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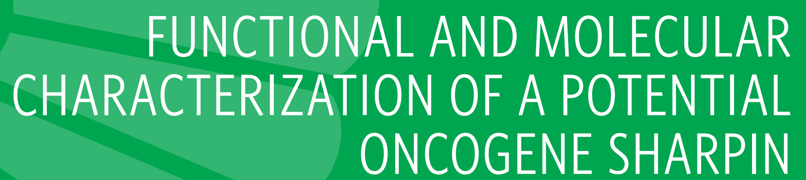

FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF A POTENTIAL ONCOGENE SHARPIN

Meraj Hasan Khan

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To Sana and Rayan

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Functional and molecular characterization of a potential oncogene Sharpin

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ABSTRACT

Sharpin (SHANK-Associated RH Domain Interactor) is a multifunctional adaptor protein that interacts with other proteins and regulates their functions. For example, Sharpin is a subunit of the linear ubiquitination assembly complex (LUBAC), which promotes NF-κB activity. Sharpin also acts as a negative regulator of PTEN, integrins, T cell receptor and Caspase 1.

In this thesis, I report the first interactome of Sharpin. The interactome identified many novel Sharpin interactors and suggested several new pathways regulated by Sharpin. In addition, I confirmed the direct interaction of Sharpin with the Arp2/3 complex, several members of which were in the Sharpin interactome. Importantly, this interaction is physiologically relevant as Arp2/3-dependent lamellipodia formation and cell migration are significantly affected if this interaction is hampered. Previously, Sharpin had been reported to be essential for the last step of granulocyte transmigration; the release of the trailing edge after crossing the endothelium. The role of Sharpin in the early steps of granulocyte transmigration has remained unknown. However, in this thesis, using intravital real-time imaging experiments on postcapillary veins in the cremaster muscles of wild-type (wt) and Sharpin-deficient mice (Sharpin-deficient mice, hereafter also called *Sharpin^{cpdm}* or Sharpin lacking mice), I showed that Sharpin deficient granulocytes are unable to switch from fast rolling to slow rolling and to firmly adhere, thus showing that Sharpin regulates the early steps of transmigration as well.

Keywords: Sharpin, LUBAC, Arp2/3 complex, Linear ubiquitination, Interactome, Integrins, Inflammation, Transmigration.

Meraj Hasan Khan

Mahdollinen onkogeeni Sharpin: molekulaarinen kartoitus ja toiminnallisten sitoutumiskumppaneiden karakterisaatio

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TIIVISTELMÄ

Sharpiini (SHANK-Associated RH Domain Interactor) proteiini toimii monissa solun prosesseissa tuomalla eri proteiineja yhteen samaan kompleksiin. Sitoutuessaan eri proteiineihin, sharpiini säätelee niiden aktiivisuutta. Sharpiini on mm. tärkeä osa NF-κB:n aktivaatiota ohjaavassa LUBAC kompleksissa. Sen lisäksi sharpiini estää sitoutuessaan PTEN:n, integriinien, T solu reseptorin ja kaspaasi-1:n toimintaa.

Väitöskirjatyössäni olen selvittänyt sharpiinin interaktomin, proteiinit, joihin sharpiini sitoutuu. Selvittämällä interaktomin, löysin uusia solun signalointireittejä, joiden säätelyyn sharpiini osallistuu. Samalla vahvistin sharpiinin ja Arp2/3-kompleksin interaktion. Osoitin, että sharpiinin sitoutuminen Arp2/3-kompleksiin on tärkeää liikkuvan solun etureunan, lamellipodian, muodostumiselle ja liikkumiselle.

Väitöskirjani toinen osatyö osoittaa, että leukosyyteissä sharpiinin puutos häiritsee leukosyyttien kulkemisnopeuden säätelyä ja kiinnittymistä muihin soluihin. Tämän tuloksen perusteella voidaan olettaa, että sharpiinilla on tärkeä tehtävä leukosyyttien ekstravasaation alkuvaiheessa. On todennäköistä, että tämä vaikuttaa immuunipuolustuksen toimintaan.

Avainsanat: Sharpiini, LUBAC, Arp2/3 kompleksi, lineaarinen ubikitinaatio, syöpä, interaktomi, integriinit, tulehdus, ekstravasaatio

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ABBREVIATIONS

Arp2/3	Actin Related Protein 2/3 complex
Cpdm	chronic proliferative dermatitis
CD40	Cluster of differentiation 40
CK666	2-Fluoro-N-[2-(2-methyl-1H-indol-3-yl)ethyl]-benzamide
DAPI	4´, 6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERK	Extracellular signal regulated kinase
EYA1	Eyes Absent 1
ECM	Extracellular Matrix
EGFP	Enhanced green flourescent protein
fMLP	N-Formylmethionine-leucyl-phenylalanine
F-Actin	Filamentous-actin
FACS	Flourescence-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green flourescent protein
GST	Glutathione S-transferase
HOIP	HOIL-1-interacting protein
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase 1
ICAM-1	Intercellular adhesion molecule
IF	Immunofluorescence
IL	Interleukin
IP	Immunoprecipitation
IKK	IκB kinase
IKKα	Inhibitor of nuclear factor kappa-B subunit alpha
kDa	Kilodalton
LUBAC	Linear Ubiquitin Assembly Complex
LPS	Lipopolysaccharides
LFA-1	Lymphocyte function-associated antigen-1 (αLβ2 integrin)
MMP	Matrix metalloprotease
MEF	Mouse Embryonic Fibroblasts
MSF	Mammary gland Stromal Fibroblasts
MS	Mass spectrometry
NF-κB	Nuclear factor-κB
NEMO	NF-κB essential modulator
NZF	Npl4 Zinc Finger

PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
PLA	Proximity ligation assay
PSGL-1	P-Selectin glycoprotein ligand 1
PTEN	Phosphatase and tensin homologue
qPCR	Quantitative polymerase chain reaction
RIPK1	Receptor Interacting Protein kinase 1
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SHANK	SH3 and multiple ankyrin repeat
SHARPIN	Shank-associated RH domain-interacting protein
siRNA	Small-interfering RNA
TIRF	Total internal reflection fluorescence
TNF	Tumour Necrosis Factor
TNFR	Tumor necrosis factor receptor
UBA	Ubiquitin-associated
UBL	Ubiquitin-like
VCAM1	Vascular cell-adhesion molecule 1
VLA-4	Very late antigen-4
WB	Western blot
wt	Wild-type

LIST OF ORIGINAL PUBLICATIONS

Following publications are included in this thesis

I. **Meraj H. Khan**, Siiri I. Salomaa, Guillaume Jacquemet, Umar Butt, Mitro Miihkinen, Takahiro Deguchi, Elena Kremneva, Pekka Lappalainen, Martin J. Humphries and Jeroen Pouwels. The Sharpin interactome reveals a role for Sharpin in lamellipodium formation via the Arp2/3 complex. J Cell Sci. 2017 Sep 15; 130(18):3094-3107.

II. Emilia Peuhu*, **Meraj H Khan***, Fumiko Marttila-Ichihara, Terhi Savinko, Liisa M Uotila, Susanna C Fagerholm, Marko Salmi, Jeroen Pouwels. Sharpin regulates granulocyte rolling and adhesion *in vivo*. (Manuscript).

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*Equal contribution

1. INTRODUCTION

Sharpin (SHANK-Associated RH Domain Interactor) is a multifunctional adaptor or scaffolding protein that does not have its own enzymatic properties, but interacts with other proteins and regulates their functions. For example, Sharpin is a subunit of the linear ubiquitination assembly complex (LUBAC), which promotes signal-induced activation of the oncogenic and pro-inflammatory transcription factor NF-κB and regulates several other pathways (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). Importantly, LUBAC is linked to several diseases, for instance, auto-inflammation and cancer (Boisson et al., 2015; Sasaki et al., 2013; Boisson et al., 2012; Iwai et al., 2014; Gerlach et al., 2011; Okamura et al., 2016; Zinngrebe et al., 2016). Direct interaction of Sharpin with PTEN, which is a known anti-tumorigenic protein, inhibits PTEN activity (De Melo et al., 2014). Sharpin also acts as a negative regulator of integrins, which are transmembrane receptors that mediate cell-cell and cell-extracellular matrix adhesions (Rantala et al., 2011). Recent reports showed that Sharpin also inactivates T cell receptor, a key regulator of immune function, and the pro-apoptotic protease Caspase 1 (Park et al., 2016; Nastase et al., 2016).

Integrins, PTEN, Caspase 1 and the NF-κB pathway are all strongly linked to cancer progression, suggesting that Sharpin could also play a decisive role in tumor progression. Importantly, Sharpin is amplified and overexpressed in many human cancers and promotes cancer cell proliferation, tumour formation and metastasis (Fu et al., 2017; Yang et al., 2017; Kumari et al., 2014; Tanaka et al., 2016; Zhang et al., 2014; De Melo et al., 2014). Even though sharpin is an adaptor and thus potentially binds to many proteins, a comprehensive approach to identify all the signalling pathways regulated by Sharpin has been missing.

To better comprehend the processes regulated by Sharpin, we set out to identify unknown proteins which interact with Sharpin. We performed a mass spectrometry screen, linking Sharpin to several novel cellular functions, such as vesicle trafficking, proteasomal degradation and RNA splicing. Also, we found that two subunits of the Arp2/3 complex and several other proteins associated with the cytoskeleton were also recruited by Sharpin in the screen. The Arp2/3 complex is an actin nucleator that catalyses actin branching and plays an essential role in many cellular processes, such as endosome trafficking, phagocytosis and lamellipodia formation (Campellone and Welch 2010; Rotty et al., 2012; Suraneni et al., 2012; Brayford et al., 2016).

In this thesis, I will describe the first interactome of Sharpin, and the identification of Sharpin as a novel direct interactor of the Arp2/3 complex. Moreover, we show that

Arp2/3-dependent lamellipodia formation and cell migration are significantly affected if this interaction is hampered.

Inflammation or infection triggers the transmigration of granulocytes through the endothelial layer of vessels towards the affected site (Ley et al., 2007; Muller, 2003). This transmigration of granulocytes is a complicated process of innate immunity, which is impaired in inflammatory diseases or cancer. Previously, Sharpin has been reported to be essential for the last step of granulocyte transmigration; the release of the trailing edge after crossing the endothelium (Pouwels et al., 2013). The role of Sharpin in the early steps of granulocyte transmigration has remained unknown. However, in this thesis, using intravital real-time imaging experiments on postcapillary veins in the cremaster muscles of wild-type (wt) and Sharpin-deficient mice, I showed that Sharpin regulated the early steps of transmigration as well. These defects lead to strongly reduced overall granulocyte transmigration upon infection in Sharpin-deficient mice, despite the fact that these mice have higher granulocyte count due to systemic inflammation (Gurung et al., 2016; Liang 2011; Potter et al., 2014).

Altogether, the results presented in this thesis have increased our knowledge of the different regulatory functions performed by Sharpin in various cellular processes inside the cell.

2. REVIEW OF THE LITERATURE

2.1 Spontaneous mutation in Sharpin gene causes chronic proliferative dermatitis phenotype

In 1993, a spontaneous variant of the C5BL/KaLawRij mice was identified that developed chronic diffused proliferative dermatitis at the age of 3–4 weeks with characteristics of psoriasis (HogenEsch et al., 1993). The other significant phenotypes observed in the mice were their small size and reduced life expectancy. These mice also developed prolonged inflammation in various organs, such as the spleen, abnormal Peyer´s patches development, and defective TH1 cytokine production (HogenEsch et al., 1993; Hogenesch et al., 2001).

