, surgery can be the last resort. The cause of disease has not been proven, and there is no pharmacological cure.

Current studies of ulcerative colitis aim at finding the faulty part, and when this is established, mending it to ultimately cure the disease. My project started with a gene expression study of colonic biopsies from ulcerative colitis patients. We aimed at elucidating the changes in protein levels in the diseased colon, and to find new potential genes that could shed more light on the pathogenesis of disease.

We confirmed results from previous reports where dysregulated genes were found with function in inflammation, epithelial barrier function, cell cycle and metabolism. Our study also describes a small subgroup of genes in membrane trafficking. Additionally 11% of the found genes were without described function, and we selected one for

further study, mostly due to its similarity to WASP, the mutated protein in the immunodeficiency disease Wiskott-Aldrich syndrome.

We called this interesting new player WASP and FKBP-like, WAFL. However, when there was no obvious direct connection to inflammation, our efforts were to reveal its biological function in attempt to further understand disease development in UC. In allegory, WASP is important in the fundamental cell function of actin polymerization and when dysfunctional, causes severe immune impairment, clearly demonstrating that cell biology is the basis of an organism's health.

Investigations led us to place WAFL in the functional context of the cytoskeleton and endocytic transport. We also found an intriguing localization to internalized bacteria. These biological processes are all interconnected and relevant in the pathogenesis of ulcerative colitis. The story of WAFL is a clear example of how a clinical problem points to disturbances at the core of a cell's machinery.

The following sections are aimed at giving the background to the setting in which WAFL operates. The focus is mainly on processes in vertebrates, since WAFL does not appear to have any homologues in lower organisms.

1.1 THE CELL

Cells in multicellular organisms come in many shapes and flavors. Differentiation into specialized cells requires different cell morphology and different activities. Some cells, like activated macrophages, exhibit highly motile behaviour, while others, such as epithelial cells of the intestine, fulfill their function partly by adhering tightly to their neighboring cells. However, in order to live and fill their purpose they need a functioning infrastructure, the cytoskeleton. Whether it is about processes common to all cells, like organelle positioning or maintaining cell shape, or more specialized processes like transcytosis, they ultimately rely on the cytoskeleton. The eternal companion of the cytoskeleton is the cell membrane in all its different variants, creating the cellular compartmentalization that is intrinsic to eukaryotic life forms.

1.2 THE CYTOSKELETON

The cytoskeleton gives the eukaryotic cell its shape and is also the framework that enables it to execute its functions. Indeed, it is working both as the "skeleton" and as the "muscles" of the cell: networks of protein filaments extend throughout the cytoplasm and create organization among the organelles and cellular compartments. Movements of the cell, as well as components within it, also depend on the cytoskeleton. There are three major components of the cytoskeleton: the microtubules, the intermediate filaments, and microfilaments. These long filaments are all made up of smaller subunits that join by polymerization: thousands of subunits assemble into long threads that can stretch from one side of the cell to another.

1.2.1 Microtubules

Microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport. They also have a crucial function in cell division where they form the mitotic spindle that segregates the chromosomes.

Tubulin polymerization
Microtubules are about 25-nm thick, polar hollow tubes with their walls made from heterodimers of the α- and β-tubulin isoforms (Nogales et al., 1999). Polymerization is GTP-dependent. There are cycles of growth and shrinkage, known as dynamic instability (Gardner et al., 2008; Mitchison and Kirschner, 1984). The dynamic behavior of microtubules is modified by interactions with microtubule-associated proteins (MAPs). Some MAPs stabilize microtubules by capping their ends and some destabilize microtubules by severing them or increasing the rate of depolymerization.

Microtubule-organizing center
In animal cells most microtubules extend from the centrosome which serves as the microtubule-organizing center which is localized next to the nucleus. In mitosis the microtubules form the mitotic spindle that organizes the alignment of duplicated chromosomes at the metaphase plate (Walczak and Heald, 2008).

Kinesins and dyneins
Microtubules mediate intracellular transport and positioning of membrane vesicles, macromolecules and organelles. The movement along microtubules is achieved by the action of two large families of motor proteins: the kinesins and the dyneins. Most kinesins move toward the plus end (in general towards the cell periphery) and dyneins toward the minus end (toward the cell center). The molecular motors possess two ATP hydrolyzing motor domains that step progressively in a hand-over-hand fashion on the microtubule track (Ross et al., 2008).

1.2.2 Intermediate filaments

The intermediate filaments mainly confer mechanical strength to the cell since they offer cushion against tensile forces. They are unique to metazoans, in contrast to microtubules and microfilaments. Intermediate filaments provide the support against mechanical stress that in plants and fungi is provided by their cell wall (Herrmann et al., 2007). Intermediate filaments are unique in that they are both strong and highly flexible and it has been shown that they can be stretched by 250-350% before they break (Kreplak et al., 2005). The intermediate filaments are organized into extensive cytoskeletal networks that span, and possibly connect, the cell surface to the nucleus. At the cell surface intermediate filaments are linked to the supporting extracellular matrix via desmosomes, hemidesmosomes, focal adhesions and linker proteins (Capetanaki et al., 2007; Green and Simpson, 2007).

Intermediate filament structure
Intermediate filaments display an enormous diversity compared to microfilaments and microtubules. This is shown on gene level as well as in filament formation and tissue expression. In humans, there are at least 65 genes coding for intermediate filament proteins, and they show great sequence diversity (Schweizer et al., 2006). The

expression of them is tissue-specific and developmentally regulated, and the filaments are apolar hetero- or homopolymers of these subunits (Goldman et al., 2008).

1.2.3 Microfilaments

Microfilaments are thin, flexible fibers made up of polymerized actin. Actin is a very abundant protein in all types of eukaryotic cells. The filaments are around 7 nm in diameter and up to several micrometers in length. They are cross-linked into networks or bundles. In most cases a shell of microfilaments supports the plasma membrane and bundles of filaments, stress fibers, span through the cell starting from the focal adhesion points where the cell is attached to its environment. When the cell is moving, actin is used to generate membrane protrusions at the leading edge. Typically one differentiates between two types of membrane protrusions: lamellipodia, which are broad and sheet-like and made up a network of actin, and filopodia (also known as microspikes), thin fingerlike protrusions supported by bundled actin fibers. In muscles, the sliding of myosin filaments along microfilaments is the basis for muscle contraction.

Actin polymerization
Actin is a polar molecule that comes in six isoforms in mammals, divided into three subclasses: α, β, and γ (Vandekerckhove and Weber, 1978). The expression of the different isoforms varies between cell types, and the isoforms also have different polymerization kinetics. However, the significance of this diversity is not entirely clear (Sheterline et al., 1995). Actin polymerization is ATP-dependent and can occur spontaneously under physiological conditions, thus forming bi-helical, polarized filaments *in vitro*. One end grows faster than the other and is referred to as the barbed, or plus end, and the slower one is the pointed, minus end (Pollard and Mooseker, 1981). *In vitro* the assembly at the plus end and the disassembly at the minus end can lead to a dynamic phenomenon known as treadmilling, when no net change in filament length occurs (Wegner, 1982).

In the cell actin turnover is enhanced by about 150-fold, compared to *in vitro*, as it is actively used in cellular processes (Didry et al., 1998) This is mediated by the action of actin-binding proteins that sever filaments, enhance subunit disassembly from the pointed end and facilitate ATP-for-ADP nucleotide exchange on free actin subunits to enable new polymerization. The rate-limiting step, the *de novo* actin polymerization, relies heavily on accessory proteins, since it is unfavorable *in vitro* (Sept and McCammon, 2001). Proteins that can initiate actin polymerization are called actin nucleators. The Arp2/3 complex was the first to be identified and is still the most studied.

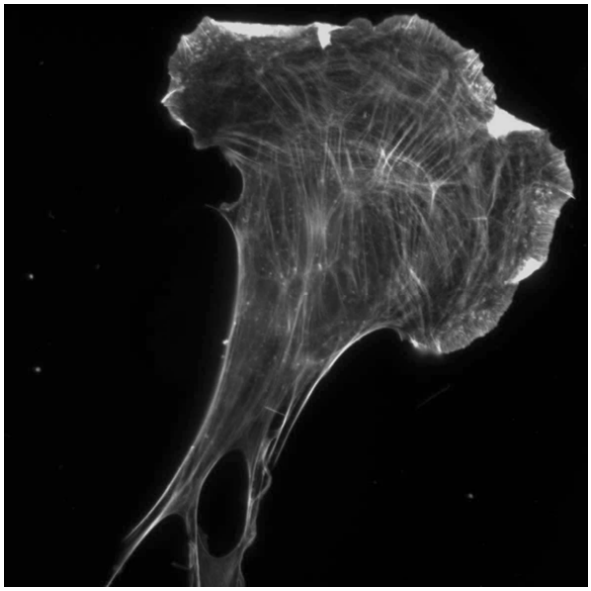

Figure 1 *Microfilaments create the lamellipodia that sense the environment as the cell moves forward. A mouse embryonic fibroblast was stained with phalloidin for actin.*

Arp2/3 complex

The Arp(Actin-related protein)2/3 complex consists of the stable assembly of two actin-related proteins (Arp2 and 3) and five accessory polypeptides (Mullins et al., 1998). They nucleate actin, i.e. initiate the start of polymerization, by mimicking the structure of two bound actin subunits, and then binding a third actin molecule. The activation of the Arp2/3 complex is primarily controlled by the WASP-family of actin regulators (see section 1.3) (Higgs and Pollard, 2000). The Arp2/3 complex has also been reported to bind to the side of pre-existing microfilaments and induce the formation of a branched, daughter filament (Mullins et al., 1998). The complex is then left incorporated at the pointed end of the filament. The branched filaments made by the Arp2/3 complex are suggested to be especially abundant in the lamella where they would function as the mechanical force that pushes the membrane forward (Pollard and Borisy, 2003). However, the existence of branched actin has been questioned. In a recent paper Koestler *et al* propose a different model whereby the actin meshwork seen at the lamella is made up of individual filaments at an angle to the front (Koestler et al., 2008). There are also indications that branched actin could be invoked by the use of the F-actin binding drug phalloidin when processing the cells (Mahaffy and Pollard, 2008).

Myosin motors

The cell takes advantage of the actin filament tracks for movements of various cargo using ATP-dependent myosin molecular motors. The human genome contains nearly 40 myosin genes, divided into 12 different classes. In general, the myosins form two-headed dimers which move progressively in a hand-over-hand fashion. Most myosins

move toward the plus end, but there are also minus-end-directed motors such as Myo6, and bidirectional such as Myo9b (Krendel and Mooseker, 2005).

1.3 WASP-FAMILY OF ACTIN REGULATORS

1.3.1 Wiskott-Aldrich syndrome

The Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive disease. Its is characterized by severe immunodeficiency and microthrombocytopenia (a decreased number of small platelets) (Aldrich et al., 1954; Wiskott, 1937). Symptoms include manifestations such as eczema, internal bleedings, recurrent infections, increased risk of autoimmune disease, and B-cell lymphomas. The only proven cure for the disease is bone marrow transplantation (Dupuis-Girod et al., 2003; Shcherbina et al., 2003; Sullivan et al., 1994).

Mutations in WASP
In 1994 the mutated gene was identified and appropriately named Wiskott-Aldrich syndrome protein (WASP) (Derry et al., 1994). WASP is only expressed in cells of hematopoietic origin (Parolini et al., 1997), and was found to be important for regulating the actin cytoskeleton downstream of the Rho GTPase Cdc42 (Aspenstrom et al., 1996; Kolluri et al., 1996; Symons et al., 1996). Defects in actin-based structures can be seen in leukocytes from patients. Functionally the deficiency of WASP leads to disturbed localization, activation, and function of leukocytes which severely impairs the immune system. Lymphocytes and antigen-presenting cells cannot migrate to their site of action, and cell-cell signaling at the immunological synapses is unproductive, as shown for T-cells (Dupre et al., 2002), and dendritic cells and NK-cells (Borg et al., 2004).

The WASP knock-out mouse exhibits WAS-like disease, with few and abnormal hematopoietic cells and T-cell signaling defects. Interestingly, they also have colitis with a Th2-cytokine profile that closely mimics that of ulcerative colitis. Colonic inflammation has also been reported in WAS patients (Nguyen et al., 2007).

1.3.2 Actin regulators via the Arp2/3-complex

WASP is the founding member of a family of actin regulators that act via the Arp 2/3-complex. There are five members which are divided into two subgroups: the WASPs; WASP and Neural (N-) WASP; and the WAVEs (WASP family verprolin homologous) also known as SCARs (suppressor of cAMP receptor): WAVE1, WAVE2, and WAVE3. They consist of ~500 amino acid residues and share similar molecular organization. The main structural difference between the groups is in the N-terminus. WAVE proteins lack the WH1 and the GBD domains of WASP and N-WASP; instead they exhibit a SCAR homology domain (SHD) in the N-terminus (Bear et al., 1998; Miki et al., 1998b; Suetsugu et al., 1999).

The WASP family of proteins is today recognized as scaffold proteins that convert signals coming from up-stream events to actin polymerization by the Arp2/3 complex. WASP/WAVE proteins will start the process by activating the Arp2/3 complex and supplying an actin monomer. This event is central in cellular events such as membrane

trafficking, podosome formation, cell adhesion, pathogen entry, neurite extension and spine formation (Takenawa and Suetsugu, 2007).

1.3.3 WASP and N-WASP

N-WASP was originally identified in neural tissue, but is now known to be ubiquitously expressed, whereas WASP is exclusively expressed in hematopoietic cells (Miki et al., 1996).

Protein architecture
WASP and N-WASP share a high sequence homology. In the very C-terminus they have the verprolin-homology domain (V, also called WASP-homology-2 domain, WH2), the cofilin-homology domain (C, also known as central domain), and the acidic (A) domain. These are collectively referred to as the VCA module, and it is responsible for the binding of the Arp2/3 complex and one molecule of monomeric actin, thus constituting the region that directly enables actin nucleation (Machesky and Insall, 1998; Machesky et al., 1999). The main structural difference between the two WASP-proteins is that N-WASP has two WH2 domains whereas WASP has one. There are conflicting opinions about the functional significance of this. One study found that it was inducing the faster rate of Arp2/3-induced actin assembly of N-WASP (Yamaguchi et al., 2000), but was later contradicted (Zalevsky et al., 2001). It is believed that the activity of the VCA module in WASP and N-WASP is regulated via an autoinhibitory loop where it folds back onto the N-terminus, and it is thus masked for actin and Arp2/3 (Rohatgi et al., 1999).

The other four domains of WASP and N-WASP bind various partners and regulate the actin polymerization in response to up-stream signaling events. In the N-terminus there is a Wiskott-Aldrich homology 1 (WH1) domain, followed by a basic region, a GTPase-binding domain (GBD, also known as CRIB-domain, Cdc42 and Rac interactive binding) and an extended proline-rich region (see Figure 2).

Activation by Cdc42 and PtdIns(4,5)P_2
The autoinhibitory loop is opened up when activated GTP-bound Cdc42 binds to the GBD (Miki et al., 1998a; Rohatgi et al., 1999). In this way Cdc42 controls the actin-polymerizing activity of WASP and N-WASP in response to extracellular signals such as growth factors. Cdc42 is a small GTPase that induces filopodia (Kozma et al., 1995; Nobes and Hall, 1995). There are data indicating that WASP also weakly binds to another small GTPase, Rac1, traditionally associated with lamellipodia (Aspenstrom et al., 1996).

Additionally, binding of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to the adjacent basic region aids in opening up the folded-back configuration (Higgs and Pollard, 2000; Prehoda et al., 2000; Rohatgi et al., 2000). PtdIns(4,5)P_2 is located in the cell surface plasma membrane and recruits and regulates many actin-binding proteins besides WASP and N-WASP, such as gelsolin, cofilin, vinculin and ezrin (Janmey and Lindberg, 2004).

The polyproline domain will bind to SH3-domain-containing protein such as Grb2, WISH, and Nck, and this will also lead to Arp2/3-activation. However, the precise mechanism remains unclear and there are many potential binding partners at this site

(Carlier et al., 2000; Fukuoka et al., 2001; Rohatgi et al., 2001). For example, a large number of membrane-deforming proteins, known as F-BAR/EFC proteins, have recently been shown to interact with N-WASP via the SH3-domain, thus coupling membrane dynamics and actin polymerization (Takenawa and Suetsugu, 2007).

The WH1 domain

The WH1 domain interacts with three proteins collectively known as the verprolins: WASP-interacting protein (WIP)(Ramesh et al., 1997), corticosteroid and regional expression-16 (CR16)(Ho et al., 2001), and WIP-related (WIRE, also known as WIP- and CR16-homologous protein (WICH))(Aspenstrom, 2002; Kato et al., 2002). The WH1 domain is often classified together with the Ena-VASP-homology-1 (EVH1) domain due to structural similarity (Callebaut et al., 1998; Symons et al., 1996). However, the binding of verprolins to the WH1 domain is exclusive and the molecular binding motifs are specific (Peterson et al., 2007; Zettl and Way, 2002).

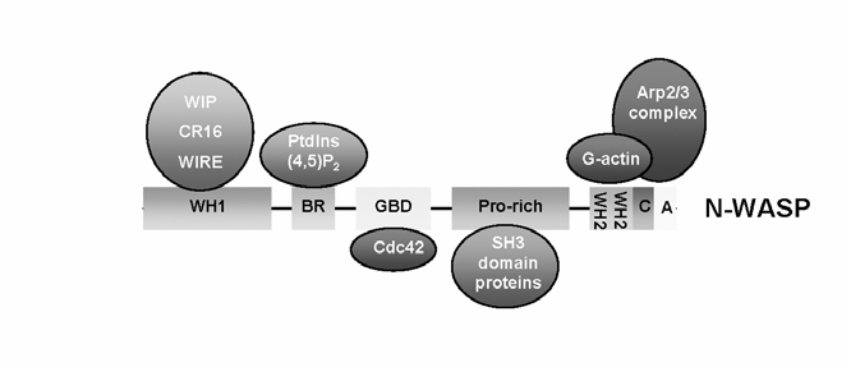

Figure 2 *N-WASP with its interacting partners. Adapted from (Bompard and Caron, 2004; Takenawa and Suetsugu, 2007)*

1.3.4 The verprolins

There are three actin-regulating proteins that constitute the mammalian verprolin family that bind to WASP and N-WASP in the WH1 domain: WIP, CR16, and WIRE (Aspenstrom, 2002; Ho et al., 2001; Kato et al., 2002; Ramesh et al., 1997; Weiler et al., 1996). They all bind to both filamentous (F-) actin and to monomeric (G-) actin (Ho et al., 2001; Kato et al., 2002; Martinez-Quiles et al., 2001). WIP and WIRE are ubiquitously expressed, whereas CR16 expression is restricted to brain, heart, lung, and colon (Aspenstrom, 2002; Kato et al., 2002; Ramesh et al., 1997; Weiler et al., 1996). They are all homologous to the *Saccharomyces cerevisiae* Verprolin, initially identified as a regulator of actin filament formation and endocytosis (Donnelly et al., 1993; Munn et al., 1995). Verprolin binds to the yeast WASP homologue Las17/Bee1, showing that the verprolin-WASP interaction is evolutionary conserved (Naqvi et al., 1998).

WIP and WASP

WIP is the member which has been most extensively studied. It binds to the WH1 domain via a C-terminal WASP-binding domain (WBD) (Peterson et al., 2007; Volkman et al., 2002). It has been estimated that more than 95% of WASP in lymphocytes is bound to WIP (Sasahara et al., 2002), and most of the mutations

associated with the Wiskott-Aldrich syndrome are located to the WH1 domain, suggesting that the WASP-WIP complex is essential for immune function, possibly since the complex appears to protect WASP from protease degradation. (Lutskiy et al., 2005).

Other functions of WIP

Also N-WASP is mostly found in a complex with WIP (Martinez-Quiles et al., 2001). WIP interaction keeps N-WASP in the autoinhibited state and stabilizes the protein. In this way WIP acts as an inhibitor of N-WASP activity, however, in other aspects WIP seems to promote microfilament formation: overexpression of WIP in lymphoid cells induces actin polymerization and cell surface projections (Ramesh et al., 1997), and microinjection of anti-WIP antiserum inhibits filopodia formation by N-WASP (Martinez-Quiles et al., 2001). Additionally, WIP shuttles WASP to areas of actin assembly in the T cell immunological synapse by binding to adaptor protein CrkL (Sasahara et al., 2002). Moreover, the WIP-WASP complex has been implicated in NK cell function (Krzewski et al., 2006), where WIP also seems to have a function independently of WASP (Krzewski et al., 2008). Indeed, the WASP-independent function of WIP is just beginning to emerge, for instance the inflammatory disorder displayed by WIP -/- mice is more severe than that of WASP-/- mice (Curcio et al., 2007; Snapper et al., 1998), it has also been demonstrated that WIP mutants unable to bind WASP still induce filopodia formation in response to PDGF stimulation (Aspenstrom, 2004). In fact, WIP binds to another Arp2/3-activating protein, cortactin via its SH3 domain, and cooperates in the formation of membrane protrusions (Kinley et al., 2003). The identification of mini-WIP, a splice form that lacks the C-terminal WASP-binding domain, is also suggesting WASP-independent functions of WIP (Koduru et al., 2007).

WIRE and CR16

WIRE and CR16 are the two other verprolin family members. WIRE binds to both N-WASP and WASP at the WH1 domain (Aspenstrom, 2002; Kato et al., 2002). CR16 has so far been shown to form a complex with N-WASP in brain and testis (Ho et al., 2001; Suetsugu et al., 2007), but there are few studies to date investigating CR16. WIRE relocalizes WASP to actin filaments via direct interaction, and ectopically expressed WIRE has been shown to produce filopodia and lamellipodia downstream of PDGF stimulation (Aspenstrom, 2002). It has also been suggested that WIRE is an actin-bundling protein, since ectopical expression will induce thick actin fibers (Kato and Takenawa, 2005). Both WIP and WIRE in complex with WASP have recently been shown to play an important role in chemotaxis of monocytes (Tsuboi, 2006). Further, WIP and WIRE probably have more actin-related functions since they both bind to cortactin, profilin, and Nck (Anton et al., 1998; Aspenstrom, 2002; Kinley et al., 2003; Ramesh et al., 1997).

1.4 ENDOCYTOSIS

Endocytosis is the uptake of extracellular material into the cell by membrane invagination and its internalization in a membrane-bound vesicle. This process controls what enters the cell, and once internalized the cargo is sorted and transported to a suitable cellular destination. Endocytosis is crucial for many biological processes such

as the immune response, nutrient uptake, development, neurotransmission, intercellular communication, signal transduction and cellular homeostasis. Generally, one differentiates between phagocytosis, the uptake of large particles, and pinocytosis, the uptake of fluid and solutes. Pinocytosis is carried out via four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and the poorly defined clathrin- and caveolae-independent endocytosis (Conner and Schmid, 2003).

1.4.1 Entry by pinocytosis

Macropinocytosis enables the cell to sample large volumes of the extracellular milieu. It involves membrane protrusions that are created by actin polymerization that then fuse to form large endocytic vesicles. The internalized material then passes through early endosomes and is either recycled back to the plasma membrane or continues to the late endosomal compartment and lysosomes where degradation takes place.

Clathrin-mediated endocytosis
The major pathway of selective receptor internalization in higher eukaryotes is via small clathrin-coated pits at the cell membrane (Ungewickell and Hinrichsen, 2007). The process occurs constitutively in mammalian cells. It has been estimated that a fibroblast will internalize membrane equivalent of its total surface area in one hour (Bretscher, 1982). Clathrin-mediated endocytosis (CME) involves the concentration of transmembrane receptors and their bound ligands into patches of the plasma membrane. These patches can be 10 to 500 nm in diameter (Heuser, 1980) and they contain clathrin, adaptors and endocytic accessory proteins. It takes from 50 to 150 s for a clathrin-coated pit to assemble and to pinch off as a coated vesicle (Bellve et al., 2006; Merrifield et al., 2005). Clathrin is a tri-legged trimer (a triskelion) which can self-

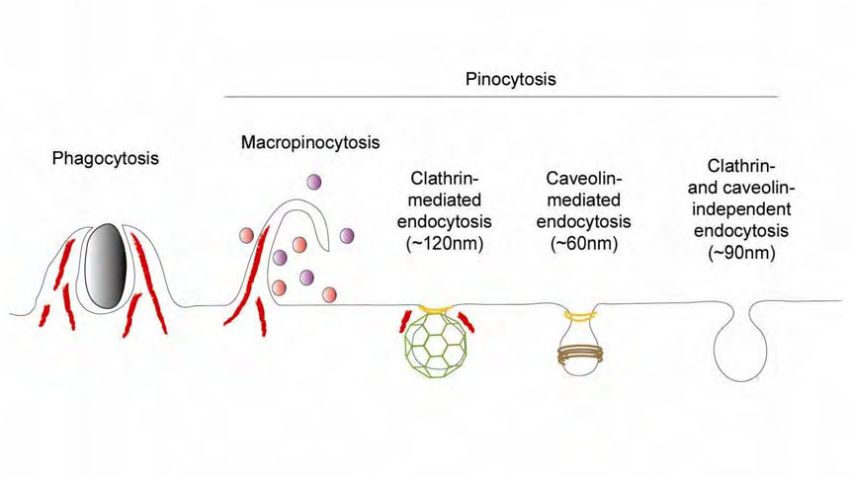

Figure 3 *Overview of the means of entry into the cell. Modified from (Conner and Schmid, 2003)*

assemble into spherical polyhedral lattices (Fotin et al., 2004) thus shaping the invaginating membrane. However, clathrin needs adaptor proteins to connect to the

plasma membrane. The main adaptor AP-2 is a four subunit complex that has a barrel-shaped core and two protruding ear-shaped appendages. AP-2 binds to phosphatidylinositol phosphates, specifically phosphatidylinositol 4,5-bisphosphate in the cell membrane (Collins et al., 2002; Gaidarov and Keen, 1999; Padron et al., 2003). AP-2 will further direct clathrin assembly into curved lattices and couple it to cargo recruitment (Brodsky et al., 2001; Kirchhausen, 1999).

Although clathrin is essential for the invagination of the coated structure, it is not sufficient on its own (Hinrichsen et al., 2006). Accessory proteins such as members of the ENTH and BAR and F-BAR/EFC protein families are known to induce, sense, and stabilize membrane curvature (Ford et al., 2002; Gallop et al., 2006; Masuda et al., 2006). F-BAR/EFC proteins are required for endocytosis of transferrin and EGF (Itoh et al., 2005; Tsujita et al., 2006) and can also recruit N-WASP and thus initiate local actin polymerization.