The responsible gene for the chronic proliferative dermatitis phenotype in mice was discovered in 2007 by Seymour et al., who showed disease-causing spontaneous autosomal recessive mutations in Sharpin (SHANK-Associated RH Domain Interactor) (Seymour et al., 2007). Two distinct spontaneous allelic mutations in the Sharpin gene were reported by them, which both caused a frameshift and subsequent loss of the protein, and led to a similar phenotype (Seymour et al., 2007).

Sharpin deficient mice suffer from progressive eosinophilic dermatitis; the skin lesions in the mice are characterized by epidermal hyperplasia, apoptosis of keratinocytes, dermal neovascularization, and increased number of eosinophils and MHC class II-positive cells in the dermis and epidermis (Hogenesch et al., 2001). To comprehend the reason behind the eosinophilic inflammation in skin, the expression of different cytokines in the skin was investigated. Elevated expression of type 2 cytokines, such as IL-4, IL-5, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF), in the skin of *Sharpin*^{cpdm} mice was observed (Hogenesch et al., 2001). Decreased expression of type 1 cytokines such as IFN-ɣ, was also seen in the same study. Type 2 cytokines can be induced by allergic reactions and increased accumulation of eosinophils (Desreumaux and Capron, 1996; Leung, 1997; Rothenberg, 1998). The increased expression of type 2 cytokines blocks Interferon gamma (IFN-ɣ) secretion from stimulated splenocytes and IFN-ɣ-dependent delayed type hypersensitivity (Pretolani and Goldman, 2018).

In summary, Sharpin-deficient mice exhibit defective secondary lymphoid organ development, and around 4 weeks of age, develop inflammation in several organs, including skin, oesophagus, lung and liver (Gijbels et al., 1996; Gijbels et al., 1995; HogenEsch et al., 1993). The *Sharpin*^{cpdm} shows upregulated production of type 2

cytokines in different organs of the mouse, such as spleen and skin, and this causes eosinophilia or eosinophilic infiltration (Hogenesch et al., 2001). *Sharpin*^{cpdm} mice can be used as a potential model system to study the pathology of chronic type 2 inflammatory disease of eosinophils in the skin (HogenEsch et al., 1993; Seymour et al., 2007; Gijbels et al., 1996).

Interestingly, not all the organs are similarly affected, which implies that Sharpin might have tissue-specific functions, and thus its deficiency shows different changes in different cells or tissues.

2.2 Domain structure of Sharpin

The Human Sharpin gene is located on chromosome 8 (Chr8q24.3) (Daigo et al., 2003), its homolog in the mouse is present on chromosome 15 (m.Chr15.D3) (Daigo et al., 2003). Sharpin is a 40 KDa cytoplasmic protein (Jung et al., 2010; Rantala et al., 2011) with three domains: a Pleckstrin homology (PH) domain on the N-terminal, a central Ubiquitin-like domain (UBL) and a Zinc finger domain, which is at the C-terminus (Fig. 1).

Sharpin interacts with other proteins using its different domains (Fig. 1). Sharpin with its PH domain forms homo-dimers (Stieglitz et al., 2012a), while through its UBL domain Sharpin interacts with many different proteins and regulates their activities, such as integrins (Rantala et al., 2011), HOIP (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011), SHANK (Lim et al., 2001), PTEN (De Melo et al., 2014), EYA1/2 (Landgraf et al., 2010). The zinc finger domain of Sharpin is involved in inactivation of T cell receptor (TCR), and is needed for Caspase 1 and LUBAC function (Ikeda et al., 2011; Park et al., 2016) (Fig.1).

The UBL domain of Sharpin is the hub of interaction, as through this domain, Sharpin interacts with integrins (Rantala et al., 2011), HOIP and many other proteins (Gerlach et al., 2011; Ikeda et al., 2011; Landgraf et al., 2010; Lim et al., 2001; De Melo et al., 2014; Tokunaga et al., 2011). Using bioinformatics tools, mutations in the Sharpin UBL domain were designed by De Franceschi et al., who used these mutants to show that the integrin-and HOIP-binding sites partially overlap (De Franceschi et al., 2015). The amino acids in the UBL domain of Sharpin (V267, L276) required to interact with integrin and HOIP, were also identified. All in all, study by De Franceschi et al., provided us important information about the UBL domain of Sharpin and revealed that integrin inhibition and NF-κB activation role of Sharpin are molecularly different (De Franceschi et al., 2015). These UBL domain mutants of Sharpin have also been instrumental in this thesis to identify specific Arp2/3-binding deficient mutants.

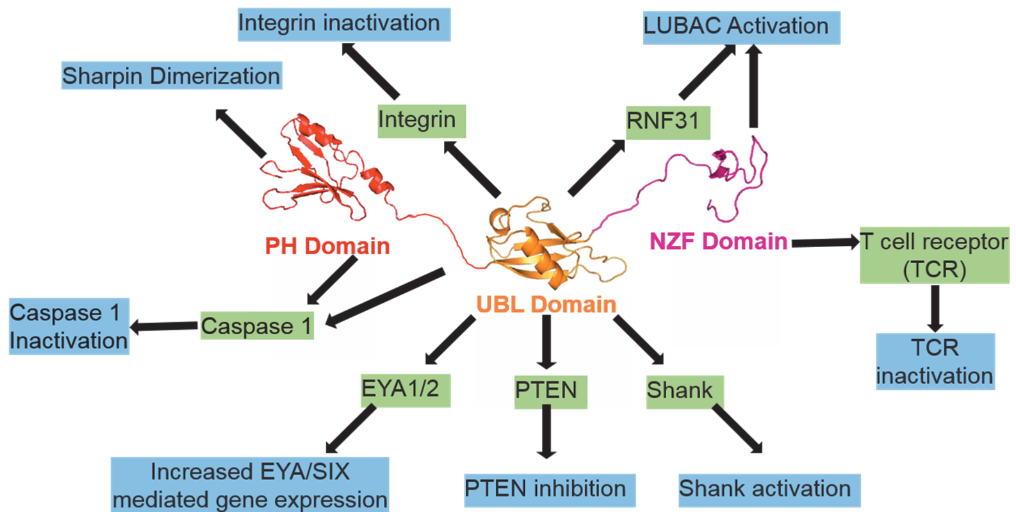

Fig. 1. Different domains of Sharpin, their interactors and functions. The three Sharpin domains: PH Domain,UBL Domain and NZF Domain are shown with the 3D models created using PyMOL Molecular Graphics system (De Franceschi et al., 2015). The linker regions between the domains have a random orientation, and thus the structure depicted here does not reflect the actual structure of Sharpin, but mainly serves as a model to illustrate different Sharpin interactors and functions. Proteins interacting with the different domains are shown in green boxes, while downstream effects are indicated in blue boxes. [Figure is modified from (De Franceschi et al., 2015)].

Recently Liu et al., published the crystal structure of the UBA domain of HOIP in complex with the UBL domain of Sharpin (Liu et al., 2017). They reported that the binding of Sharpin or HOIL-1L UBL domain to the UBA domain of HOIP could potentially release the auto-inhibitory state of HOIP and activate it (Liu et al., 2017). Though Sharpin UBL and HOIL-1L UBL interact with different regions of HOIP UBA domain, this binding brings conformational changes in HOIP UBA domain and help the E2 loading of HOIP for its activation (Liu et al., 2017).

The PH domain of Sharpin was crystalized by Stieglitz et al., (Stieglitz et al., 2012a). It comprises 170 amino acids at the N terminal and has small heptad repeat motif across residues 36 to 49. The immunoprecipitation assay revealed that the N terminal is responsible for Sharpin dimerization although evidence for Sharpin dimers in cells remains lacking. Their crystal structure confirms that Sharpin is capable of forming homodimers; however, this dimerization was not mediated via the coiled-coil present in the N terminal (Stieglitz et al., 2012). Sharpin uses the pleckstrin homology (PH) superfold for dimerization (Stieglitz et al., 2012a).

The full Sharpin crystal structure is not yet published, rather the PH and UBL domains were individually crystallized by different labs (Stieglitz et al., 2012; Liu et al., 2017).

How UBL domain binds so many different proteins, and how these interactions are regulated via different amino acids present at the surface of UBL domain?

2.3 Sharpin and its interactors

2.3.1 Sharpin is required for the activation of SHANK

Sharpin was reported for the first time in 2001 by Lim et al., using yeast two-hybrid method they reported a novel protein (Sharpin), which interacted with the N-terminal region of SHANK1 having ankyrin repeats and the SH3 domain (Lim et al., 2001) (Fig. 2). Different domains or regions of SHANK1 were tested with Sharpin to determine the minimal part responsible for the interaction. The seven ankyrin repeats from amino acids 102 to 348 were required to interact with Sharpin. The region of Sharpin sufficient for binding to SHANK1 consists of amino acids from 172 to 305, which are part of the Sharpin UBL domain (Fig. 2).

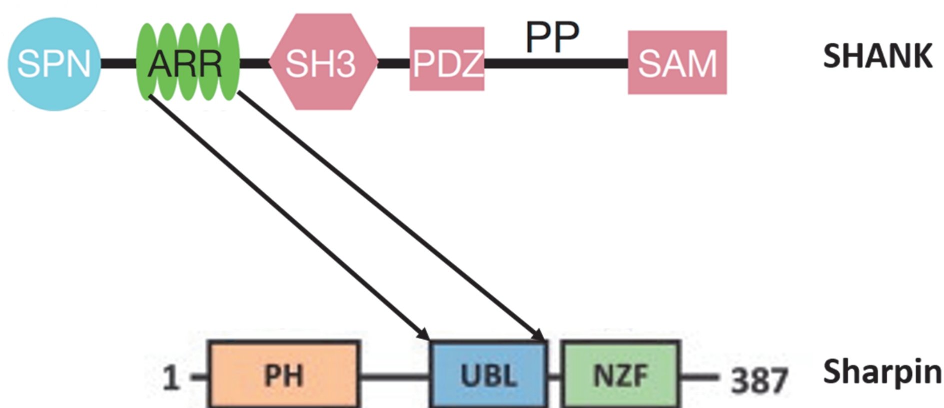

Fig. 2. Different domains of SHANK and Sharpin proteins and the domains which interact with each other. [Figure is redrawn from (Lilja et al., 2017)]

SHANK protein family consists of three members: SHANK1, SHANK2, and SHANK3 (Naisbitt et al., 1999). All three isoforms are scaffolding proteins, enriched in the postsynaptic density (PSD) of excitatory synapses and essential for the generation of dendritic spines and postsynaptic synapses (Baron et al., 2006; Roussignol, 2005; Sala et al., 2001). These processes are required for normal brain development (Kreienkamp, 2008; Sheng and Kim, 2000). SHANK proteins are also highly expressed in peripheral organs, where their function is not known. If the SHANK 3 gene gets mutated, it causes neurological diseases like autism spectrum disorders (ASDs), intellectual disability (ID) and manic-like behaviour (Betancur and Buxbaum, 2013; Carbonetto, 2014; Gauthier

et al., 2010; Grabrucker et al., 2014; Guilmatre et al., 2014; Han et al., 2013; Leblond et al., 2014).