Actin in endocytosis

The contribution of actin in clathrin-mediated endocytosis has been studied extensively in *Saccharomyces cerevisiae*, where it is needed for vesicle invagination (Kaksonen et al., 2003) but has only recently been appreciated in higher eukaryotes after the advent of fluorescence live cell imaging techniques (Kaksonen et al., 2006; Ungewickell and Hinrichsen, 2007), although it was first indicated already in 1980 (Salisbury et al., 1980). It has been shown that bursts in actin polymerization occur in 80% of the internalizing clathrin-coated pits (Merrifield et al., 2002). However, in contrast to the studies of yeast, in mammalian cells it appears that actin is not required for invagination but rather for the scission of the vesicle from the plasma membrane (Yarar et al., 2005). The main mediator of vesicle scission is the GTPase dynamin which forms a ring around the neck of the pit (Merrifield et al., 2002). Also recruited are N-WASP, Arp2/3 (Benesch et al., 2005), actin (Merrifield et al., 2002) and the actin-binding protein cortactin (Le Clainche et al., 2007). Thus actin could supply the force needed to constrict the neck of the vesicle and enable scission. Alternatively, myosin motors have been located to the vesicle neck, and these could produce a strain by pulling in opposite directions (Ungewickell and Hinrichsen, 2007). As the endocytic vesicle buds of from the plasma membrane it also sheds it clathrin-coat.

Caveolin-dependent endocytosis

In caveolin-dependent endocytosis flask-shaped invaginations known as caveolae are formed in membrane microdomains enriched in cholesterol and sphingolipids (Anderson, 1998). The most abundant structural components are oligomers of caveolin-1 that are believed to shape the caveolae (Fernandez et al., 2002). Dynamin and dynamic rearrangements of microfilaments are also needed in caveolin-dependent endocytosis. However, caveolae differ from clathrin-coated vesicles in many ways: they are very slowly internalized, (their half-time is more than 20 min), the vesicles are small (50-60 nm), and their cargo is different (Conner and Schmid, 2003). Known caveolar ligands are simian virus 40 (SV40) (Anderson et al., 1996; Stang et al., 1997) and Cholera toxin (Orlandi and Fishman, 1998; Parton et al., 1994). The cellular fate of the vesicle after budding, of the caveosome, also seems different than that of clathrin coated vesicles, as will be briefly discussed below.

1.4.2 Sorting for recycling or degradation

Incoming vesicles merge with each other and with pre-existing early endosomes. Early endosomes, sometimes also referred to as sorting endosomes, are tubular-vesicular structures with a luminal pH of ~6.0 (Maxfield and McGraw, 2004). The pH is established via the action of a membrane-bound proton pump, the V-ATPase, which is also present on late endosomes and lysosomes (Galloway et al., 1983). The cargo delivered to early endosomes is either returned to the plasma membrane or passed on to lysosomes via late endosomes. There is also communication of biosynthesized molecules with the trans-Golgi network.

The major sorting task during endocytosis is to separate membrane from contents in the incoming vesicles. Since large quantities of membrane are constitutively internalized, there must be an efficient process to return this to the cell surface. Receptors that are positioned in the membrane can also be recycled, after their ligands have been dissociated due to the lower pH in the early endosomes (Pattni and Stenmark, 2006).

Recycling routes
Cargo destined for recycling can return to the plasma membrane via two routes, either rapidly from early endosomes, or via recycling endosomes. Recycling endosomes are highly tubular, perinuclear structures that rarely contain fluid-dissolved macromolecules (Mellman, 1996; Yamashiro et al., 1984). The “recycling part” of early endosomes and recycling endosomes has a pronounced tubular architecture with a high membrane-to-volume-ratio, thus ensuring enrichment of membrane over soluble content. Rapid recycling of receptors and membrane by budding takes place from the early endosomes (3-4 min) while receptors that reach recycling endosomes require longer time to return the plasma membrane (20-30 min) (Hopkins, 1983; Sheff et al., 1999). The recycling is controlled by the small GTPases Rab4 in early endosomes and Rab11 in recycling endosomes (Zerial and McBride, 2001). Indeed, the majority of receptor recycling, probably more than 65%, goes straight from early endosomes to the plasma membrane (Sheff et al., 1999).

Transcytosis
Transcytosis is a specialized form of recycling in polarized cells where cargo is shuttled between the apical and the basolateral surface. These cells control selective recycling by recognizing the same cytoplasmic and luminal sorting signals on basolateral and apical proteins as is used in the secretory pathway (Matter et al., 1993) (Aroeti and Mostov, 1994; Mellman, 2006). Most recycling will be directed to the original surface compartment, but there is also the transcytic delivery of cargo such as immunoglobulin receptors in epithelia (Maxfield and McGraw, 2004).

Endosome maturation
Early endosomes accept incoming vesicles for about 5-10 minutes, then they lose their tubular extensions, translocate along microtubules and become increasingly acidic. This is part of the maturation process into late endosomes, which also involves acquiring acid hydrolases (Gur et al., 2006; Maxfield and McGraw, 2004). The cargo that remain in the endosomes, such as fluid-phase particles and some receptors, are destined for lysosomal degradation. A means to down-regulate receptors such as growth factor receptors is to target them for degradation after activation and internalization. Mono- or multi-ubiquitination of the cytoplasmic portion of a receptor is a dominant signal for

degradative protein sorting (Haglund et al., 2003; Hicke and Dunn, 2003; Mosesson et al., 2003; Reggiori and Pelham, 2001). However, while luminal content of the endosome is directly accessible to lysosomal degradation, receptors in the membrane require the formation of intraluminal vesicles. These are formed by the sequential action of four distinct protein complexes known as ESCRT (endosomal sorting complex required for transport) 0 to III (Babst, 2005). It starts when Hrs, a constituent of ESCRT-0, is recruited to the endosomal membrane via its binding to membrane component phosphatidyl inositol 3-phosphate, and then recognizes the ubiquitylated receptor (Bilodeau et al., 2002; Misra and Hurley, 1999; Shih et al., 2002). The ubiquitin is shed before budding into the endosome proceeds by unclear actions of the ESCORT complexes (Hurley and Emr, 2006; Williams and Urbe, 2007).

Late endosomes

With the formation of the intraluminal vesicles the early endosome has matured into a multivesicular body (MVB), also known as late endosome. Furthermore, the late endosome has lost the early endosomal key regulatory protein Rab5 and recruited its late endosomal counterpart Rab7. The transition from early to late endosomes is characterized by many fusion and fission events between early endosomes. Gradually degradative cargo accumulates in fewer and bigger endosomes, and the Rab5 to Rab7-conversion then enables fusion events with late endosomes (Rink et al., 2005). There is, however, still controversy in the field regarding the nature of early to late endosome transition. An alternative scenario of early to late endosomal transition would be that the cargo is delivered from early endosomes by transport vesicles that fuse with pre-existing late endosomes (Aniento et al., 1993; Gruenberg et al., 1989).

Lysosomes

There has also been controversy regarding the transition of late endosomes to lysosomes; whether it proceeds via a maturation model or a carrier vesicle model. However, today there is good evidence that late endosomes fuse directly with lysosomes (Bright et al., 1997; Futter et al., 1996; Mullock et al., 1998; Ward et al., 2000). The organelles will transiently fuse, “kissing”, or undergo permanent fusion, where kissing often precedes full fusion. Additionally, contents are sometimes mixed by protruding tubules (Bright et al., 2005). However, little is known about what “prepares” a late endosome to merge with a lysosome (Luzio et al., 2007). After fusion a hybrid organelle is formed which contains all lysosomal enzymes. It is in this compartment that the actual degradation of endocytosed macromolecules takes place. It is probable that lysosomes are, fundamentally, storage compartments for lysosomal enzymes, and that it is with the fusion of late endosomes that these come to use (Griffiths, 1996; Luzio et al., 2007). Lysosomes are then re-formed from the hybrid organelle by maturation processes.

1.4.3 Endosomes as regulators of signaling programs

Early endosomes are important in regulating receptor signaling since they represent the site where the decision to degrade or recycle the receptors is made. However, additional ways in which endosomes are of importance are beginning to emerge. Internalized receptors can still be active, and thus spatial and temporal regulation of signaling can be controlled at the level of the endosome (Polo et al., 2006). Spatial regulation is apparent

in the case of NGF and its receptor TrkA (Howe et al., 2001). If receptor-ligand complex occurs at the tip of the axon, the complex has to be transported by endocytosis to the cell body in order to achieve modification of gene transcription.

Additionally it seems like some receptors require internalization for the signaling to occur. Signaling by the Notch receptor in *Drosophila* requires endocytosis, both for signaling in the Notch expressing cell, as well as for the neighboring cell expressing the transmembrane ligand (Le Borgne et al., 2005; Seugnet et al., 1997).

Some receptors can be internalized by different endocytic routes, and this has been shown to be one way of regulation. The TGFβR is internalized through both clathrin-coated pits and caveolae. CME leads to increased receptor signaling from early endosomes, while the caveolar pathway causes rapid receptor degradation (Di Guglielmo et al., 2003). Routing an activated receptor to clathrin-independent internalization could be an effective way to down-regulate signaling in the presence of excess ligand. The example of EGFR demonstrates this: when stimulated with low doses of ligand, EGFRs are almost exclusively internalized by CME. At higher doses a shift occurs when there is also use of the clathrin-independent pathway, which leads to ubiquitination and degradation (Sigismund et al., 2008; Sigismund et al., 2005).

1.4.4 Regulation of endocytic trafficking

The endocytic system is characterized by constant membrane remodeling, which can be described as continuous fusion and fission events linked into a network in time (Rink et al., 2005). These processes are tightly regulated, and they require the organization of the organelle membranes into functional domains. The above mentioned small GTPases of the Rab family have been recognized as the key regulators of membrane trafficking.

Rabs

There are more than 60 members of the Rab family and they regulate virtually all membrane trafficking steps within the secretory and endocytic pathways, such as formation of vesicles, motility along cytoskeletal filaments and docking and fusion with target membranes (Miaczynska and Zerial, 2006). Rabs are membrane-anchored proteins that cycle between active GTP-bound form and inactive GDP-bound form controlled by GDP/GTP-exchange factors (GEFs) and GTPase activating proteins (GAPs). The active Rabs will interact with specific effector proteins, and thus exercise control of downstream processes. Rab proteins are restricted to specific intracellular compartments and have thus been used as organelle markers (see Figure 4). The Rabs are often located within distinct domains on the same organelle. Examples are early endosomes where cargo destined for degradation is located with Rab5, and cargo which is to be recycled with Rab4 and Rab11. Transferrin, which is recycled, has been shown by light microscopy to first colocalize with Rab5, and then Rab4 and Rab11 (Sonnichsen et al., 2000). Similarly, in late endosomes Rab7-enriched domains direct cargo to degradation in lysosomes whereas Rab9-domains regulate transport with the trans-Golgi network (TGN) (Barbero et al., 2002). Rab proteins exert their effect by a large number of effector proteins and by mediating local changes in membrane composition via interaction with lipid-modifying enzymes.

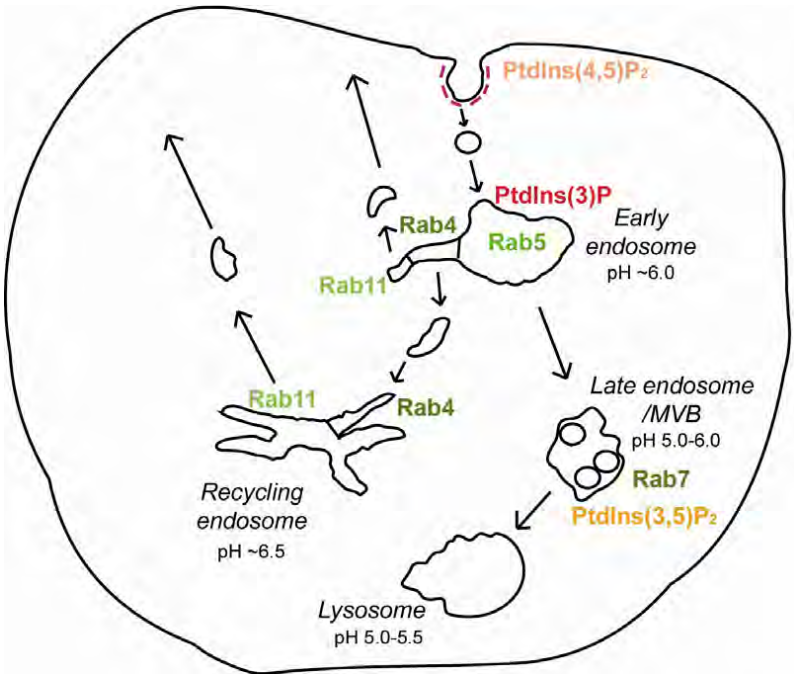

Figure 4 *Overview of the endocytic compartments. Adapted from (Maxfield and McGraw, 2004; Miaczynska and Zerial, 2006; Vicinanza et al., 2008)*

Rab5

One of the best studied Rabs is Rab5 which is located to early endosomes and clathrin-coated vesicles (Bucci et al., 1992). Rab5 has been shown to be able to interact with over 30 proteins (Christoforidis et al., 1999a; Christoforidis and Zerial, 2000), one of its main functions being to control homotypic endosome fusion, and docking of incoming clathrin-coated vesicles (Bucci et al., 1992; Gorvel et al., 1991). Rab5 activates phosphatidylinositol-3-kinase (PI3K) thus initiating the local production of membrane lipid phosphatidyl inositol 3 phosphate (PtdIns(3)P) (Christoforidis et al., 1999b) which further recruits effector proteins such as EEA1 (Simonsen et al., 1998). EEA1 is crucial for endosome tethering and docking (Christoforidis et al., 1999a) via interaction with the SNARE machinery (McBride et al., 1999).

SNAREs

SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) are a family of membrane-tethered coiled-coil proteins that are central players in membrane fusion (Jahn and Scheller, 2006; Martens and McMahon, 2008). These proteins undergo an assembly-disassembly cycle, and when appropriate SNAREs are combined they form a stable α-helical bundle, the SNARE complex (Fasshauer et al., 1997). It is composed of four α-helices that come from three or four different SNAREs, situated on the two opposing membranes (Antonin et al., 2002; Sutton et al., 1998). The assembly of the complex pulls the membranes together, to the point where they can fuse. The SNARE-complex formation releases energy, and it has been proposed that this energy is transduced though the transmembrane domains into the lipid bilayers, thus causing membrane destabilization that enables fusion (Li et al., 2007; Martens and McMahon, 2008). *In vitro*, the appropriate sets of SNAREs in mixed liposomes will

cause membrane fusions, although rather slowly (McNew et al., 2000; Schuette et al., 2004; Weber et al., 1998). However, *in vivo* other molecules probably work in concert with the SNARE complex to trigger the fusion. Different combinations of SNAREs are likely to direct membrane fusions between different compartments, but it has been difficult to unequivocally assign these, especially in mammalian cells (Brandhorst and Jahn, 2006).

Phosphatidylinositol phosphates

Regulation of endosome function relies on recruiting the right effectors to the right place at the right time. This complex task is facilitated by the presence of integral membrane components, such as the phosphatidylinositol phosphates (PIPs). They function as organelle markers that can be changed in order for the membrane compartment to take on a new identity. Phosphatidylinositol (PtdIns) is the basic building block for the PIPs, and the seven different variants are made by reversible phosphorylation on three sites on the inositol ring headgroup (Vicinanza et al., 2008).

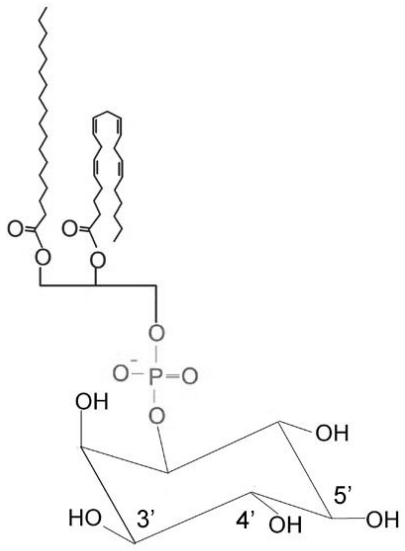

Figure 5 *Phosphatidylinositol. The three possible sites of phosphorylation are in position 3', 4', and 5' on the inositol ring*

Through controlled recruitment and activation of different PI kinases and phosphatases PIPs are transformed, and thus also the membranes that they reside in. PtdIns(4,5)P_2 is found at the plasma membrane where it sequentially promotes recruitment of the coat adaptors, dynamin and other components of the fissioning machinery and finally the actin-based machineries with e.g. N-WASP (see section 1.3.3) (Haucke, 2005). In order for the vesicle to be released into the cell, the PtdIns(4,5)P_2 needs to be dephosphorylated. On early endosomes and in the intraluminal vesicles in MVBs, one finds PtdIns(3)P, which could be produced from PtdIns from the PtdIns(4,5)P_2.

PtdIns(3)P acts as a membrane organizer for the actions of e.g. the above described EEA1 and the ESCORT complexes. Target proteins will bind via PtdIns(3)P-binding domains, such as the FYVE and the PX domains (Stenmark et al., 2002). As endosome maturation proceeds to late endosomes PtdIns(3)P is phosphorylated to PtdIns(3,5)P_2. Additionally, PtdIns(4)P is found at the Golgi complex (Vicinanza et al., 2008). The PIPs work in concert with the Rab proteins (and also other small GTPases), and they exhibit mutual regulatory feed-back loops: PIPs regulate membrane recruitment of GAPs and GEFs by direct binding, and in their turn Rabs control the action of many PIP-metabolizing enzymes (Di Paolo and De Camilli, 2006; Shin et al., 2005; Wenk and De Camilli, 2004). PIPs and Rabs also work as co-receptor in the recruitment of specific cytosolic proteins.

1.4.5 Transport of endocytic vesicles

The movement of endocytic vesicles is either based on microtubules or actin filaments. In animal cells microtubules are used for long-range movement, and actin filaments for short-range transport (Rogers and Gelfand, 2000). Dyneins and kinesins are the molecular motors employed on microtubules, and myosins are used for actin-filament movement. Myosins could drive initial movement through the actin-rich cell cortex before the switch to microtubules. For short-range movements endosomes can also harness the power of *de novo* actin polymerization to propel themselves (Soldati and Schliwa, 2006).

Movement with molecular motors
Both plus-end and minus-end directed molecular motors have a role in vesicle movement, since endosomes are frequently observed to move bidirectionally, in particular in the late endosomal stage (Gross, 2004). The rationale for this "jerking" motion is not clear but several functional explanations have been proposed: to keep the vesicles dispersed in the cytoplasm (Brown et al., 2005), to enable fusion between different compartments (Harrison et al., 2003), or to facilitate fission (Bananis et al., 2003).

The motor proteins can associate with its cargo either directly via non-motor domains or via accessory proteins. The most common mechanism is via scaffolding complexes. Dynein is an example which needs the phospholipid-binding dynactin for cargo association (Schroer, 2004). Rabs have been found to regulate the recruitment of both actin and microtubule molecular motors to membranes (Caviston and Holzbaur, 2006). Bidirectional movement of early endosomes along microtubules *in vitro* was found to depend on Rab5 (Nielsen et al., 1999). For late endosomes *in vitro*, these were seen to move primarily toward the minus end of microtubules and to bind Rab7 (Bananis et al., 2004). Rab5 also seems to regulate switching from actin- to microtubule-based motility. The Rab5-effector HAP40 will recruit Huntingtin to early endosomes which favors interaction with actin rather than microtubules. However, as Rab5-bound GTP is hydrolyzed, HAP40 is released while Huntingtin remains and now recruits binding partners for microtubule-based movement, such as dynein or HAP1 which binds directly to dynactin and kinesin (Caviston et al., 2007; Engelender et al., 1997; McGuire et al., 2006; Pal et al., 2006).

Movement by actin rocketing

The requirement for actin polymerization in the transport of endosomes is beginning to be established, just as it has been in the formation of the endocytic vesicle. It was previously known that invading bacteria such as *Shigella* or *Listeria*, or *Vaccinia* virus, could use the actin polymerization machinery to move intracellularly, displaying "actin comet tails" (see section 1.5.2). But the first observation of tails on the cells own organelles was in 1999 when Frischknecht *et al* in a study of *Vaccinia* tails also observed tail-like actin structures on clathrin-coated vesicles in *Xenopus* egg extracts (Frischknecht et al., 1999). In the same year Merrifield *et al* reported actin tails on endocytic vesicles from mast cells (Merrifield et al., 1999). Since then similar structures have been reported in different systems (Girao et al., 2008; Kaksonen et al., 2000; Taunton et al., 2000). The driving force appears to be by the Arp2/3 complex, and members of the WASP-family have been reported to localize to the vesicles (Benesch et al., 2002; Chang et al., 2003; Taunton et al., 2000). Additionally, it has been shown that the recruitment and activation of WASP and N-WASP to these vesicles involved WIP (Benesch et al., 2002).

1.4.6 Phagocytosis and autophagy

In mammals it is mainly specialized cells such as macrophages and neutrophils that ingest extracellular matter by phagocytosis, but it can also occur in other cells. Internalized objects are for example bacteria or yeast, or cell debris and apoptotic bodies. The mechanism involves specific cell-surface receptors and actin polymerization that enable large membrane extensions that engulf the particle. After the particle is internalized it is confined to a membrane-bound structure known as the phagosome. Just as endosomes, phagosomes sequentially acquire different proteins, such as Rabs, that enable maturation and acidification (Smith et al., 2007). One of the earliest maturation events is the recruitment of Rab5, followed by PtdIns(3)P and then Rab7. Eventually the phagosome fuses with lysosomes for full degradation of its cargo (Kinchen and Ravichandran, 2008). In cells like macrophages and dendritic cells this results in the generation of peptide antigens that are loaded onto MHC molecules and presented at the cell surface (Ramachandra et al., 2008).

When the internalized particle is a bacterium, the phagosomal processing should end in destruction and elimination. However, pathogens have found ways to escape into the cytoplasm (see section 1.5). These bacteria then have to face another line of defense: autophagy. Autophagy is a conserved pathway that sequesters cytoplasmic material and delivers it to lysosomes for degradation. This is important for recycling of nutrients and disposal of organelles. However, this system is also used for degradation of cytoplasmic bacteria, although the triggering signals remain unclear. When a bacterium is targeted for destruction by autophagy the resident autophagy compartment wraps itself around it and elongates until it is sealed into a double-membrane bound structure known as the phagophore. This process is executed by a ubiquitin-like conjugation system controlled by the Atg proteins. Fusion with lysosomes then creates the autolysosome in which degradation is completed (Sanjuan and Green, 2008).

1.5 BACTERIAL INVASION

The sophisticated endocytic system is designed to exert control over the traffic into the cell. However, pathogens have found ways to use it for their own purposes. To evade immunosurveillance, pathogens such as *Salmonella* and *Shigella* enter into cells and then use the cell as a shelter for propagation. In contrast to the phagocytosis by phagocytes, where the bacteria is passive, the pathogen in bacterial –induced phagocytosis is actively manipulating the entry system into cells such as the intestinal epithelial cells of the gut (Cossart and Sansonetti, 2004).

1.5.1 Strategies of entry

There are two main strategies that a bacterium can use to invade a non-phagocytic cell, the zippering mechanism and the triggering mechanism. They both involve initial contact with the host cell followed by membrane protrusions and cytoskeletal remodeling that causes the bacterium to be caught and internalized. In the zippering model the membrane protrusions are less dramatic than in the triggering process. The zippering process also makes use of host endocytic proteins such as clathrin and dynamin, which does not seem to be the case for triggering entry (Veiga et al., 2007).

Zippering entry
Yersinia and *Listeria* use the zippering mechanism. Here transmembrane cell-adhesion proteins are used as receptors for entry. For *Listeria* its two proteins internalinA (InlA) and InlB bind to the host E-cadherin and hepatocyte growth factor receptor (Met) (Cossart and Veiga, 2008). In *Yersinia* the protein is invasin, and it binds to β1-integrin receptors (Fallman and Gustavsson, 2005). The binding leads to Arp2/3-dependent actin polymerization, in the case of *Yersinia* via N-WASP action (McGee et al., 2001) and for *Listeria* probably both via Rac-activated WAVE1 and WAVE2, and Cdc42-activated N-WASP (Seveau et al., 2007). The cell membrane wraps tightly around the bacterium and encloses it into a vacuole. However, it should be noted that the biological significance of *Yersinia* entry in epithelial cells is uncertain. The usual entry route for *Yersinia* in the intestine is via M-cells over Peyer's patches (Shao, 2008).

Triggering entry
In the triggering mechanism the bacteria manipulates the host cell by delivery of effector proteins via the type three secretion system (T3SS). The T3SS is a syringe-like structure that assembles on the bacterial surface and which upon contact with the host cell membrane triggers translocation of bacterially pre-stored effectors (Cornelis and Van Gijsegem, 2000). These effectors instigate major rearrangements of the actin cytoskeleton which manifest in cell extensions raising several micrometers that engulf the bacterium into a large vacuole (Galan, 2001). For *Salmonella*, the bacterial effectors SopE and SptP acts as GEFs and GAPs toward Cdc42 and Rac thus triggering the process (Galan, 2001). For *Shigella* activation of Rac and Cdc42 is indirect via its effectors IpaC and IpgB1. Also, *Shigella* invasion will recruit cortactin in a Src-dependent manner (Nhieu et al., 2005; Rottner et al., 2005).

1.5.2 Intracellular lifestyle

Once inside the cell, the pathogens strategies diverge. The default program of the phagosome is to undergo maturation toward lysosomal degradation, and the bacteria have different mechanisms to avoid this. They can either block the phagosomal maturation and reside and multiply inside the organelle, or they can destroy the vacuole and escape into the cytoplasm (Gruenberg and van der Goot, 2006).