SHANK1 and SHANK3 have similar domains for protein-protein interaction: an N-terminal SPN (Shank/ProSAP N-terminal) domain, multiple ankyrin repeats, a Src homology 3 (SH3) domain, a PDZ domain (Post-synaptic density protein, Drosophila disc large tumour suppressor, and Zonula occludens-1 protein), a proline-rich region, and a SAM (sterile alpha motif) domain (Fig. 2). SHANK2 lacks the SPN domain and multiple ankyrin repeats, but all the other domains are present in the same order as in SHANK1 and SHANK3 (Lim et al., 1999). The domain arrangement of Shank proteins seems to be organized by alternative splicing (Lim et al., 1999).

As mentioned previously, point mutations in SHANK 3 have been observed in autistic patients, a study by Mameza et al., analyzed several disease-causing mutations in SHANK3 N-terminus (SPN+ARR) (Mameza et al., 2013). The SHANK/ProSAP N-terminal (SPN) domain is located at the N-terminal region before the ankyrin repeats; it is a highly conserved region (Fig. 2). These mutations affect the binding of Sharpin and alpha-fodrin to SHANK3 and one of the mutations (L68P) increased the binding of these ligands to the ARR domain of SHANK3. Interestingly, the SPN domain of SHANK3 is involved in an inhibitory interaction with the ARR domain (Mameza et al., 2013). Nuclear magnetic resonance (NMR) based analyses revealed that the ARR and the SPN domains are connected by a short linker or a linker immobilized in the SPN-ARR double domain (Mameza et al., 2013). This indicates that a stable contact is established between the SPN and the ARR domains, thus allowing them to function as a single unit. In cells, the binding of SPN domain inhibits the availability of the ARR domain to its ligands (alpha-fodrin and Sharpin). This study shows that ligands, such as alpha-fodrin and Sharpin compete with SPN to bind to the same interaction site of the ARR domain.

Han et al., reported that overexpression of SHANK3 enhances F-actin levels via Arp2/3 complex in excitatory synapses of transgenic neurons (Han et al., 2013). The authors of this stduy performed a mass-spectrometry analysis using EGFP-SHANK3 and compared the results with their previously published Yeast two-hybrid screen (Sakai et al., 2011). Two subunits of the Arp2/3 complex (ARPC2 and ARPC5L) were the new direct interactors of SHANK3 common in the two different screens. Arp2/3 complex is required to initiate the branching of actin filaments (Campellone and Welch, 2010). Previous report already confirmed the interaction of WASF1 and cortactin with SHANK3 (Naisbitt et al., 1999; Proepper et al., 2007). Based on their findings and previous reports, Kihoon et al., proposed that SHANK3 functions as an adaptor protein, which provides a platform to WASF1, cortactin and Arp2/3 to eventually facilitate the F-actin polymerization (Han et al., 2013).

Sharpin is a known interactor of the SHANK3 protein, does Sharpin plays any role in actin polymerization or Arp2/3 dependent processes, is an interesting question?

2.3.2 Sharpin act as an endogenous inhibitor of integrins

Rantala et al., for the first time showed that Sharpin is an endogenous integrin inhibitor (Rantala et al., 2011). Sharpin inhibits integrin activity in many cell types, and its depletion causes increased integrin-activity. Sharpin binds to the conserved membrane-proximal part of α-integrins, which reduces binding of integrin activators talin and kindlin to the β1-subunit. Importantly, in line with the well-established role for integrins in cell adhesion and migration (Vicente-Manzanares et al., 2009), Sharpin-mediated integrin inhibition regulates β1-integrin-dependent cell adhesion and migration in different cells types. Sharpin deficient mice also have increased β1-integrin activity *in vivo* (Peuhu et al., 2017a; Rantala et al., 2011). Thus, Sharpin regulates integrin activity, where Sharpin acts as a physiological switch between integrins activation and inactivation.

Integrins are found in metazoans, no homologs in prokaryotes, plants and fungi (Whittaker, 2002) and have two subunits, α and β, that form a heterodimer. These heterodimers cross the cell membrane only once. The eight β subunits can group with 18 α subunits to form 24 distinct integrins (Hynes, 2002) (Fig. 3). Tissue distribution and substrate specificity of these integrin heterodimers are sometimes specific and sometimes overlapping (Hynes, 2002). Some integrins are specific to certain cell types or tissues, such as αIIβ3 to platelets, α6β4 to keratinocytes, αEβ7 to T cells, dendritic cells and mast cells in mucosal tissues, α4β1 to granulocytes, α4β7 to a subset of memory T cells, and the β2 integrins to granulocytes. Many integrins are expressed widely, for example, αVβ3 is expressed on endothelium. Mammalian integrins can be grouped according to the ligand they bind; laminin-binding integrins, collagen-binding integrins, granulocyte integrins, and RGD-binding integrins (Fig. 3) (Takada et al., 2007).

Integrins are multifunctional molecules that act as a bridge between the extracellular matrix and the cytoskeleton (Vicente-Manzanares et al., 2009). Integrins can mediate signalling in both directions due to the fundamental change in structure that can be triggered by extracellular matrix binding or binding of proteins which act as integrin activators in the cytoplasm, such as talin and kindlin. This leads to the formation of a protein complex which links the extracellular matrix with the actin cytoskeleton (Zaidel-Bar et al., 2007). Focal adhesion kinase (FAK) and steroid receptor coactivator (Src) are tyrosine kinase, which are activated once recruited to this protein complex (Fig. 4). Furthermore, this leads to the activation of signalling pathways involving ERK, Jun N-terminal kinase (JNK) or Rho-family small GTPases. These signalling pathways are important for several cellular

processes, such as cell adhesion, migration, proliferation, gene expression and tyrosine kinase signaling (Ivaska and Heino, 2010; Zaidel-Bar et al., 2007).

Integrins play a significant role in many biological processes and diseases, for instance, maintaining homeostasis, immune responses, immune cell traffic and cancer. Their considerable contribution makes them the chosen target to treat diseases like thrombosis and many inflammatory disease s (Li et al., 2003; Edelson et al., 2004; Moser et al., 2009; Legate et al., 2009; Ley et al., 2016).

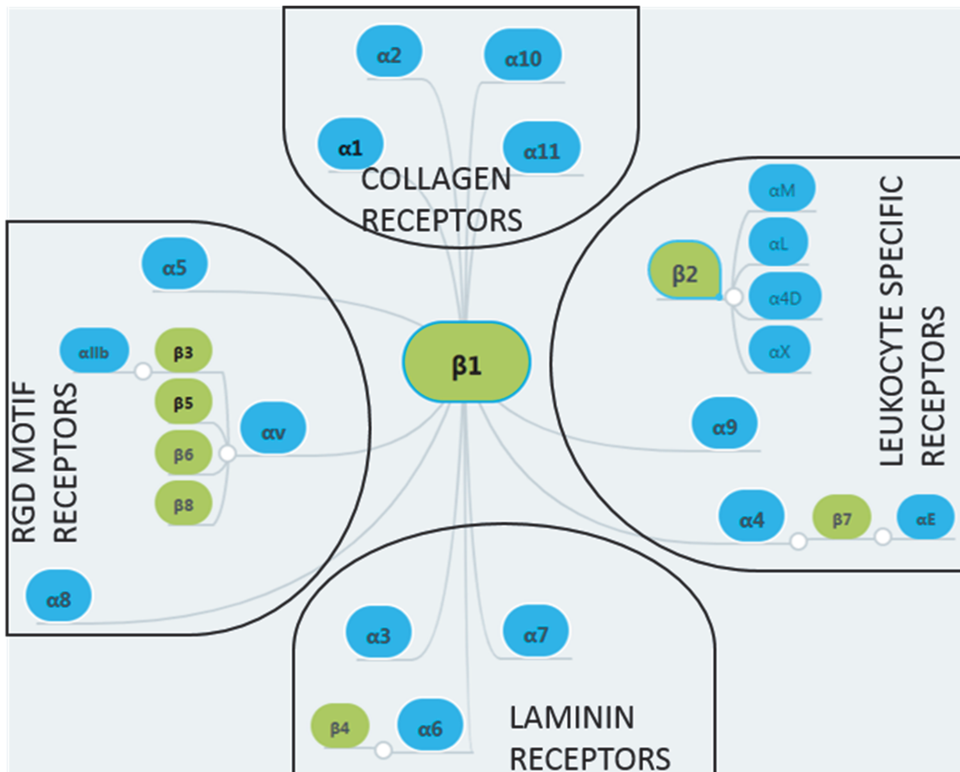

Fig. 3 Integrin superfamily based on its heterodimers [Figure is redrawn from (Hynes, 2002)]

In active integrin state, talin directly binds to the integrin cytoplasmic domain and actin filaments, thereby providing the first indirect link between the integrin and the actin cytoskeleton (Fig. 4). Subsequently, myosinII-mediated contraction forces pull on talin, which exposes hidden vinculin-binding sites within the talin carboxyl end. The subsequent binding of vinculin reinforces the connection between the ECM and the actin cytoskeleton (Springer and Dustin, 2012) (Fig. 4).

Integrin inactivators comprise of two types of classes: Direct inhibitors that directly bind to the integrins and obstruct the recruitment of integrin activators, such as talin

and kindlin; and indirect inhibitors that cause integrin inhibition through different mechanisms.

The integrin cytoplasmic domain is the site for direct integrin inhibitors to bind and obstruct the binding of integrin activators. The known inhibitors bind either to the talin and kindlin interaction domains in the β-integrin cytoplasmic domain (Calderwood et al., 2003; Chang et al., 1997; Kiema et al., 2006; Wegener et al., 2007), such as filamin and DOK1 (Anthis et al., 2009; Kiema et al., 2006; Millon-Frémillon et al., 2008; Wegener et al., 2007), or they target the conserved amino acids of the α-integrin cytoplasmic tails, such as Sharpin (Nevo et al., 2010; Pouwels et al., 2012).