Salmonella stays in the phagosome, and modifies it to become a Salmonella-containing vacuole (SCV). The first step is to produce a phosphoinositide phosphatase, SopB, that preferentially removes the 5-phosphate of PtdIns(3,5)P_2 and the 4-phosphate of PtdIns(3,4,5)$_3$ (Marcus et al., 2001). This leads to sustained PtdIns(3)P production on the SCV, and SopB thereby alters trafficking to prevent further maturation (Hernandez et al., 2004). The SCV is neither a late nor an early endosome (Holden, 2002) and it will transiently acquire Rab5, PI3-kinase, EEA1 and finally Rab7 (Meresse et al., 1999). Shortly after invasion the SCV migrates to the perinuclear region close to the MTOC where it associates with the Golgi (Salcedo and Holden, 2003). This movement takes place via microtubules and the Rab7-interacting lysosomal protein (RILP) which associates with dynein (Cantalupo et al., 2001; Harrison et al., 2003; Jordens et al., 2001). The SCV is then maintained in its position by the action of the effector SifA which via SifA- and kinesin-interacting protein (SKIP) prevents the recruitment of kinesin (Boucrot et al., 2005). SifA is also required for the formation of Sifs, tubular extensions of the SCV of disputed function (Garcia-del Portillo et al., 1993).

Other invasive bacteria such as *Listeria* and *Shigella* escape the phagosome, replicate in the cytosol and move by hijacking the cell's actin polymerization machinery (Cossart and Sansonetti, 2004). Twenty minutes after internalization *Listeria monocytogenes* escapes the vacuole by secretion of listeriolysin O (LLO) which generates transmembrane pores in cholesterol-containing membranes in an acidic environment (Myers et al., 2003; Tweten, 2005). The *Shigella* mechanism of vacuole escape is less clear but appears to depend on the action of effector IpaB (Cossart and Sansonetti, 2004). Once free in the cytoplasm both *Shigella* and *Listeria* take control of the cells Arp2/3-dependent actin polymerization to create force for movement. From one pole of the bacteria surface protein ActA (*Listeria*) or IcsA (*Shigella*) is expressed (Goldberg and Theriot, 1995; Kocks et al., 1993). ActA mimics the C-terminal VCA module of the WASPs and thus directly recruits the Arp2/3 complex and actin monomers (Welch et al., 1998) (Boujemaa-Paterski et al., 2001). IcsA of *Shigella* will instead recruit cellular N-WASP and with it actin and the Arp2/3 complex (Egile et al., 1999; Suzuki et al., 1998). The result is fast intracellular movement of the bacteria, manifest by prominent actin tails, which can generate the force for cell-to-cell spread in the epithelial cell layer. Movement is also a strategy to avoid degradation by the autophagy route. In *Listeria* the rate of movement is estimated to be 10-87 μm/min and in *Shigella* 3-26 μm/min (Stevens et al., 2006).

1.6 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract. Its two major forms are ulcerative colitis (UC) and Crohn's

disease (CD). UC and CD differ in the location and type of inflammation. There is no pharmacological cure for IBD today; treatment is solely to alleviate the symptoms. It is believed to be elicited by an abnormal inflammatory reaction to the resident gut flora in genetically susceptible individuals. Although UC and CD manifest themselves as different diseases, it can clinically be difficult to tell the difference. Therefore a subgroup of the IBD patients are diagnosed as having "indeterminate colitis".

1.6.1 Clinical features

IBD represents a major burden of morbidity in Western countries. The prevalence in North America and Europe range from 21 to 246 per 100 000 for UC and 8 to 214 per 100 000 for CD (Loftus, 2004). The typical disease development for IBD is reoccurring attacks with more or less symptom-free remissions. Although great hope is raised for development of new therapeutic agents, the standard regimen is still corticosteroids for acute flares and 5-ASA for maintenance of remission. Surgery is the only resort for full-blown disease. For longstanding, extensive inflammation in UC patients, there is a significant risk of colorectal cancer, up to 40% lifetime risk (Ekbom et al., 1990).

1.6.2 Disease pathogenesis

The key feature in IBD pathogenesis appears to be an imbalance between microbes and host defensive responses at the mucosal epithelial barrier. Inflammation in CD can include the whole gastrointestinal tract from the mouth to the anus. It is patch-wise and intramural and may give rise to fistulas and stenosis. It is characterized by aggregation of macrophages that frequently form granulomas. The pathogenesis of UC shows a different pattern, the inflammation is mucosal and constrained to the colon; always starting at the rectum and extending proximally in a continuous fashion. Histopathological features include the presence of neutrophils within the lamina propria and the crypts, forming micro-abscesses. Symptoms common to both UC and CD are bloody feces and diarrhea. Some patients also suffer from extra-intestinal manifestations affecting the joints, eyes, skin, mouth, and liver (Bouma and Strober, 2003; Xavier and Podolsky, 2007).

1.6.3 Genetics and gene expression in IBD

Epidemiological studies have revealed that the risk factors for IBD are both environmental and genetic, in an intricate interplay. Studies showed concordance rates for UC in monozygotic and dizygotic twin pairs to be 6-18% and 0-5%. For CD monozygotic twins showed up to 58% concordance while dizygotic twins had no higher risk than for other siblings. These results are indicative of genetic predisposition, especially in CD (Orholm et al., 2000; Thompson et al., 1996; Tysk et al., 1988), and familial clustering further reveal genetic susceptibility (Orholm et al., 1991; Peeters et al., 1996).

Susceptibility genes

Nine IBD susceptibility loci have been identified and confirmed, however only one

gene has been identified with certainty, and this is the Nod2 gene which is an intracellular sensor of bacteria (Muise and Rotin, 2008). Nod2 appears to be specific to CD. On chromosome 5 polymorphisms in the OCTN1 and OCTN2 cation transporter genes have been proposed to be associated with CD (Peltekova et al., 2004). A large number of candidate genes have been found in by linkage analysis or genome-wide association searches and by candidate gene approach, a selection of which are discussed below.

TLR4 is an important sensor of LPS and thus pathogen recognition and activation of innate immunity. It has been shown to be associated with both UC and CD, thus underlining the importance of microbial sensing, as also seen with Nod2 (Oostenbrug et al., 2005). Two genes involved in autophagy have been associated with CD, ATG16L1 and IRGM (Hampe et al., 2007; Parkes et al., 2007; Rioux et al., 2007). Further, IL23R polymorphism has been associated with both UC and CD (Duerr et al., 2006; Tremelling et al., 2007), in addition to other autoimmune diseases such as ankylosing spondylitis and multiple sclerosis (Burton et al., 2007). IL23R has been proposed to be involved in the recruitment and activation of neutrophils and macrophages (McGovern and Powrie, 2007). Early on, the MHC complex was suggested to give an important contribution to IBD susceptibility and this association was again shown in a recent genome-wide association study of UC. Interestingly, this study also implicated the immunosuppressive cytokine IL-10 and ARPC2 in UC susceptibility. ARPC2 is a component of the Arp2/3 complex, thus linking UC to fundamental disturbances in actin dynamics (Franke et al., 2008). Interestingly, Arp2/3 is regulated by WASP, and the mouse WASP knock-out has UC-like colitis in addition to WAS-like disease (Nguyen et al., 2007).

Dysregulated pathways

Global gene expression study is a powerful method to characterize the complex interaction of molecular players in IBD. Investigations of UC gene expression have identified some main dysregulated cellular pathways. These are epithelial barrier proteins, inflammatory and immune mediators, cell fate factors, and metabolism and ion transport. These were replicated in our study by use of the SSH methodology (Paper I). We also identified proteins involved in membrane trafficking as a new small category. Langmann and colleagues have additionally found genes involved in cellular detoxification to be down-regulated in UC, this could be an aspect of down-regulation of genes involved in cellular metabolism and ion transport (Langmann et al., 2004). An early study from 2001 performed with micro-arrays has shown the two IBD manifestations to have distinct, but partially overlapping, gene expression profiles (Lawrance et al., 2001). Important differences include the differential expression of cancer-related genes in UC, consistent with the higher risk of colorectal cancer, as compared to CD.

1.6.4 Bacteria

The microbiota and IBD

The gut mucosal surfaces are characterized by a vast and complex ecosystem of commensal and symbiotic bacteria: 10^{12} bacteria/g feces in the colon, consisting of

more than 400 different species (Farrell and Peppercorn, 2002). The inflammation in IBD is believed to be triggered by an aberrant immune response to the resident gut flora. It is known that IBD patients suffer from a higher colonization of bacteria at the epithelial cell lining and mucous layer (Darfeuille-Michaud et al., 1998; Macfarlane et al., 2004; Schultsz et al., 1999; Swidsinski et al., 2002; Swidsinski et al., 2005). It is also clear that the parts of the GI tract most crowded with bacteria, the terminal ileum and colon, are also those more prone to develop inflammation (Campieri and Gionchetti, 1999). Additionally, antibiotic treatment has been observed to alleviate disease both in UC and CD (Prantera, 2008). The most compelling argument for a bacterial role in the disease comes from rodent models of IBD. Interleukin (IL)-10 deficient mice or chemically treated rats will not develop IBD if raised under germ-free conditions (Rath et al., 1996; Sellon et al., 1998).

Sensing of bacteria

In 2001 Nod2 was identified as a susceptibility gene for CD (Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001a). Nod2 senses intracellular bacteria by binding to the peptidoglycan component muramyl dipeptide, and its binding leads to activation of the pro-inflammatory regulator NFκB (Ogura et al., 2001a; Ogura et al., 2001b). Since then a SNP in the autophagocytic gene ATG16L1 has been associated with CD (Hampe et al., 2007; Rioux et al., 2007), and also a second autophagy gene, IRGM (Parkes et al., 2007). It has been proposed that Nod2 binding to cytoplasmic bacteria targets them for degradation through the autophagy pathway (Sanjuan and Green, 2008). These findings point to a dysregulated response to invasive bacteria, which could then lead to inappropriate immune reactions.

1.6.5 Epithelial integrity

One of the hallmark characteristics of IBD is disruption of the intestinal epithelial barrier, and a defective barrier is speculated to cause a loss of tolerance to the normal enteric flora. Indeed, abnormal intestinal permeability have been found in patients with Crohn's (Arnott et al., 2000; Mankertz and Schulzke, 2007; Wyatt et al., 1997; Wyatt et al., 1993) prior to relapse, an even in some first-degree relatives to Crohn's disease relatives, underlining the importance of the epithelial barrier in disease predisposition (Buhner et al., 2006; Irvine and Marshall, 2000). The apical junctional complexes (AJC) between the epithelial cells that line the intestine are known to be dynamically regulated by cytokines, and expression analysis in IBD biopsies have revealed a downregulation of junctional complexes (Gassler et al., 2001). Further, polymorphisms in several AJC genes have been shown to play a role in the pathogenesis of IBD through disruption of the barrier defense (Muise and Rotin, 2008).

1.7 FK506-BINDING PROTEINS

The FK506-binding proteins (FKBPs) are diverse family of proteins that exhibit various cellular functions such as apoptosis (FKBP38) (Shirane et al., 2008), regulation of steroid receptors (FKBP52 and FKBP51) (Davies and Sanchez, 2005) and stabilization of the ryanodine receptors in skeletal and cardiac tissue (FKBP12 and FKBP12.6) (Chelu et al., 2004). In humans there are 17 FKBPs described, but these proteins are found in all levels of life, also in bacteria. FKBPs can bind to and mediate the effects of the immosuppressant drugs FK506 (Tacrolimus) and rapamycin, and they are peptidyl-prolyl *cis-trans* isomerases (PPIases). These two functions are mediated by the FKBP domain, and seem to depend on the same structural conformation, although the drug-binding is a gain-of-function, probably one of Nature's coincidences (Galat, 2004; Harrar et al., 2001).

2 AIMS OF THESIS

Gene expression studies are powerful tools to get a global picture of pathological processes in cells and tissues. From this starting point our objective was to describe overall protein changes in ulcerative colitis and to find and characterize previous unknown players in the disease. The major finding of this project was the identification and characterization of novel protein WAFL:

Specific aims:

- To investigate the differences in gene expression profile between inflamed vs. non-inflamed biopsies from ulcerative colitis patients.
- To identify novel genes that could be involved in ulcerative colitis pathogenesis
- To functionally and structurally characterize the novel gene WAFL

3 METHODOLOGICAL HIGHLIGHTS

3.1 SUBTRACTIVE SUPPRESSION HYBRIDIZATION (SSH)

SSH is a method to selectively amplify differentially expressed cDNA between two populations, in our case between inflamed and non-inflamed tissue. The two cDNA populations are known as the tester (the inflamed) and the driver (non-inflamed). The tester is divided into two pools that are tagged with different 5'adaptors. Each pool is then mixed with excess driver, denatured and allowed to hybridize. Single-stranded cDNAs will emerge from transcripts that are more abundant in the tester population. This stage functions as a normalization mechanism, since abundant cDNAs tend to homohybridize, with less abundant cDNAs more likely to stay single stranded. cDNAs common to both populations will form heterohybrids.

The two hybridization reactions are mixed and more driver is added (without denaturation). Thus single-stranded cDNA of the two pools can anneal. The resulting tester hybrids will have both kinds of 5'tags. The overhangs that these create are filled in, and these are then the sites for the primers in the subsequent PCR amplification step. Only the templates with both sites for both primers will be amplified exponentially. cDNAs from tester homohybrids will have the same site at both ends, and these will self-hybridize faster than the primers can bind, causing suppression of amplification. As an additional enrichment step the finished PCR reaction is diluted and a nested PCR is performed.

Finally, the sites at the end of the cDNAs are used as sites of restriction digest, and for subsequent ligation into vectors for clone amplification. Both an up- and a down-library were created for each tested patient, and 10 000 clones were picked, amplified by PCR and spotted onto membranes. The original patient libraries were then radioactively labeled and hybridized. Clones of up- or down-regulated in at least 3 out of 8 patients were sent for sequencing.

3.2 PROTEIN EXPRESSION IN E. COLI AND INSECT CELLS

E. coli

E. coli expression of human proteins is an elegant system since it will give high yields at a relatively low cost. The eukaryotic gene is inserted into a plasmid that contains the genetic elements appropriate for bacterial gene expression, such as promoter and ribosomal binding site. As the plasmid is delivered into the bacteria, protein production can be performed on a large scale.

However, the expression of eukaryotic proteins in a prokaryotic host presents some difficulties. If the protein does not fold properly, it will be degraded or it will be insoluble within the bacterium as aggregates in inclusion bodies. If this protein is to be used, it has to be refolded after purification. Large multidomain proteins are usually difficult to fold correctly, since bacterial proteins are normally small.

Another drawback is that *E. coli* cannot do the proper post-translational modifications to eukaryotic proteins. In particular glycosylation is extremely uncommon in bacteria, and recombinant proteins will never be glycosylated in *E. coli.* This contributes to the

problematic folding, and can also impair the functionality of the recombinant protein (Baneyx and Mujacic, 2004).

Insect cells
Insect cells can also be used for protein production, but it is more labor-intense and is not as easily scaled up. But since it is a eukaryotic host it will make proteins at a higher fidelity to the original organism than *E. coli*. For example glycosylation is performed at a style close to the mammalian. In order to achieve high yields of protein, the gene is delivered via a baculoviral system. These viruses are specific to insect cells and will induce high production of the protein.

The insertion of the gene into the viral genome can be achieved in some different ways. One method is known as Bac-to-Bac (Invitrogen). Here the baculovirus DNA is propagated in *E. coli* as a single copy plasmid known as bacmid. These *E. coli* also contain a plasmid encoding a transposase. The gene of interest is cloned into a shuttle plasmid which, as the bacmid, contains sites for transposition. Recombination between the bacmid and the shuttle plasmid will insert the transgene into the bacmid. After purification, insect cells can be transfected with the bacmid to start the first production of recombinant viral particles that make the cells express the transgene (Condreay and Kost, 2007).

3.3 MATERIALS AND METHODS FOR PRELIMINARY DATA

A few results from preliminary/unpublished experiments are discussed in the Results and Discussion section. Here are closer descriptions of these:

Rotamase assay
The rotamase assay uses a purified FKBP (or another PPIase) and a substrate peptide with a proline and a chromophore linked, that can be cleaved by chymotrypsin only when in the *trans* conformation. The cleaved off chromophore can be quantified by spectrophotometric reading. Thus the presence of a functional FKBP will speed up the reaction.

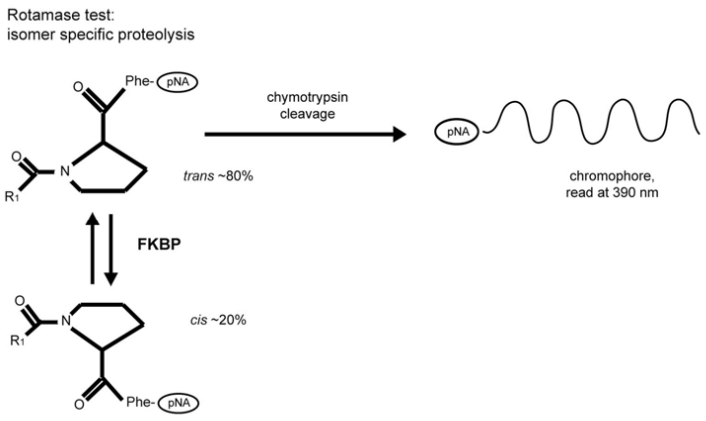

Figure 6 *The rotamase assay*

Of the prolines in the peptide substrates, 20% are normally in *cis* and 80% in *trans* conformation. The prolines naturally flip between the two conformations. PPIases enhances the rate of this flipping. α-chymotrypsin selectively cleaves the *trans* peptides. 80% is then consumed at once, releasing the chromophore p-nitroaniline. This first phase of the reaction is too fast to be recorded. The remaining 20% that were in the *cis* position then flip into *trans* at a fixed rate, being instantly consumed by α-chymotrypsin until all substrate has been consumed, making up the second phase of the reaction. If a PPIase is present, the first phase is still the same, but the kinetics of the second reaction is faster, producing a steeper curve and a faster consumption of the substrate.

α-chymotrypsin was diluted in 0.1M Tris HCl pH 7.8 buffer (2 µl in 200µl Tris buffer /1ml rx) to a final concentration of 2.4µM and aliquoted into plastic cuvettes. Tris buffer, peptide (final concentration 20µM) and PPIase were mixed in eppendorph tubes and incubated for 1h at RT. To start the reaction, the cuvette containing α-chymotrypsin was positioned in the spectrophotometer chamber, the other components were added and the reading performed at 390 nm. The peptides used were ALPF and AAPF. Positive control FKBP12 was used at final concentration 50nM for ALPF and 250nM for AAPF. WAFL peptides were used in final concentrations from 0.5µM to 4µM.

Bacterial invasion

Salmonella typhimurium serovar enterica strain TT16729LT2, kindly provided by Prof Mikael Rhen, was used in these experiments. Bacterial culture was started by inoculating a colony grown on ampicillin-LB plate into fresh LB and left o/n at 225 rpm at 37°C. This culture was diluted 1:10 into fresh LB, tightly capped, and left for an additional 2h at 225 rpm and 37°C. *Yersinia pseudotuberculosis* YIII (pIB102), and plasmid-cured, were a generous gift by Prof Hans Wolf-Watz. A fresh colony was inoculated to an o/n culture in LB with kanamycin at 26°C 225 rpm, and then at 37°C for 2h. *Shigella flexneri* M90T (Sansonetti lab) was grown o/n in TCS broth with spectinomycin and placed on a roller at 37°C. A subculture was then added to fresh TCS broth with spectinomycin and rolled at 37°C for 2h.

Bacteria giving MOI 1:100 to 1:10 were added to cell medium containing 20mM HEPES without FCS or antibiotics. HeLa or SW480 cells transfected with EGFP-WAFL and grown on cover slips were incubated for 5 min to 1h at 37°C with this medium, and when incubated longer it was replaced by complete cell medium with 20mM HEPES containing 50µg/ml gentamicin to kill extracellular bacteria. Cells were fixed in warm 4% formaldehyde, permeabilized in 0.1% Trition X-100, and stained with appropriate antiserum to the bacteria according to standard protocol.

Phosphatidylinositol phosphate binding

A PIP Array (Echelon Biosciences) spotted with the seven kinds of PIPs, and PtdIns, was probed with 10 µg/ml WAFL coiled-coiled His-tagged protein according to the manufacturer's recommendations. Bound protein was detected by immunoblotting with anti-His antibody. Positive control peptide was LL5-α from the manufacturer, which should bind all the PIPs except PtdIns.

4 RESULTS AND DISCUSSION

The WAFL project spans from identification of a novel gene in diseased tissue to its functional characterization *in vitro*. It has been a challenging task, that has demanded employment of many different methods, and left some questions unanswered along the way. In order to fully grasp the magnitude of this project, a number of unpublished results will be presented.

I will discuss the results we gathered divided into two parts. The first is the disease context in which it was identified, and some of our initial attempts of characterization. Then follows the story of the cell biological role of WAFL, and the implications it has on the normal cell machinery. Finally, I will present the theories we have to connect the role of WAFL in the cell, to a putative role in IBD.

4.1 IDENTIFICATION OF WAFL IN ULCERATIVE COLITIS AND INITIAL CHARACTERIZATIONL

(PAPER I: IDENTIFICATION OF A NEW WASP AND FKBP-LIKE (WAFL) PROTEIN IN INFLAMMATORY BOWEL DISEASE: A POTENTIAL MARKER GENE FOR ULCERATIVE COLITIS, AND PAPER II: EXPRESSION AND PURIFICATION OF DOMAINS OF THE WASP AND FKBP-LIKE PROTEIN, A NOVEL MEMBER OF THE WASP FAMILY INVOLVED IN EARLY ENDOCYTOSIS, AND UNPUBLISHED RESULTS)

As UC is still a disease with unknown cause there is a great interest to discover the etiology of the disorder. Additionally, diagnosis is difficult since there is much similarity with CD. There has previously been some success in identifying dysregulated genes in Crohn's disease, notably the breakthrough finding of NOD2, but a similar step forward has not been achieved for ulcerative colitis, although some promising candidate genes have been proposed.

Gene expression in ulcerative colitis (Paper I)

We employed the SSH method in an attempt to characterize the gene expression profile of diseased tissue in ulcerative colitis. This study differs from previous work in two ways: First, paired inflamed and non-inflamed biopsies from the same patients were compared, in order to avoid the background noise that arises from the normal, individual, variation in baseline gene expression. Secondly, the SSH method is an open system where any mRNA that is present in the sample will be processed, in contrast to the predetermined set of genes in microarray experiments. The method is also sensitive to rare transcripts since they are enriched by SSH.

We identified 331 differentially expressed genes, approximately half of them up-regulated. After grouping these into functional groups we found changes in four main categories: cell metabolism and ion transport, cell fate, structure and ECM, and inflammatory pathways. Previous studies have shown similar results, corresponding to the clinical manifestations of inflammation, epithelial barrier disruption, and increased risk of cancer development (Costello et al., 2005; Dieckgraefe et al., 2000; Lawrance et

al., 2001; Wu et al., 2007). The change in cell metabolism indicates disturbances in general epithelial homeostasis. Indeed, for UC, there is generally a repeatable pattern of functional groups that appear in gene expression studies, rather than on the level of individual genes. However, it should be noted that 18 % of the identified genes in our study have previously been reported in UC.

One category is a novel finding by us, which consists of genes functional in membrane trafficking. This group involves genes such as members of the Rab family and sorting nexins. This result is indeed in line with the newly discovered function of autophagy in IBD, although this is so far mainly established in CD.

Identification and initial characterization of WAFL (Paper I)
Eleven percent of the genes were of unknown function. These genes represent interesting new players in a disease where little is still known on the molecular level. One up-regulated gene was especially interesting. Initial bioinformatic analysis revealed the gene to have sequence similarity with both the WASP-family of proteins and the FKBPs. This was a highly intriguing combination since the WASP knock-out mouse is a model for ulcerative colitis (Nguyen et al., 2007), and WASP is the mutated protein in the Wiskott-Aldrich immunodeficiency syndrome. WASP proteins are also manipulated by invasive bacteria for entry and intracellular movement. FKBPs mediate the immunosuppressive effect of the drug Tacrolimus. Tacrolimus is used to prevent graft rejection in transplant patients, but can also be used to treat IBD patients, although this was not the case for any of the patients in our study. We selected this gene for further investigation, and called it WASP and FKBP-like (WAFL) with respect to these sequence similarities.

Quantitative RT-PCR on a new set of IBD patients confirmed WAFL to be preferentially up-regulated in all investigated UC biopsies but not in the CD biopsies. This indicates that WAFL expression is not elevated in inflamed regions in general. Whether the lack of induction in CD or augmented levels in UC represents the normal or pathological response is not clear. The results display the strength of another aspect of our set-up: it was only in comparing inflamed and non-inflamed tissue in the same patients that the up-regulation could be seen. Individual levels varied, and if using pooled samples or normal controls for comparison, it would probably have gone undetected.

Initial investigations into WAFL gene expression on mRNA and protein level revealed a ubiquitous profile, and still left us without clues to the function. Cytokine stimulation in cell culture assays did not affect WAFL expression (unpublished data), however, differentiating monocytes to macrophages induced a 2.5 fold induction. Why would WAFL be expressed in macrophages compared to suspension monocytes? The mature macrophage is very actively sampling the environment and is crawling through tissues. These processes demand active membrane reorganization and microfilament dynamics. These clues foretell the results found in Paper III, where WAFL is placed in the functional context of membrane trafficking and cytoskeletal dynamics.

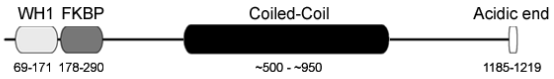

Figure 7 *Domain organization in the WAFL protein*

Structure of WAFL (Paper II)
As WAFL was selected as our candidate gene for further characterization, we also aimed for a structural investigation. WAFL is predicted to have a unique combination of a WH1 domain and a FKBP domain in close proximity in its N-terminus. The structure of these domains have already been resolved but the combination of the two could generate a novel fold, and potentially also give functional clues. However, WAFL is a large protein of 1219 residues with several potential sites for post-translational modifications. This proved to be too challenging for *E. coli* expression of the full-length protein. Attempts to express the full-length protein in a baculovirus/insect cell system also failed due to degradation or aggregation during purification. Nonetheless, deletion constructs of WAFL were successfully expressed, as described in Paper II. Circular dichroism indicated that the expressed FKBP-domain and the central coiled-coil region had the predicted folds, and purification was very successful, yielding 95% pure, folded protein.

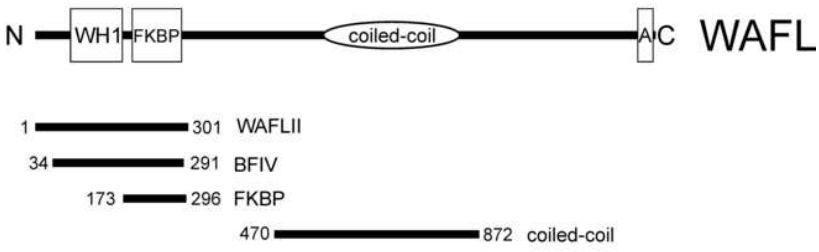

Figure 8 *Four deletion constructs of WAFL have been successfully expressed and purified*

Yet, hitherto attempts to crystallize these purified peptides have been unproductive. They have however, been used for functional tests, one to test whether the FKBP domain has a predicted enzymatic activity (below), and one to test the PIP binding of the coiled-coil domain (see section 4.2).