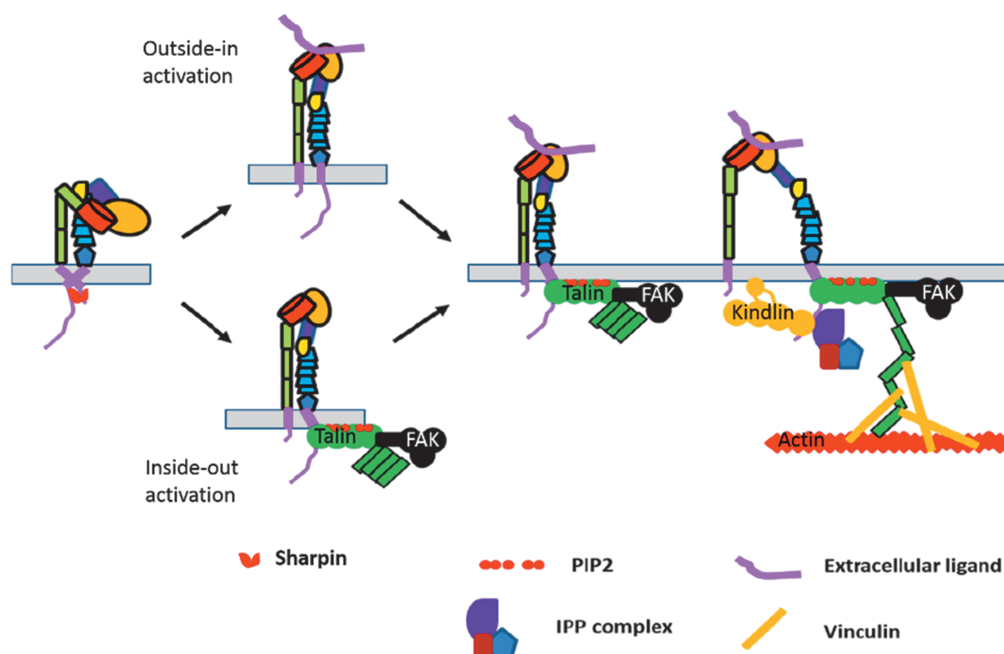

Fig. 4. Three different conformational states of integrins. Inactive state or bent conformation, prime state or extendend conformation, and active state. Outside-in activation is initiated upon binding of an extracellular ligand to the integrins, which brings change in its conformation from bent to a primed state. This change in conformation facilitates the binding of cytoplasmic factors, such as the integrin activator talin. Inside-out activation of integrins involves the binding of intracellular integrin activators, such as talin and kindlin, to the cytoplasmic tail of integrins. Binding of these intracellular poteins changes the bent conformation to the primed conformation. Subsequently, the extracellular head domain of integrins adapts an open conformation, which increases the affinity for extracellular ligands. Both inside-out and inside-out signalling result in recruitment of several cytoplasmic proteins that together form a platform that mediate the interaction between the integrin and the actin cytoskeleton. Finally, mechanical forces extend the integrin into its fully activated state [Redrawn from (Bouvard et al., 2013)].

2.3.3 Role of Sharpin in the transmigration of granulocytes

During transmigration, the trailing end of the granulocyte, the uropod, needs to be uncoupled to move forward (Smith et al., 2005). Pouwels et al., showed that Sharpin accumulates in the uropod, where it is required for uropod release through inactivation of LFA1 (Pouwels et al., 2013).

Granulocytes circulating in the blood start migrating towards the sites of infection or inflammation to clean the source of inflammation and they also contribute to tissue repair. This process is started by invading microorganisms and damaged or inflamed cells, which release pathogen-associated molecular patterns (PAMPs), such as LPS, and damage-associated molecular patterns (DAMPs), respectively (Medzhitov, 2008).These DAMPs and PAMPs act as chemo-attractants for the innate immune system. Additionally, memory T cells are activated by antigens, which start the secretion of various primary inflammatory cytokines and this initiate the granulocytes recruitment. The granulocyte recruitment is further promoted by mast cells, macrophages, and dendritic cells (DCs) when they release pro-inflammatory mediators (Fig. 5).

The transient interaction between granulocytes and endothelial cells close to the inflamed tissues is crucial for granulocyte transmigration. The whole process, known as the granulocyte-adhesion cascade, comprises four major steps: rolling, activation, arrest and transmigration which are mainly facilitated by two adhesion receptor families, selectins and integrins (Ley et al., 2007).

Granulocyte rolling on the endothelial cells, the first step in the cascade, is mediated by selectins (L-selectin, P-selectin and E-selectin) and P-selectin glycoprotein ligand 1 (PSGL1) (Kansas, 1996; McEver and Cummings, 1997) (Fig. 5). Granulocytes express L-selectin and PSGL, while E-selectin and P-selectin are expressed on the surface of endothelial cells upon inflammation. PSGL1 is the primary ligand for all three selectins (Alon et al., 1995). PSGL1 expresses on all granulocytes (Martins et al., 2007; Rivera-Nieves et al., 2006). Once activated by glycosylation, PSGL1 interacts with the L-selectin on other granulocytes, and this causes granulocyte-granulocyte interactions, which results in capture or tethering. Glycosylated CD44 and E-selectin ligand 1 (ESL1), which is present on the granulocyte also interacts with the E-selectin of the endothelium (Hidalgo et al., 2007) (Fig. 5).

Under blood flow conditions, interactions between selectins and their respective ligands allow granulocytes to adhere to the inflamed endothelium (Alon et al., 1995), causing the granulocytes to slow down and roll along the endothelium. Shear stress is necessary for selectin-based adhesion (Finger et al., 1996; Lawrence et al., 1997), as the rolling cells get detached if the flow is removed. This phenomenon is related to the

catch bond character of selectins and the transport phenomenon of selectin ligands relative to that of selectins. As shear stress is applied, each bond strengthens due to the bond character of selectin. Furthermore, the rolling motion of the cells allow new bonds to form prior to breaking of the old bonds (Marshall et al., 2003; Yago et al., 2007).

The selectin-mediated interactions between granulocytes and endothelium are relatively weak. Upon inflammation or infection the endothelium secretes classical chemoattractants and chemokines that stimulate the specific arrangement of heterotrimeric GPCRs in the granulocytes (Shamri et al., 2005), which subsequently causes activation of granulocyte integrins. Activation of these integrins, such as αLβ2-integrin (LFA-1) and α4β1-integrin (VLA-4), stimulates their interactions with their respective endothelial cell ligands, such as ICAM-1 and VCAM-1 (Block et al., 2012; Sriramarao and Broide, 1996; Stadtmann et al., 2011; Zarbock et al., 2008). These bonds strengthen the granulocyte-endothelium interaction and cause the rolling granulocyte to slow down. Subsequent formation of additional integrin-substrate bonds causes the firm arrest of granulocytes on endothelial cells (Berlin et al., 1995; Chan et al., 2001; Huo et al., 2000; Singbartl et al., 2001).

After successful arrest, these activated granulocytes start crawling on the endothelial cells, which is dependent largely on integrins, in search of exit points (Phillipson et al., 2009). Once these granulocytes find an exit cue, they transmigrate across the endothelium towards the inflamed tissues, which is the final step in the granulocyte-adhesion cascade. Granulocytes can follow paracellular and transcellular routes for transmigration (Fig. 5). In the paracellular route, a granulocyte squeezes between two neighboring endothelial cells, which is the preferred transmigration during inflammation. In the transcellular route, the granulocyte crosses through the body of an endothelial cell (Shaw et al., 2001; Huang et al., 1993). The transmigration process is dependent on αMβ2 (Mac-1) integrin (Schenkel et al., 2004) of the granulocyte. The integrin-dependent granulocyte adhesion initiates clusterring of endothelial ICAM-1 and recruitment of VCAM-1 and this provides a platform for transmigration (Barreiro et al., 2008; Carman and Springer, 2003).

The trailing end of granulocytes have inactive αLβ2 integrins, and previously, it was presumed that the inactive αLβ2 (LFA-1) integrin is the preferred default state of the LFA-1. Pouwels et al., showed that Sharpin interacted with LFA-1 and keeps the LFA-1 in an inactive state or inactivates the active LFA-1 to keep the dynamic balance between the active and inactive integrins intact (Pouwels et al., 2013). Thus, the final step in granulocyte transmigration is regulated by Sharpin (Pouwels et al., 2013), but the role of Sharpin in earlier steps were not reported. How Sharpin regulates these early steps will be discussed in the “result and discussion section” of this thesis.

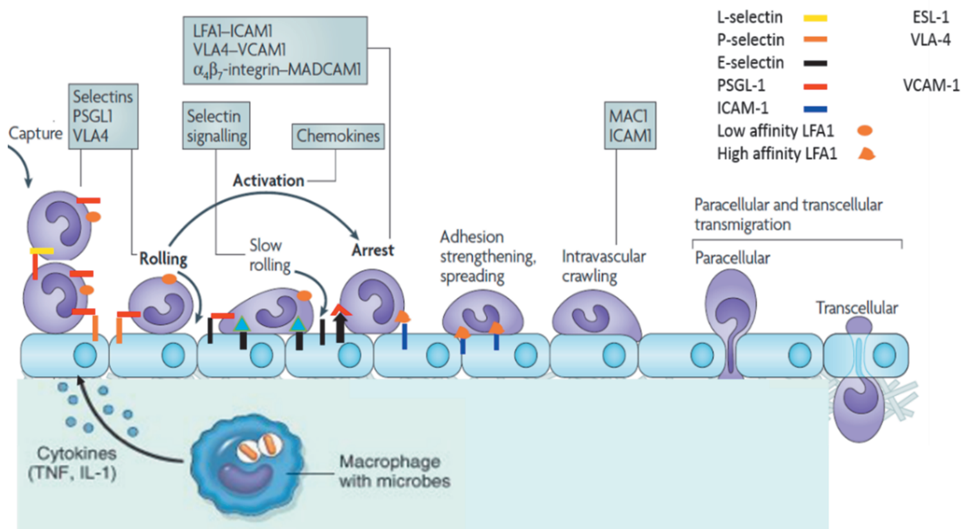

Fig. 5. Granulocyte adhesion cascade comprises four major steps: rolling, activation, arrest and transmigration. Rolling is mediated by selectins, such as L-seletin, E-and P-selectin. Activation is induced by chemokines and arrest and transmigration are dependent on integrins. Important players of each steps are shown in boxes and also mentioned on the right upper corner of the figure. ICAM1, intercellular adhesion molecule 1; LFA1, lymphocyte function-associated antigen 1 (also known as αLβ2-integrin); ESL-1,E-selectin ligand-1; MAC1, macrophage antigen 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as α4β1-integrin). [Figure is modified from (Ley et al., 2007)]

2.3.4 Sharpin is an essential part of the linear ubiquitin assembly complex

HOIP is the only E3 ligase, adept to polymerize linear chains of ubiquitin, which is a post translational modification discovered in 2006 (Kirisako et al., 2006). The sequence similarity between Sharpin and HOIL-1L domains proposed that Sharpin, like HOIL-1L may be a component of LUBAC. In 2011, three different labs simultaneously reported that Sharpin is a novel and a non-catalytic member of LUBAC (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). The UBA domain of HOIP interacts with UBL domains of HOIL-1L and Sharpin (Liu et al., 2017; Rittinger and Ikeda 2017; Ikeda et al., 2011) (Fig. 7).

Post translational modifications, such as phosphorylation and ubiquitination, are a way to modulate or extend protein functions. It can lead to increased or decreased enzymatic functions, changes in protein-protein interactions, as well as shifts in protein conformations. Ubiquitination is one of the most common and diverse post-translational modifications. It has an important role in almost all different types of cellular processes, for example DNA damage responses, trafficking and intracellular

signalling. Ubiquitination is an ATP-dependent three-step enzyme reaction requiring E1, E2, and E3 ligases. During the early steps E1 enzyme, also known as ubiquitin activating enzyme, activates the ubiquitin conjugating enzyme E2. The E3 ligase binds to the substrate and E2 enzyme (Komander, 2009) (Figure 6), thus provides substrate specificity. The E3 ligase then joins the C-terminal glycine of ubiquitin to the ε-amino group of lysine (K) residue of the target protein (Kulathu and Komander, 2012).

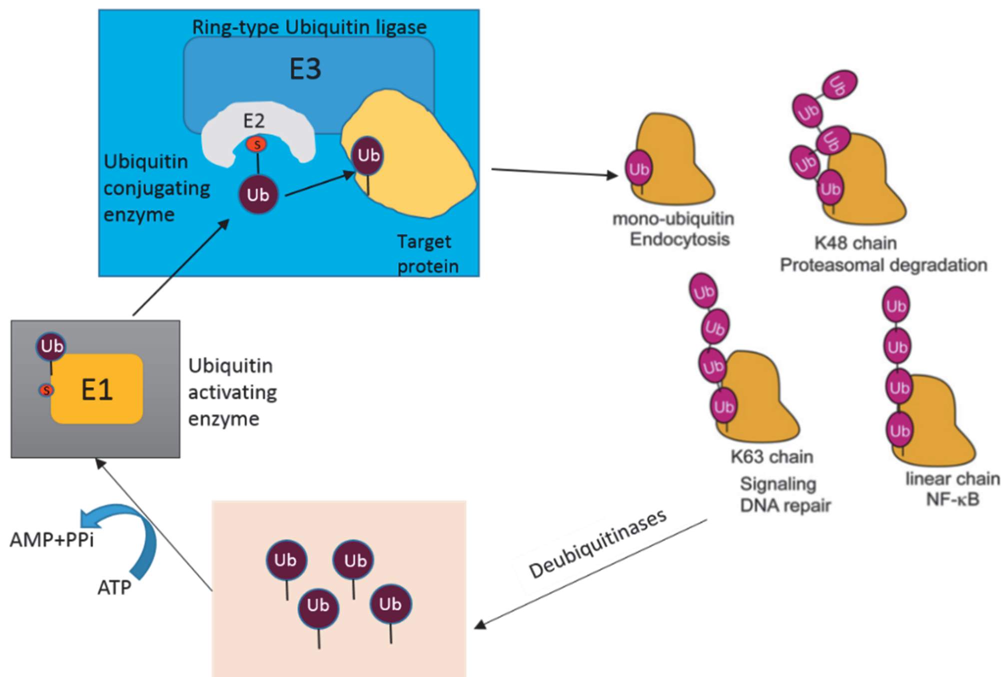

Fig. 6. Schematic representation of different steps involved in the protein ubiquitination. E1 is ubiquitin activating enzyme, E2 is ubiquitin conjugating enzyme and E3 is ring-type ubiquitin ligase. These three enzymes are required for the successfull conjugation of ubiquitin to the target proteins. Ubiquitination of proteins govern several cellular processes. [Figure is modified from (Tokunaga and Iwai, 2012)].

Different types of ubiquitination exist, for example, proteins can be modified with a single ubiquitin molecule at one or more locations (mono- or multi-mono-ubiquitination) or with poly-ubiquitin chains. Poly-ubiquitin chains are the result of single ubiquitin molecules linked to another ubiquitin molecule. This is achieved by linking their C-terminal carboxyl group to one of the 7 ubiquitin lysine residues (K6, K11, K27, K29, K33, K48, and K63). K48 is the most predominant type of ubiquitin chain (Xu et al., 2009) found in cells, and triggers degradation of the substrate by the proteasome (Swatek and Komander, 2016). Heterogeneous and branched/forked chain linkages of ubiquitination also exist (Park and Ryu 2014). Thus, many different types of poly-

ubiquitin chains can be made with several different combinations of mixed and branched ubiquitin chains (Komander and Rape, 2012).

Interestingly, in 2006 a totally new form of poly-ubiquitination was discovered, linear ubiquitination (Kirisako et al., 2006). The novelty was that ubiquitin chains are not added to lysine residues, but to the N-terminal methionine of ubiquitin (Behrends and Harper, 2011). Linear ubiquitination is mediated by the Linear Ubiquitin Assembly Complex (LUBAC), which consist of several proteins. The central player is HOIP (HOIL1-interacting protein; also known as RNF31), which is the catalytic subunit of LUBAC. In addition, two adaptor proteins are essential for LUBAC function; Sharpin (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011) and HOIL-1L (Kirisako et al., 2006) (haem-oxidized IRP2 ubiquitin ligase1L; also known as RBCK1). The UBA domain of HOIP interacts with the UBL domains of both HOIL-1L and Sharpin (Liu et al., 2017; Rittinger and Ikeda 2017; Ikeda et al., 2011) (Fig. 7), which stabilizes the complex. In addition to proteins contributing to linear ubiquitination, LUBAC carries deubiquitinase (DUB) enzymes (OTULIN or CYLD, only one interacts at a time), as well as an adaptor protein (SPATA2, spermatogenesis-associated protein 2) that mediates the interaction between LUBAC with CYLD. OTULIN is a linear-ubiquitin-specific DUB, while CYLD removes linear and K63-linked chains (Elliott et al., 2016; Keusekotten et al., 2013; Kupka et al., 2016; Rivkin et al., 2013) (Fig. 7). The continuous presence of DUBs on LUBAC suggests that it is essential that LUBAC is kept strictly inactive under normal conditions.

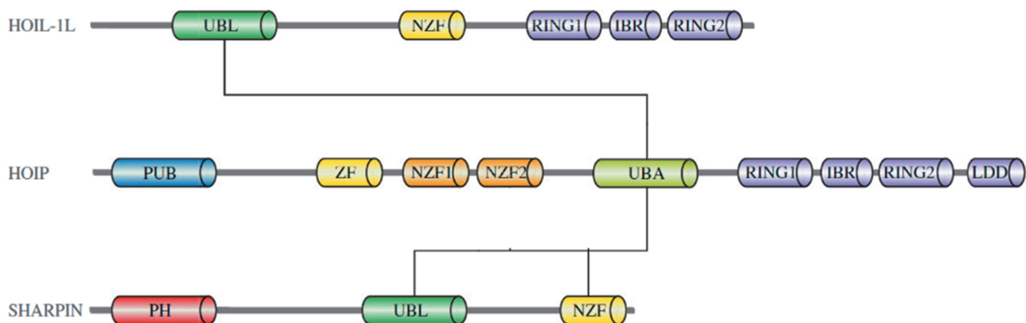

Fig. 7. Different domains of Sharpin, HOIP and HOIL-1, and how different domains mediate the interactions between the proteins. The UBL domain of HOIL-1L binds the UBA domain of HOIP, UBL and NZF domain of Sharpin interact with UBA domain of HOIP. [Figure is modified from (Rittinger and Ikeda, 2017)]

The significance of linear ubiquitination was realized when the immune system was directly linked to LUBAC activity (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011) and LUBAC was shown to propagate the canonical NF-κB pathway by

ubiquitination of RIPK1 (receptor interacting protein kinase 1) and NEMO (NF-κB essential modulator, also called IKKγ) (Fujita et al., 2014; Haas et al., 2009; Tokunaga et al., 2009) (Fig. 8).

2.3.4.1 NF-κB signalling via TNF receptor (TNFR)

LUBAC has mostly been studied downstream of the TNFR, and deregulated in TNFR signalling. Deregulated LUBAC triggers the complicated phenotype of Sharpin-deficient mice (Gerlach et al., 2011; Kumari et al., 2014; Rickard et al., 2014). Therefore, here the role of LUBAC in TNF-induced NF-κB signalling will be described. NF-κB is a family of inducible transcription factors that play vital roles in regulation of cellular processes like cell survival and death, cell proliferation, acquired immune response and mobilization and development of lymphoid tissues (Ben-Neriah and Karin 2011; Oeckinghaus et al.,2011; Hayden et al., 2006). NF-κB complexes consist of combinations of the NF-κB family members NF-κB 1 (p105/p50), NF-κB 2 (p100/p52), c-REL, RELB and RELA (p65). All 5 members of the NF-κB family possess a conserved RHD (N-terminal REL-homology) domain, which plays a role in facilitation of nuclear trafficking, dimerization, interaction with IκB (Inhibitor of kappa B) and DNA binding. Downstream gene activation and expression is activated due to the presence of a TAD (transcriptional activation domain) at the C-terminus of c-REL, RELB and RELA. Without stimulation, the NF-κB dimer stays in the cytoplasm, and thus inactive, through direct interaction with IκB proteins, which include BCL3, IKBε, IKBβ, and IKBα (Fig. 8).

Depending on the signalling inhibitors involved, the NF-κB signalling can be classified in two groups; the canonical and the non-canonical signalling pathway (Oeckinghaus et al., 2011; Matthew et al., 2008). The canonical pathway occurs in most cells as the dominant pathway and, for example, can be triggered by B and T cell receptors, LPS (lipopolysaccharides) and TNF-α.

Trimerization of the TNFR1 is induced by the ligation of TNF. This leads to the recruitment of TRADD, TRAF2/5, RIPK1 and cIAP1/2 to the TNFR1 cytoplasmic death domain (Fig. 8). This subsequently results in the formation of a multi-unit complex called TNF/RSC (TNF receptor signalling complex) (Fig. 8). Importantly, several ubiquitin ligases, including LUBAC, are recruited to this complex, where they ubiquitinate many key proteins (Draber et al., 2015). For example, LUBAC ubiquitinates RIPK1 and NEMO (Fig. 8). These ubiquitin chains critically contribute to TNFR signalling as they form signalling scaffolds and help activate several of the kinases in the pathway. For example, the linear ubiquitin chains are specifically recognized by NEMO (Tokunaga et al., 2009). This intricate interplay of different kinases and ubiquitin ligase leads to activation of the IKK kinase complex, consisting of IKKα, IKKβ and NEMO. This kinase subsequently phosphorylates IκB-α, which triggers its proteasome-mediated

degradation, thus releasing the NF-κB heterodimer allowing its nuclear translocation resulting in the transcription of target genes (Fig. 8). These target genes consist of genes required for activating immune cells and production of cytokines, which are needed to regulate immune and inflammatory response (Hayden et al., 2006). For example, downregulation of the canonical NF-κB pathway causes defects in spleen architecture, Peyers patches, development of nasopharyngeal associated lymphoid tissue and follicular dendritic cells (Hayden et al., 2006).