Functional test of the FKBP-domain (unpublished data)
The purified N-terminal peptides (WAFLII, BFIV, FKBP) were used for a functional assay to test the activity of the predicted FKBP domain. FKBPs are peptidyl-prolyl isomerases (PPIases) also known as rotamases or immunophilins. This means that they enable a proline residue to flip between *cis* and *trans* orientation with respect to the peptide bond. This reaction does occur naturally, but since there is a great energy barrier to overcome it is a rather slow process, typically many minutes, which can be greatly accelerated by PPIases (Lu et al., 2007). The binding of the drugs Tacrolimus

and Rapamycin to the FKBP domain leads to the inhibition of the PPIase activity, although the actions of these drugs do not seem to be related directly to the inhibition.

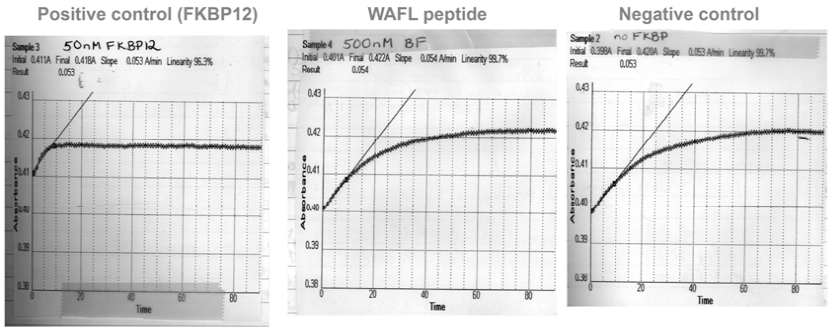

Figure 9 *WAFL does not appear to have PPIase activity, since the kinetics of the reaction is similar to the negative control.*

All three constructs produced that contain the FKBP domain have been tested at various concentrations, and with two variants of the substrate peptide. So far no PPIase activity has been detected. This is in agreement with the results of Nakajima and colleagues in their study of the mouse homologue of WAFL (noted as data not shown in their paper). However, our tests were carried out with truncated WAFL, and it could be that full-length protein or additional cofactors are required for activity. This is the case for FKBP39 that is only functional when bound to a calcium-calmodulin complex (Edlich et al., 2005). A recent example is FKBP36 that will only exhibit PPIase activity after a residue modification (Jarczowski et al., 2008).

4.2 FUNCTIONAL CHARACTERIZATION OF WAFL

(PAPER III: WAFL, A NEW PROTEIN INVOLVED IN REGULATION OF EARLY ENDOCYTIC TRANSPORT AT THE INTERSECTION OF ACTIN AND MICROTUBULE DYNAMICS, AND UNPUBLISHED DATA)

In order to elucidate the function of WAFL, the gene was cloned and fused to the GFP-protein. After transfection, a dotted cytoplasmic staining was seen, similar to the stainings for the endogenous protein.

Localization to intracellular bacteria (unpublished data)
Since no activity could be detected in WAFL's FKBP domain, attention was diverted to the other domain of interest, the WH1 domain, previously only found in the WASP-family of proteins. The WASP-proteins are hi-jacked by pathogenic bacteria for colonization, invasion, and intracellular movement and spread. Since UC is a disease with suspected bacterial involvement, experiments were launched to test if WAFL could also play a part in bacterial interactions. Initial experiments involved colonic epithelial cell line (SW480) that was challenged with *Yersinia pseudotuberculosis* and then fixed and stained for microscopic analysis. We found WAFL to relocalize to the internalized bacteria. It was a sporadic observation among cells of a sample, but it was consistent and reproducible in several experiments. Subsequent experiments involved trials with two other invasive pathogens: *Salmonella typhimurium* and *Shigella flexneri.* These bacteria also resulted in WAFL recruitment, although it was an even rarer event for *Shigella*. The brief, and early, localization to *Shigella* could be because of its quick (a few minutes) escape from the phagosome. However we have so far not been able to find any functional effect of WAFL on bacterial invasion, to date what we have observed is the relocalization.

WAFL localizes to early endosomes (Paper III)
Why would WAFL localize to phagosomes? Could it be that it was the phagosome, and not the bacteria in itself, which recruited WAFL? We speculated whether the WAFL recruitment was part of a normal intracellular membrane localization, since it apparently was not a species-specific event. Phagocytosis is akin to endocytosis where the WASP-family of proteins is also involved. The WAFL dots could then represent endosomes.

By using the EGFP-WAFL construct we could monitor the protein in live cell imaging, revealing that the dot-shaped structures were indeed motile within the cell, indicating a vesicular localization. Co-movement was also seen when cells were fed with fluorescently labeled dextran, as a marker for the endocytic pathway. Initial co-stainings for EEA1, a marker for early endosomes, proved positive, although not complete. There seemed to be more of an association than a perfect overlap. However, this is not surprising, these organelles display a highly mosaic organization of surface proteins into dynamic domains, e.g. proteins such as Rab5 and Rab4 both stain to early endosomes, but in different parts (Miaczynska and Zerial, 2006).

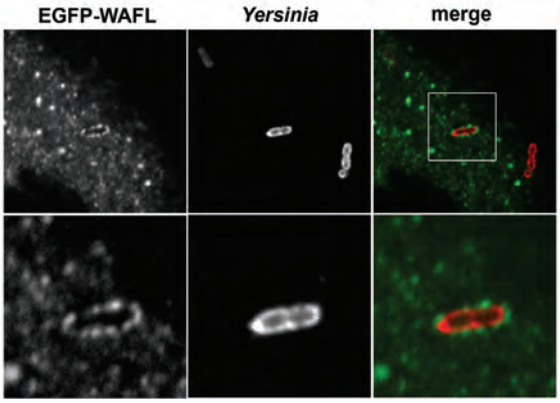

Figure 10 *SW480 cell 2h after Yersinia pseudotuberculosis uptake*

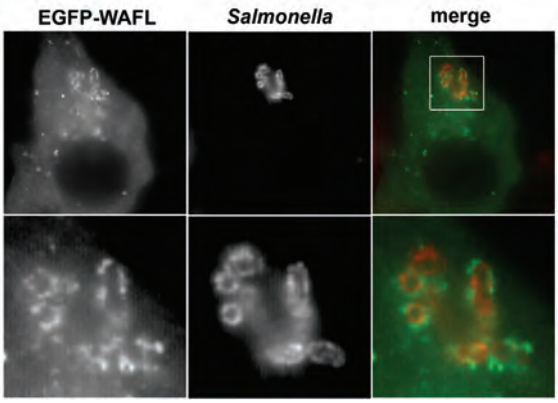

Figure 11 *HeLa cell 2h after infection with Salmonella typhimurium*

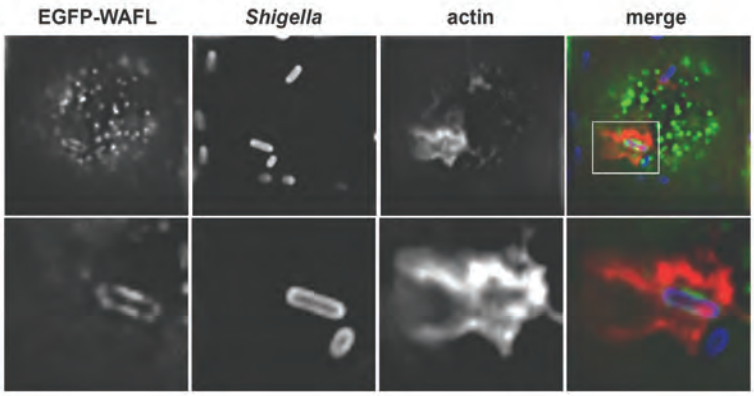

Figure 12 *HeLa cell 5 min after invasion with Shigella flexneri*

Further studies with markers of other stages of the endocytic pathway revealed that WAFL indeed associated with early endosomes. In addition, this association was mathematically verified by an image quantification program in which 30 cells per marker were captured by confocal imaging and assessed in 3D.

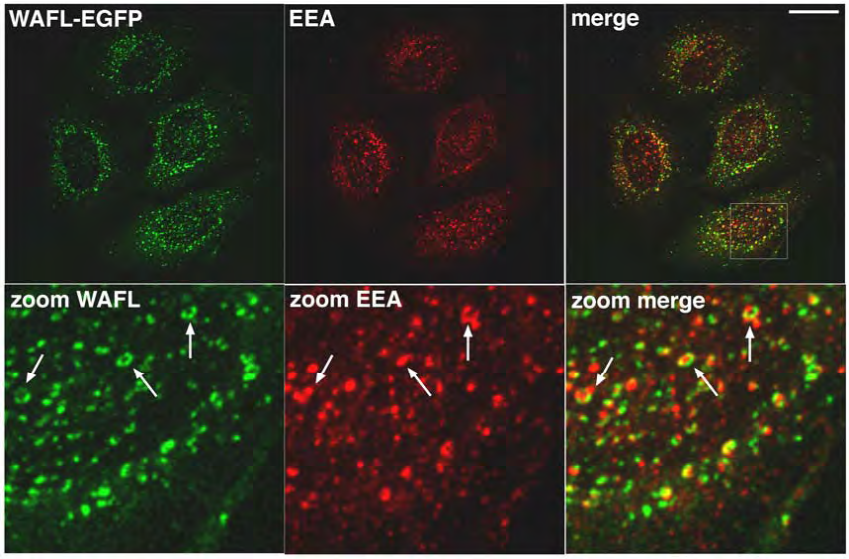

Figure 13 *WAFL and EEA1 display partial co-localization (picture not included in Paper III)*

The coiled-coil domain is essential and sufficient for endosomal localization (Paper III)
We cloned deletion constructs of EGFP-WAFL that enabled us to investigate the necessary domains that interact with endosomes. Just from the subcellular expression pattern of the deletion mutants it was clear that the central coiled-coil region was essential for vesicular targeting. Co-staining for EEA1 confirmed this observation. Indeed, the coiled-coil region in itself was sufficient for endosomal localization.

WAFL depletion leads to disturbed endocytic trafficking (Paper III)
In order to test the functional relevance of WAFL on endosomes, we set up an RNAi-dextran-feed experiment. Cells were fed with fluorescently labeled dextran. The processing of dextran through the degradative route could then be monitored by FACS, since fluorescence is quenched at lower pH. The results consistently showed a higher signal for WAFL-depleted cells after 30 min chase, an effect that was wearing off at 2 h. Our interpretation is that WAFL is not essential for delivery to lysosomes, but is needed for correct processing earlier on, and that the lack of WAFL will cause disturbances.

WAFL and actin and microtubules (Paper III and unpublished data)
The similarity with the WASP-proteins additionally called for investigations into a potential role for WAFL in actin regulation. Early endosomes are known to employ both microfilaments and microtubules for intracellular movement. Therefore parallel

studies into microtubule-associated aspects were pursued. HeLa cells were co-stained for endogenous WAFL and actin, or microtubules. Moreover, live cells expressing EGFP-WAFL were treated with actin or microtubule-disrupting drugs, and monitored before and after treatment, to investigate the mean of transport for WAFL-labeled endosomes. The experiments gave interesting results: WAFL did not show any colocalization with the large stress fibers of the cell, but rather with actin clusters in the central cell region, however, the movement of WAFL structures was not inhibited by the latrunculin A-inhibition, although relocalization was seen towards the middle of the cell. For microtubules a subtle but clear alignment in the orientation of the filaments was seen, and movement was in complete arrest after nocodazol-treatment. These results reflect that the movement of WAFL-labeled vesicles is mainly on microtubules, but that a contribution of actin can not be excluded. An additional result that further indicates WAFL in both of these cytoskeletal systems is the phenotype of RNAi WAFL-depleted cells (unpublished data):

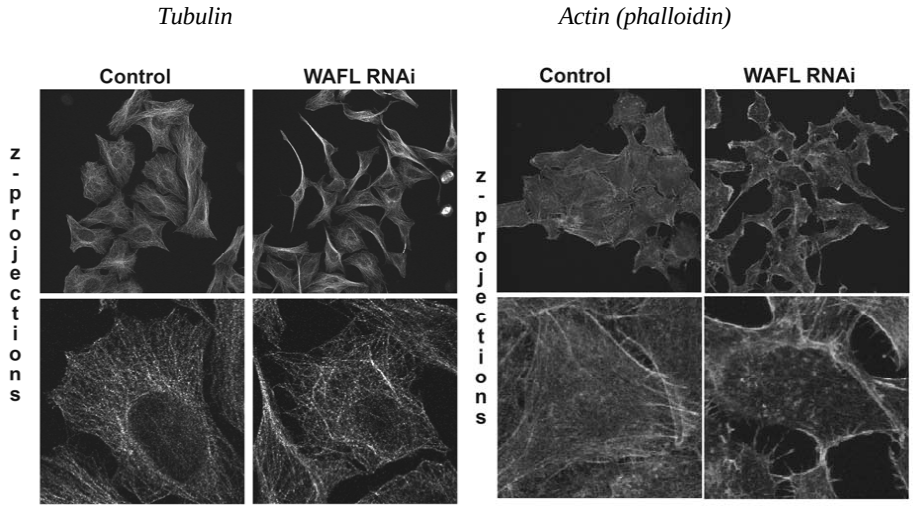

Figure 14 *Phenotype of WAFL-RNAi treated HeLa cells*

The RNAi-treated cells display a highly elongated shape, in itself an indication of a flaw in cytoskeletal function. Staining for actin reveals cells with fewer stress fibers and an increase in central actin clusters, similar to those that WAFL previously was seen to localize with. The microtubule staining is less revealing, but there seems to be some disorganization among the microtubules.

The actin interaction was studied more specifically, since WAFL has a WH1 domain that could potentially bind to WIP. Also binding to actin was assayed, although no apparent actin-binding domain has been identified in the WAFL sequence. By co-transfection and co-immunoprecipitation we did indeed see WAFL-WIP interaction. Deletion mutants of WIP further showed that binding was dependent on the WASP-

binding domain, indicating a similar way of interaction as with WASP and N-WASP. Also actin binding was positive, although one cannot conclude from the IP whether binding was to monomeric or filamentous actin, or if it is a direct interaction, or via another protein, perhaps WIP.

If the WH1 domain of WAFL seems functional, what about the other similarity to the WASP-proteins: the acidic C-terminus. Below is an alignment of this region in WAFL together with the equivalent region in four other proteins in which it is known to bind to the Arp2/3 complex:

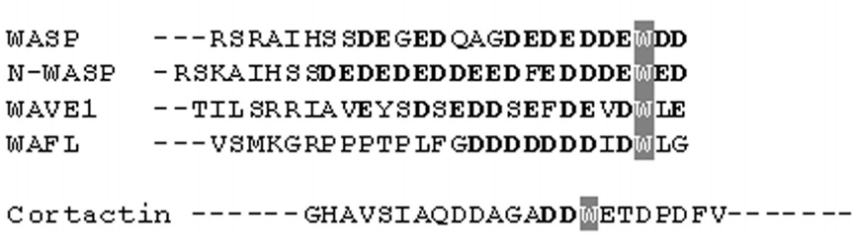

Figure 15 *WAFL C-terminus aligned to the acidic (A) portion of other Arp2/3-binding proteins. Acidic residues are marked in bold and the conserved tryptophan in gray.*

WAFL has a stretch of aspartic acid residues and the third last residue is a tryptophan. These are the basic similarities that unite these regions in the various proteins. Especially the tryptophan is essential for Arp2/3complex binding and functionality (Marchand et al., 2001). However, we have investigated the potential function of this region in WAFL, with negative results. Arp2/3 binding by immunoprecipitation has been tested with negative results, and also direct pyrene-actin *in vitro* polymerization assays with purified c-terminal WAFL was unfruitful. Notably, in the highly similar mouse homologue, alternative splicing has been reported for this very region (Nakajima et al., 2006).

WAFL binds to PIPs (unpublished data)

WAFL was found to interact with endosomes via its coiled-coil region. Membrane anchorage can be achieved via different mechanisms, one is binding to PIPs, which are also very specific markers of membrane organelles. The His-tagged coiled-coil protein described in Paper II was used to investigate this. It was probed onto a membrane spotted with the seven kinds of PIPs and PtdIns. Clear signal was detected in the three monophosphorylated lipids, although weak signal was detected for all lipids except PtdIns and PtdIns(3,4)P_2. The strongest signal appeared with PtdIns(3)P, which is found in the membrane of early endosomes. The WAFL coiled-coil protein also binds clearly to PtdIns(4)P and PtdIns(5)P. PtdIns(4)P is located to the Golgi whereas the localization of PtdIns(5)P remains poorly characterized (Di Paolo and De Camilli, 2006). This is an additional possible arena for WAFL in vesicle transport. Indeed WAFL staining quite often displays a clustering of vesicles around the juxtanuclear space.

Interestingly, the PIP interaction also gives clues to the phagosomal localization that WAFL exhibits. Invasive pathogens frequently manipulate the PIP profile of its

intracellular compartment. This is especially apparent in the case of *Salmonella*, which induces sustained production of PtdIns(3)P by action of its effector SopB (Hernandez et al., 2004).

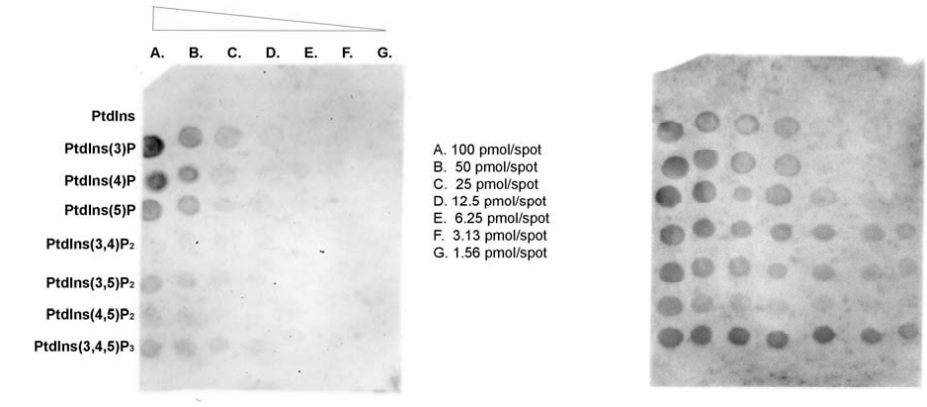

Figure 16 *PIP binding of the WAFL coiled-coil domain (left) and control peptide (right).*

Potential WAFL function

Altogether these results present WAFL as a membrane-binding protein that interacts with early endosomes and phagosomes and affects the proper processing of endocytic cargo. Further, WAFL appears to operate at the intersection of actin and microtubule dynamics. Direct interaction with WIP and actin points to an active role in microfilament regulation, whereas the microtubule connection remains more obscure.

Since WIP is known to bind to both N-WASP and WASP to a very high degree, it is tempting to speculate that WAFL interaction is a regulatory mechanism to sequester WIP. Alternatively, binding could be a means to recruit WIP to endosomes. If WAFL-depletion disturbs the transition of endosomes from actin to microtubule-based transport, this could offer an explanation for the altered delivery to lysosomes seen in Paper III. Perhaps the increase in actin clusters seen in WAFL-depleted cells would be in accordance with this speculation.

	WASP and N-WASP	**WAVE1,2,3**	**WAFL**
Actin-binding	Yes	Yes	Yes
Binding to PIPs	PtdIns(4,5)P_2 (N-WASP)	PtdIns(3,4,5)P_3 (WAVE2)	PtdIns(3)P, PtdIns(4)P, PtdIns(5)P
Organism expression	Homologues from yeast to mammals	In multicellular organisms (e.g. Dictyostelium)	Vertebrates
Tissue/Cell expression	WASP: hematopoietic cells N-WASP: ubiquitous	WAVE1: brain WAVE2: ubiquitous, WAVE3: ubiquitous and high in brain	Ubiquitous
Binding to Rho-family of small GTPases	Cdc42, possibly Rac	Indirectly to Rac (WAVE2)	nd
Effect of knock-out in mice	WASP: immunosuppression and UC-like colitis, N-WASP: embryonic lethal	WAVE1: defects in balance, memory and learning, WAVE2: embryonic lethal	nd
Clinical importance	WASP is mutated in Wiskott-Aldrich syndrome	nd	Up-regulated in ulcerative colitis

Figure 17 *WAFL as a new member of the WASP family. References are in the main text in the introduction, and (Soderling et al., 2003; Yamazaki et al., 2003)*

4.3 ENDOCYTIC PROTEINS IN ULCERATIVE COLITIS

We have shown that WAFL is involved in endocytic transport. Interestingly, 6% of the differentially expressed genes found in our SSH-screen were involved in membrane trafficking. This points to a general involvement of this category of proteins in ulcerative colitis, where WAFL is one component.

There are a number of potential sites of action for these proteins in the disease, and probably they work at several of them. Let's speculate from the starting point of some of the main clinical features of UC: inflammation, barrier dysfunction, and bacterial interaction.

Inflammation is a process that requires vast membrane remodeling. From the motile macrophages and neutrophils that engulf extracellular particles and microbes, to the processing of antigens for presentation at the cell surface on MHC molecules. Defects in apoptotic cell clearance can potentially result in autoimmune disease. Additionally, regulation of response to receptor signaling takes place by membrane remodeling and receptor internalization.

The disruption of the intestinal epithelial barrier is a key feature of IBD. Its integrity depends on the apical junctional complexes (AJC). The disruption of these structures is believed to be initiated by cytokines, and this process has been modeled *in vitro*. The tight junction and apical junction proteins of the AJC are removed from the cell surface by clathrin-mediated endocytosis, and are delivered to a cytosolic compartment that stains for early endosomal markers Rab5 and EEA1 (Ivanov et al., 2004). Dysregulation at the level of the endocytic machinery could potentially cause increased epithelial permeability.

It has been shown that IBD patients have a higher bacterial colonization at the epithelial cell lining and mucous layer. In general it is believed that the inflammation in IBD is response to the normal gut flora due to a dysfunctional epithelial barrier. Also pathogens have been implicated, although it remains unanswered whether they would initiate the inflammation or take advantage of the damaged host. What is clear however is that the host has a very delicate task in balancing tolerance to commensals with an efficient inflammatory response to pathogens. In IBD, this balance is lost. Handling bacteria can be executed by receptors like TLR4 and Nod2 that senses the environment and then signals when bacteria are present. Interestingly, Nod2 is an intracellular sensor, and in intestinal epithelial cells,TLR4 has been shown to require the internalization of LPS for signaling. This is in contrast to macrophages which have cell-surface recognition (Hornef et al., 2003). Possibly this is a reflection of tolerance to the commensals in the intestine. Another feature is the handling of bacteria once they have reached inside the cell, which is executed in phagosomes and autophagosomes. Needless to say both of these strategies are dependent on endomembranes. Genes related to the function of the autophagosome have recently been identified in IBD.

5 CONCLUDING REFLECTIONS

This thesis is a description of a scientific journey that started with an unknown gene sequence from a SSH library, and ends in its functional characterization as WAFL, a protein involved in endosomal trafficking. Every time a new feature of WAFL has been revealed, another ten new questions appeared. Finding a new piece of the puzzle forced us to constantly reconsider and revise our initial hypothesis.

The scientific literature has offered material for much speculation around the role of WAFL, and one of the most challenging tasks has been to select what experiments to prioritize. After many endeavors we have now a glimpse of the function of WAFL. The full potential of the biological and functional relevance of WAFL is still waiting to be revealed, but a lot is to be expected of a ubiquitously expressed, multidomain protein with implications in membrane dynamics. The potential scope of its function is vast.

Endocytosis is crucial to maintain the body and its cells at health. When specific receptor endocytosis is affected particular diseases arise. Examples are familial hypercholesterolemia when the LDL receptor is dysfunctional which leads to early onset of atherosclerosis and an increased risk of cardiac disease. General problems in endocytic trafficking cause other, multiple tissue-diseases. These are often neurological disorders, pigmentation defects, or developmental problems. But also immune dysregulation is reported, as in Griscelli syndrome, where immune impairment arises due to defective secretion of lytic granules by cytotoxic T lymphocytes (Stinchcombe et al., 2001).

WAFL was isolated in the context of the intestinal barrier of IBD. First line defense cells, such as epithelial cells, are constantly under stress imposed by luminal content in the intestinal canal, including high load of gut microbiota. Intestinal epithelial cells already have high membrane turnover due to their absorptive and transcytic function. An inadequate handling of internalized cargo could potentially lead to additional stress signals and activation of inflammatory signaling pathways that disrupt normal barrier function. If this occurs in intestinal epithelial cells, this may render the gut prone to leakage with subsequent invasion of gut microbiota into the lamina propria. As a consequence an unwanted innate immune response will be mounted coupled to prolonged inflammation.

Our SSH expression profile revealed that UC is characterized by differential expression of many metabolic proteins, indicating an imbalanced epithelial homeostasis. A cell in this condition is more likely to accumulate stress signals that can trigger an inflammatory state. In allegory it has recently been shown in a study by Kaser *et al* (Kaser et al., 2008) that deletion of a gene involved in handling ER stress is sufficient to elicit intestinal inflammation in mice.

Membrane trafficking is a central component in the cell machinery. It is therefore tempting to speculate that the chronic inflammation displayed in inflamed tissue from UC biopsies may in part arise from a non-optimal intracellular transportation system. Why WAFL and other endocytic proteins are differentially expressed in ulcerative colitis remains to be investigated. But considering the multitude of potential sites of action, it is likely that more connections are to be expected.

Finally, WAFL function in the body at large is exciting to envision. A functional role for WAFL in brain biology has been proposed. The mouse homologue of WAFL was identified in brain development and was found to affect the growth of axons. In addition, WAFL may also be involved in regulation at the synapse, where correct and timely vesicle shuttling is crucial for neurotransmission. Our observation of checker-board expression of WAFL in heart muscle cells is intriguing and investigations into muscle function are highly warranted. The proposed function of WAFL involvement in intracellular transportation indicates that immune cells regulating phagocytosis may also be of interest to study. Indeed, our data show that WAFL expression can be induced in macrophages.

In conclusion, I believe WAFL to be a candidate for many key cellular processes. It is with pride that I introduce this novel star into the scientific lime light. *Take a bow*.

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