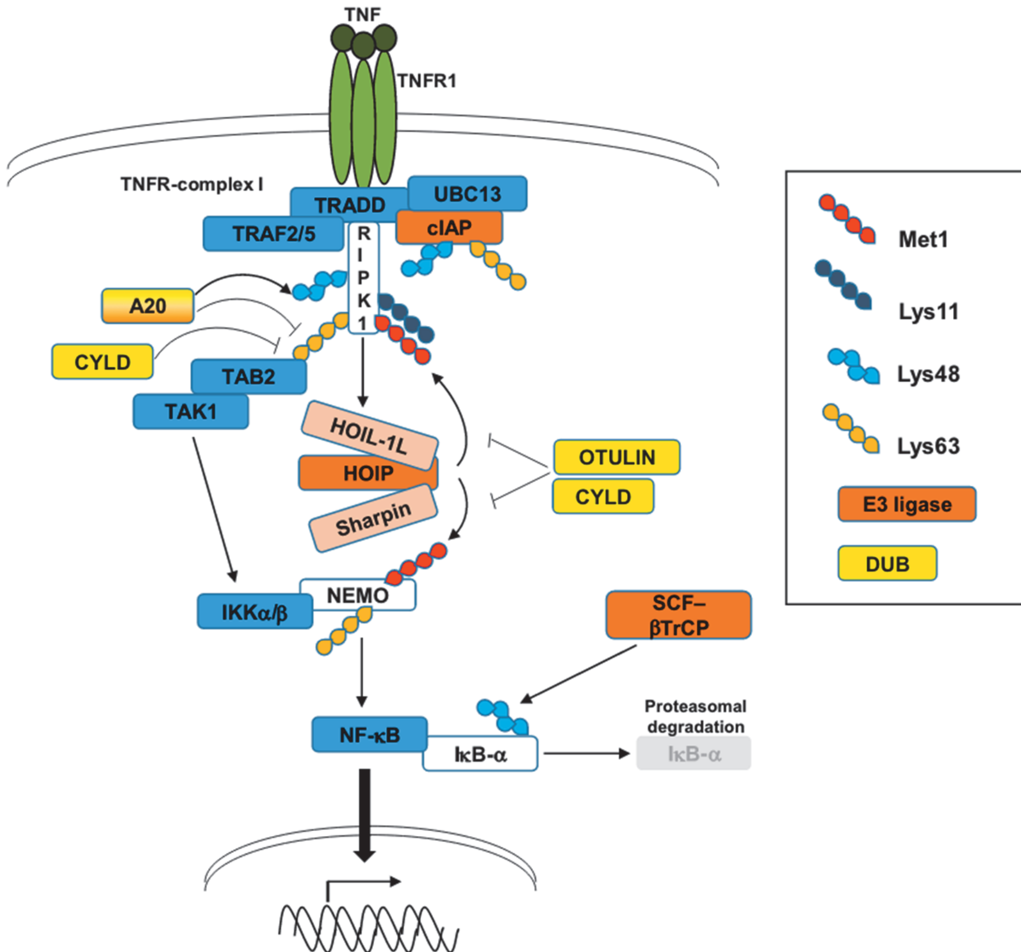

Fig. 8. Role of Sharpin/LUBAC in TNF induced NF-κB activation. TNF ligation induces the trimerization of TNFR1, which causes recruitment of TRADD, TRAF2/5, RIPK1 and cIAP1/2 to the TNFR1 cytoplasmic death domain. Many important proteins are ubiquitinated by different ubiquitin ligases, such as LUBAC. LUBAC linearly ubiquitinates RIPK1 and NEMO (indicated as Met1). The combined activity of the different kinases and ubiquitin ligase leads to activation of the IKK kinase complex (IKKα, IKKβ and NEMO), which phosphorylates IκB-α, leading to IκB-α degradation and release of NF-κB heterodimer. The NF-κB heterodimer subsequently moves into the nucleus and initiates the transcription of target genes. [Figure is copied from (Ikeda, 2015)].

In comparison to the canonical pathway, the non-canonical NF-κB pathway induction is achieved by stimulation of members of the TNF receptor superfamily, although they also induce the canonical pathway. Examples of ligands that can trigger the non-canonical NF-κB pathway are Lymphotoxin (LT), CD40 ligand and B cell activating factor BAFF. The non-canonical pathway induces phosphorylation of NIK and IKK, causing the activation of a kinase cascade. These kinases phosphorylate NFKB2, which results in the partial degradation of NFKB2 at the C terminus. This partial degradation transforms the 100 kDa NFKB2 into p52, the 52 kDa N terminal RHD domain of NFKB2. This allows the exposed p52 to interact with RELB resulting in the formation of a functional transcription factor, allowing its nuclear translocation for expression of target genes. The non-canonical pathway plays a vital role in the development and maintenance of secondary lymphoid tissues as well as B cell maturation and activation (Hayden et al., 2006; Weih and Caamano 2003).

An interesting study published in 2014 by Wu et al., reported that depletion of Arp2/3 complex causes increased expression of secretory proteins, such as chemokines, growth factors and matrix metalloproteinases, the expression profile very similar to the SASP (senescence-associated secretory phenotypes) gene expression profile (Wu et al., 2013). Cells undergo irreversible cell cycle arrest, DNA damage, oncogene activation and cellular stress. When cellular senescence is initiated, this phenotype is termed as senescence-associated secretory phenotypes (SASP), and is regulated by NF-κB (Freund et al., 2011; Watanabe et al., 2017). The authors of this study proposed that loss of Arp2/3 complex is the main reason behind the activation of NF-κB pathway and reported that Arp2/3-induced activation of NF-κB pathway takes place via CCM2-MEKK3 pathway. Sharpin is an important regulator of NF-κB pathway (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011) and it is also an important molecule for Arp2/3 complex. Therefore, it is assumed that the Arp2/3 complex requires Sharpin to avoid the activation of SASP as some studies reported that SASP act as a barrier to cancer (Collado and Serrano, 2010; Kuilman et al., 2010).

2.3.5 Sharpin regulates downstream proteins in Toll-like receptor 2 (TLR2) signalling

Toll like receptors (TLRs) play a significant role in the innate immune system. They are expressed on macrophages, dendritic cells, B and T cells, (Kawasaki and Kawai, 2014) and specifically recognize conserved molecular motifs of the infectious agents, which lead to priming of antigen-specific adaptive immunity (Kawai and Akira, 2010). A systems biology analysis suggested that Sharpin might play a significant role in the TLR pathway (Zak et al., 2011). In this study, Zak et al., linked two mutations, chronic proliferative dermatitis mutation (CPDM) and L153P/panr2, to the TLR pathway. CPDM

is a spontaneous null mutation in the Sharpin gene (Seymour et al., 2007) and L153P/panr2 is a chemically induced hypomorphic mutation in the *Ikbkg* gene encoding NEMO (NF-κB Essential Modulator) (Siggs et al., 2010). Zak et al., showed impaired IL-12 production upon activation of the Toll-like receptors in Sharpin-deficient macrophages (Zak et al., 2011). Thus, similar to TNFR, TLR2-mediated NFκB activation depends on Sharpin/LUBAC, which plays a decisive role to ultimately control the production of pro-inflammatory cytokines, such as IL12 (Zak et al., 2011).

A very recent study by Liu et al., reported the role of TLR 2 in colorectal cancer cell growth (CRC) (Liu et al., 2018). The authors of this study observed the effect of TLR 2 activation on growth, migration and invasion in CRC cell lines. TLR 2 mediates invasion via activating NF-κB in breast cancer cells is already known (Xie et al., 2009). Sharpin is an important regulator of NF-κB pathway (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011), what is the role of Sharpin in cancer cell migration?

2.3.6 Sharpin interacts with T cell receptor and down regulates downstream signalling

Recently, Park et al., reported that Sharpin negatively regulates T cell receptor (TCR) signalling (Park et al., 2016). Regulatory T cells (Treg cells), a subtype of T cells, keep the immune response under control by suppressing the immune reaction (Ohkura and Sakaguchi, 2010). A transcription factor known as Foxp3 is the marker of these Treg cells, and Foxp3 plays significant roles in development and functions of Treg cells (Sakaguchi et al., 2008; Fontenot et al., 2003; Hori et al., 2003). Compromised generation of Treg cells lead to lethal autoimmune and inflammatory diseases (Hori et al., 2003; Bennett et al., 2001; Brunkow et al., 2001). Signalling through the T- cell antigen receptor (TCR) is essential for Foxp3 expression, peripheral differentiation of Treg cells, and their immuno-suppressive activity (Ohkura and Sakaguchi, 2010). Activation of TCR causes the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCRζ chain. This leads to the binding of the signalling kinase Zap70 to TCRζ. This interaction is required for the activation of downstream molecules for T cell activation (Brownlie and Zamoyska, 2013). Zap70 plays a vital role in Treg development and mutations in Zap70 lead to autoimmune diseases (Chuck et al., 2010; Koonpaew et al., 2006; Siggs et al., 2007; Tanaka et al., 2010). Mutations in TCRζ inactivate it and cause increased Treg numbers or Tregs with increased immunosuppressive activity (Hwang et al., 2012).

Park et al., studied the previously unknown role of Sharpin in Tregs. They showed that the inflammatory phenotype in Sharpin-deficient mice is partly due to defective or poor development of Treg cells, rendering them functionally inactive, which thus removes the break on the immune response (Park et al., 2016). Park et al., reported that Sharpin

inhibits binding of TCRζ to Zap70 in a ubiquitin-dependent fashion (Park et al., 2016) (Fig. 9). K63-linked ubiquitination of Sharpin at K312 causes its binding with TCRζ, which inhibits TCR signalling by inhibiting the binding of ZAP70 to TCRζ (Fig. 9). In Sharpin-deficient mice, Zap70 constitutively interacts with TCRζ, causing over-phosphorylation of TCRζ in Tregs. Active TCR signalling in Sharpin-deficient thymocytes leads to lower self-reactivity selection-shift by eliminating more self-reactive T cells, which eventually lead to reduced number of Treg cells. These findings showed that Sharpin is required for normal Treg cell development by negatively regulating TCR signalling (Park et al., 2016).

In summary, K63 ubiquitination of Sharpin causes Sharpin to interact with the TCR subunit TCRζ, which prevents recruitment of Zap70 and, thus, TCR downstream signalling. Whether Sharpin interferes with binding of ZAP70 to TCRζ because of structural interference of the ubiquitin chain of K63 or if Sharpin recruit some other factors which ultimately regulate this interaction, remains unknown (Park et al., 2016). The ubiquitin ligases responsible for the K63 ubiquitination of Sharpin which further impaired the TCR downstream signalling is not known. The first experimental chapter in this thesis provided us a list of many ubiquitin ligases, such as STUB1 and RNF114, which ubiquitin ligase is involved here can be investigated by validating these putative interactors available in the publication I of this thesis.

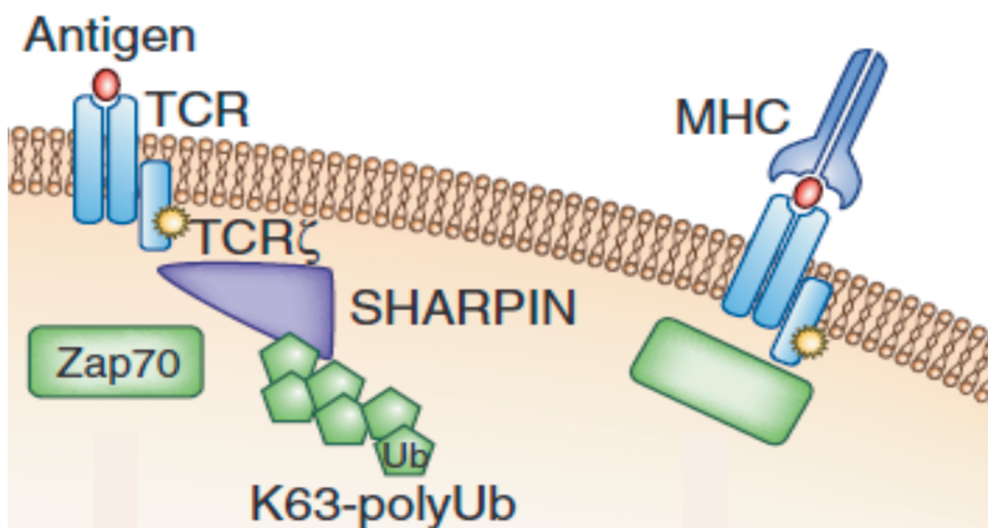

Fig. 9. K63 polyubiquitination of Sharpin promotes interaction of Sharpin with TCRζ, which prevents the interaction between Zap70 to TCRζ and thus inhibits TCR signalling. Therefore, absence of Sharpin leads to over-active TCR signalling [Figure is modified from (Bowman et al., 2016)].

2.4 Physiological consequences of Sharpin

2.4.1 Sharpin interacts with Caspase 1 and inhibits its activity

Nastase et al., established that Sharpin directly interacts with Caspase 1 and negatively regulates its activity in sepsis (Nastase et al., 2016). Sepsis is a systemic inflammatory condition caused by pathogens, which leads to death if left untreated (Fink and Warren 2014). IL-1β plays an important role in sepsis (Merline et al., 2011; Schaefer et al., 2005; Ando et al., 2000; Zhang et al., 2007), and blocking of IL-1β receptor has shown promise to treat sepsis in clinical trials, especially in combination with inhibition of IL18 (Opal et al., 1997; Berghe et al., 2014).

Caspase 1 or interleukin (IL)-1 converting enzyme is a protease, which cleaves the proinflammatory cytokine pro-interleukin (IL)-1β and pro-IL-18 into their mature forms (Cerretti et al., 1992; Mariathasan et al., 2004; Strowig et al., 2012; Thornberry et al., 1992). The N-terminal domain of Sharpin binds to the active Caspase 1 hetero-tetramer, thereby disrupting the p20/p10 dimer, thus leading to inhibition of Caspase 1 activation and prevention of the release of mature cytokines (Fig. 10). This role of Sharpin is LUBAC-independent as evidenced by the absence of HOIL-1L and HOIP in the Sharpin-Caspase 1 complex (Nastase et al., 2016). In addition, Nastase et al., showed that a known synthetic Caspase 1 inhibitor in mice inhibits Caspase 1 enzymatic activity, decreases IL1β/IL18 levels and prevents death of splenic cells due to inflammation (Nastase et al., 2016).

Both IL-1β and IL-18 are required during the early steps of transmigration of granulocytes under inflammatory conditions (Morel et al., 2001; Weirather and Frantz, 2015). Sharpin downregulates Caspase 1 and impaired the maturation of IL-1β and IL-18, Sharpin knockout mice show defective transmigration of granulocytes (Pouwels et al., 2013), Whether this granulocyte transmigration is linked to Caspase 1 regulation via Sharpin needs further attention.

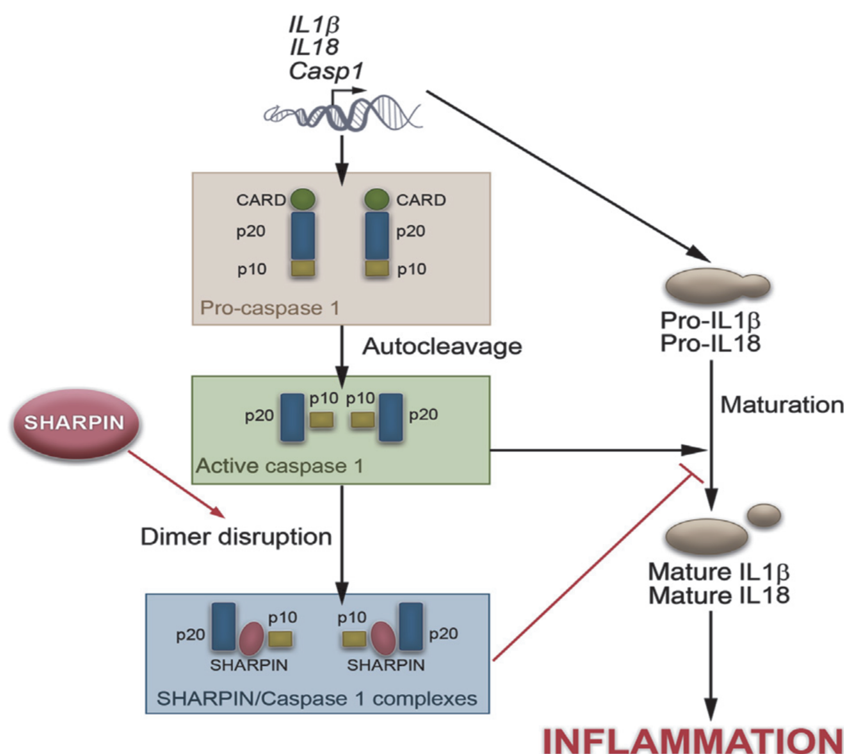

Fig. 10. Sharpin causes inhibition of Caspase-1 activation by destabilizing p20p10 dimers and this inhibits the maturation of IL1β and IL18. CARD, Caspase recruitment domain. [Copied from (Nastase et al., 2016)].

2.4.2 Sharpin promotes p53 degradation

Recently, Yang et al., have shown that Sharpin regulates p53 (Yang et al., 2017). p53 is a transcription factor that acts as a tumour suppressor (Matlashewski et al., 1984) and regulates expression of genes, which are responsible for the cessation of the cell cycle and regulate apoptosis (Bouvard et al., 2000). p53 gets activated by stress signals, for example DNA damage, oxidative stress or overexpression of some oncogenes (Böhnke et al., 2004). In many cancers, such as ovarian cancer and lung cancer, p53 is the most commonly mutated gene (Olivier et al., 2010; Mullany et al., 2015; Cai et al., 2015).

Ubiquitination, phosphorylation, acetylation and methylation are post-translational modifications that regulate p53 expression (Bode and Dong, 2004; Zhou et al., 2014). Ubiquitination is the most thoroughly investigated post-translational modification in p53 (Shi et al., 2016; Zou et al., 2017). It has been reported that E3 ligases, such as MDM2, COP1 and P300 are responsible for the ubiquitination of p53 (Hock and Vousden, 2014). One of the most studied E3 ligases is MDM2, which suppresses p53 expression under unstressed (normal) condition by targeting it for proteasome-

mediated degradation (Shangary and Wang, 2008), thus p53 increases MDM2 expression and, therefore, p53 controls its own expression through a feedback inhibition mechanism (Feng et al., 2016; Lahav et al., 2004). Other E3 ligases, for example RNF31/HOIP, ubiquitinate MDM2, which stabilize MDM2 and leads to increased p53 ubiquitination and degradation. Knockdown of RNF31/HOIP leads to the activation of p53 signalling pathway, which causes cell cycle arrest and promotes cisplatin-induced apoptosis (Zhu et al., 2016), cisplatin being in clinical use as chemotherapy medication. It is important to note here that the proteasomal degradation of p53 does not involve linear ubiquitination.

Yang et al., have shown that Sharpin is an indirect regulator of p53 (Yang et al., 2017). Sharpin binds directly to MDM2, increasing its stability. Knockdown of HOIP does not interfere with Sharpin-MDM2 binding, which shows that this function of Sharpin is independent of LUBAC. Yang et al., proposed two Sharpin-mediated models of stabilization of MDM2 (Yang et al., 2017). Sharpin might promote the stability of MDM2 by promoting mono-ubiquitination of MDM2 by an as-of-yet unknown ubiquitin ligase. Alternatively, Sharpin binds MDM2 and blocks the access for degradative E3 ubiquitin ligase to bind to MDM2 (Yang et al., 2017).

Wang et al., reported that the p53 nuclear import due to the DNA damage is negatively regulated by actin polymerization (Wang et al., 2013). Sharpin also indirectly regulates p53 functions (Yang et al., 2017). Does Sharpin also regulate actin polymerization and Arp2/3 will be discussed in the result and discussion part of this thesis.

2.4.3 Sharpin stabilizes estrogen receptor α

Zhuang et al., revealed a role of Sharpin in ERα signalling (Zhuang et al., 2017). Immunohistochemistry (IHC) of 133 breast cancer samples was performed to observe the expression of Sharpin, ERα, progesterone receptor (PR), and HER2. The authors of this study showed that Sharpin is highly expressed in breast cancer and that Sharpin expression correlates with ERα expression (Zhuang et al., 2017). Sharpin promotes mono-ubiquitination of ERα, which leads to the stabilization of ERα as poly-ubiquitination of ERα is inhibited (Zhuang et al., 2017). This subsequently leads to increased ´tamoxifen´ (tamoxifen is used to treat estrogen receptor-positive breast cancer) resistance.

Breast cancer is the most common cancer form among females (Siegel et al., 2016). Estrogen receptor α (ERα) is upregulated in many breast cancer cases, and anti-estrogen therapies have shown great benefits in those patients (Bernard et al., 2009). In due course, however, breast cancer patients deteriorate (Musgrove and Sutherland, 2009), thus, it is required to comprehend the possible mechanisms of estrogen

signalling. Previous studies have shown that ERα is directly or indirectly regulated by oncogenic signalling pathways, such as human epidermal growth factor receptor-2 (HER2), epidermal growth factor receptor (EGFR) and NF-κB (Nakshatri et al., 1997; Shou et al., 2004). Ubiquitination, SUMOlyation and phosphorylation of co-activators or regulators of ERα govern the expression of ERα in breast cancer (Zhuang et al., 2015; Donley et al., 2014; Karamouzis et al., 2008). However, the detailed mechanism how these co-activators or post translational modification of the regulators of ERα control the expression and signalling of ERα remains elusive. Estrogen signalling in breast cancer is promoted by different ubiquitin ligases, such as BRCA1, CHIP and HOIP (Eakin et al., 2007; Zhang et al., 2011; Zhu et al., 2014; Zhu et al., 2016).

ERα stability and activity are also regulated by different ubiquitination events (Lonard et al., 2000), which have different outcomes depending on the type of ubiquitination and the residues being modified (Zhang et al., 2011; Lonard et al., 2000; Berry et al., 2008). For example, ERα ubiquitination can promote its degradation (Berry et al., 2008) or stabilization (La Rosa et al., 2011). Sharpin being a part of LUBAC is involved in linear ubiquitination of downstream proteins involved in NF-κB pathway, it is interesting to note that Sharpin pull down screen discussed in the results and discussion part has many putative ubiquitin ligases. It is possible that Sharpin has a specific role in ubiquitination of other proteins or it helps ubiquitin ligases to bind to their target proteins. The cellular factors that trigger and recognize this type of modification need to be further characterized.

2.4.4 The role of Sharpin in mammary gland development

The recent work of Peuhu et al., shows that Sharpin-deficient mice have defective mammary ductal outgrowth (Peuhu et al., 2017). Postnatal development of the mammary gland is stimulated by steroid hormones, growth hormones and local growth factors. During growth and development of mammary ducts, the mammary epithelium starts invading the surrounding fat pad stroma to form a mammary gland, which further grows and terminally differentiates to develop during pregnancy and lactation. An outgrowth of mammary duct starts from the terminal end buds at the site of active cell division, and hollow ducts are formed due to luminal apoptosis (Ewald et al., 2008; Hinck and Silberstein, 2005). Luminal and basal mammary epithelial layers are formed as a result of cell differentiation (Ewald et al., 2008; Hinck and Silberstein, 2005). The mammary ductal outgrowth and its branching through the adipose tissue involve intricate signalling between the epithelium and the stroma (Howard and Lu, 2014; Sternlicht et al., 2006). Epithelial cells adhere to the ECM through integrins, which play a significant role in mammary ductal outgrowth, and regeneration of mammary epithelia (Klinowska et al., 1999; Levental et al., 2009; Taddei et al., 2008). Changes in

ECM organisation and stiffness stimulate invasion and metastasis in breast cancer (Acerbi et al., 2015; Robertson, 2016; Schedin and Keely, 2011).

Development of mammary ducts and the crosstalk between epithelial and stromal cells (Howard and Lu 2014; Zhu et al., 2014) are used as a tool to study the mechanism of cell invasion. Peuhu et al., have shown that Sharpin plays a role in cell traction force generation on collagen matrices and in collagen fibre arrangement, it´s contraction and its degradation by mammary gland stromal fibroblasts (MSFs) (Peuhu et al., 2017). In line with *in vitro* ECM-related remodelling defects seen in *Sharpin*^{cpdm} MSFs, mammary ductal outgrowth was reduced in *Sharpin*^{cpdm} mice, which have lower stromal stiffness (Peuhu et al., 2017).

Altogether, this study by Peuhu et al., reported that mammary ductal outgrowth is strongly inhibited in *Sharpin*^{cpdm} mice (Peuhu et al., 2017). This phenotype is most likely due to deregulation of ECM structure in the stroma of mammary gland ducts, as loss of Sharpin results in rearrangement of collagen bundles *in vivo* and *in vitro,* as a result of failure of Sharpin deficient MSFs to contract, pull and degrade collagen.

The extracellular matrix and the actin cytoskeleton of a cell are linked via integrins, and these structures mediate cellular force transmission. The role of integrins in contracting the extracellular matrix by altering traction forces is previously reported (Schwarz and Gardel, 2012) and these reports have identified Sharpin to be an endogenous inhibitor of integrins (Rantala et al., 2011; Pouwels et al., 2013). Importantly, mammary gland stromal fibroblast from *Sharpin*^{cpdm} mice demonstrated abnormal focal adhesion and traction force when plated on collagen (floating 3D collagen plug assay) (Peuhu et al., 2017). Whether the deficient matrix production in the absence of Sharpin is due to hyperactivation of integrins (Rantala et al., 2011) or this phenotype of *Sharpin*^{cpdm} mice is mediated through one of the other Sharpin functions, remains to be established.

3. AIMS OF THE STUDY

Being an adaptor or scaffolding protein, Sharpin (SHANK-Associated RH Domain Interactor) regulates a plethora of signalling pathways by modulating many cellular functions either as a negative or a positive regulator. For example, Sharpin is a subunit of the linear ubiquitination assembly complex (LUBAC), which promotes signal-induced activation of the oncogenic and pro-inflammatory transcription factor NF-κB. In addition, Sharpin acts as negative regulator of PTEN, Integrins, T cell receptors and Caspase 1. All these proteins regulate many functions, which are vital for normal functioning of a cell.

Furthermore, recently Sharpin has been linked to several new cellular functions. However, a systematic approach to identify all the pathways regulated by Sharpin was missing. This study was dedicated to find out the novel interactors of Sharpin and to study the Sharpin interactome. In addition, earlier Pouwels et al., 2013, reported a novel role of Sharpin in the last step of transmigration, as a dynamic inactivator of LFA-integrin at the trailing end of granulocytes. However, as Sharpin´s role in earlier steps of transmigration has not been reported, this was investigated in the second aim.

The specific aims of this PhD thesis were:

1. *Identification and characterization of novel interactors of Sharpin using mass spectrometry (Sharpin interactome).*
2. *Investigate the role of Sharpin during the early steps of granulocyte transmigration.*

4. MATERIALS AND METHODS

4.1 The Sharpin interactome reveals a role for Sharpin in lamellipodium formation via the Arp2/3 complex (Publication I, hereafter called I)

4.1.1 Mass spectrometry

GFP-Trap beads (ChromoTek) were used to perform GFP pulldowns according to the manufacturer's protocol. The cells were either plated on fibronectin (10 µg/ml; Sigma) or kept in suspension for 1 h prior to lysis. Separation of protein samples was performed using SDS-PAGE followed by staining with InstantBlue (Expedeon). The gel lanes were sliced and digested using in-gel digestion with trypsin as previously described (Shevchenko et al., 1996) with modifications (Byron et al., 2015). The digested samples were analysed using liquid chromatography tandem MS (LC-MS/MS) using an UltiMate 3000 Rapid Separation LC (Dionex Corporation) coupled to an Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer. Peptide mixtures were separated using a gradient from 92 % A (0.1 % formic acid in water) and 8 % B (0.1 % formic acid in acetonitrile) to 33 % B, in 44 min at 300 nl/min, using a 75 mm×250 µm internal diameter 1.7 µM BEH C18 analytical column (Waters). Automatic selection of peptides for fragmentation was done by data-dependent analysis. A Proteome Discoverer (1.4) connected to in-house Mascot (v. 2.4) software was used for protein identification. The data were compared with the SwissProt database (release 2015_08). Carbamidomethylation of cysteine was set as a fixed modification parameter whereas oxidation of methionine was allowed as a variable modification. Only tryptic peptides were considered, with up to one missed cleavage permitted. Monoisotopic precursor mass values were used, and only doubly and triply charged precursor ions were considered. Scaffold (version 3.6) was used to validate the data. A threshold of identification of at least 99 % probability at the protein level, at least 50 % probability at the peptide level and assignment of at least two unique, validated peptides was used. These criteria resulted in an estimated false positive rate of 0.1 % for all datasets. PRIDE Converter (version 2.5.5) (Barsnes et al., 2009) was used to convert the data. PRIDE Inspector (version 2.5.2) (Perez-Riverol et al., 2016) was used to validate the data.

For each GFP pulldown, two biological replicates were performed. The unweighted spectral count of a given protein normalized to the total number of spectra observed in the whole sample and to the molecular mass of that protein (normalized spectral count) was used to calculate the relative protein abundance. Three thresholds

reflecting the specificity and quality of the binding to GFP-Sharpin were used to list and score putative Sharpin interactors. 690 proteins detected with at least four spectra and enriched twofold in GFP-Sharpin datasets (suspension or adherent) over control were assigned low confidence. Two other thresholds were used to provide the list of putative Sharpin binders that are likely to contain fewer false positive. A medium confidence was assigned to 297 proteins detected with at least five spectra and enriched fourfold in the GFP–Sharpin datasets (suspension or adherent) over control, while a high confidence was assigned to 48 proteins detected with at least ten spectra and enriched fourfold in both GFP–Sharpin datasets.

DAVID (version 6.8) (Huang et al., 2009) was used to perform a Gene Ontology analyses. The Cytoscape plugin ‘Enrichment Map’ (Merico et al., 2010) was used to create a Gene Ontology map. Using Cluster 3.0 (Clustering Library, version 1.50) (de Hoon et al., 2004) on the basis of uncentred Pearson correlation, the proteins were hierarchically clustered. JavaTreeView (version 1.1.6r2) (Saldanha, 2004) was used for visualization. Cytoscape (version 3.4.0) (Smoot et al., 2011) was used to perform Protein–protein interaction network analyses. Proteins were mapped onto a merged human interactome consisting of protein–protein interactions reported in the Protein Interaction Network Analysis platform Homo sapiens network (Wu et al., 2009) integrated within Cytoscape using PINA4MS (version 2.0.1).

4.1.2 Antibodies

The antibodies used in this study are described in table 1.

Table 1. Primary antibodies used in this study

Antibodies	Description (Catalogue no. and provider)	Application	Used in
Rabbit RNF31/HOIP	ab46322, Abcam	WB	I
Rabbit GST	91G1, Cell Signalling Technology	WB	I
Rabbit GFP	A11122, Molecular Probes	PLA	I
Mouse GFP	ab1218, Abcam	PLA	I
Rabbit GFP	A11122, Molecular Probes	WB & PLA	I
Mouse GFP	ab1218, Abcam	PLA	I
Mouse Sharpin	ab69507, Abcam	IF & PLA	I
Rabbit Sharpin	14626-1-AP, Proteintech	WB & IP	I
Mouse cortactin	(05-180, Merck Millipore	IF & WB	I
Rabbit Arp2	ab47654, Abcam	IF & WB	I
Rabbit Arp3	58182, One World Lab	WB	I
Rabbit paxillin	SC-5574, Santa Cruz Biotechnology	TIRF	I
Mouse GAPDH	5G4MaB6C5, HyTest	WB & PLA	I
Rabbit Sharpin	14626-1-AP, Proteintech	WB & IP	I
Mouse cortactin (p80/85)	05-180, Merck Millipore	IF & WB	I
Rabbit Arp2	ab47654, Abcam	IF & WB	I
Rabbit Arp3	58182, One World Lab	WB	I
Rabbit paxillin	SC-5574, Santa Cruz Biotechnology	TIRF	I
Mouse GAPDH	5G4MaB6C5, HyTest	WB & PLA	I
Mouse β-actin	A2228, Sigma	WB & PLA	I
Rabbit α2-integrin	ab1936, Chemicon	PLA	I
Rabbit phospho-p44/42 MAPK (Erk1/2)	4370, Cell Signalling Technology	PLA	I
Rat 9EG7	553715, BD Biosciences	FACS	I
Mouse P5D2	Hybridoma bank	FACS & TIRF	I
Mouse 12G10	ab30394, Abcam	FACS & TIRF	I
Rabbit p65	8242, Cell Signalling Technology	PLA	I
Rabbit Arpc3	57646, OneWorld Lab	WB	I

The secondary antibodies were Alexa Fluor 488- or Alexa Fluor 555-conjugated IgGs (Invitrogen; IF), horseradish peroxidase (HRP)-conjugated IgGs (GE Healthcare; WB), DyLight 680- or 800-conjugated anti-mouse and rabbit IgGs (Thermo Scientific; WB).

4.1.3 Plasmids and siRNAs

Generation of the Sharpin mutant plasmids (De Franceschi et al., 2015) as well as the GST–Sharpin and siRNA1-insensitive GFP–Sharpin (Rantala et al., 2011) have been previously described. Addgene Plasmid #8462 (deposited by Matthew Welch (Welch et

al., 1997)) was used to express Arp3-GFP. Construction of Arp3-TagRFP was done by introducing EcoRI and BamHI sites using primers. This was followed by cloning into pTagRFP-N (Evrogen). In order to obtain pmCherry, the GFP in GFP-C1 (Clonetech) was replaced with mCherry by making use of primers introducing NheI and EcoRI restriction sites. The coding sequence for siRNA1-resistant Sharpin was cloned into pmCherry using primers introducing EcoRI and BamHI restriction sites to obtain mCherry-Sharpin. Addgene plasmid #54148 (deposited by Michael Davidson) was used to express mEmerald-Lifeact (mEmerald-Lifeact-7). pcDNA3-EGFP-Rac1-Q61L was Addgene plasmid #12981 deposited by Gary Bokoch (Subauste et al., 2000). pRK5-Myc-PIP5KIb (Rozelle et al., 2000) was kindly donated by Laura Machesky (CRUK Beatson Institute, Glasgow, UK). pSpCas9(BB)-2A-GFP (PX458) was Addgene plasmid #48138 deposited by Feng Zhang (Ran et al., 2013).

4.1.4 Synthetic peptides and recombinant proteins

E. coli strain Rosetta BL21DE3 was used to produce and purify recombinant GST and GST-Sharpin according to the manufacturers protocol (BD Biosciences). The Arp2/3 complex from bovine brain and the GST-tagged human WASP VCA domain were purchased from Cytoskeleton (Cytoskeleton Inc.). Purification of the Rabbit skeletal muscle actin has been described previously (Pollard and Cooper, 1984).

4.1.5 Cells and transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 2 % HEPES, 1 % MEM non-essential amino acids, 1 % L-glutamine, 1 % penicillin-streptomycin and 1 % sodium pyruvate. HEK-293 and U2OS cells were cultured in DMEM supplemented with 1 % L-glutamine, 10 % FBS and 1 % penicillin-streptomycin. NCI-H460 cells were cultured in RPMI1640 supplemented with 1 % penicillin-streptomycin, 1 % MEM non-essential amino acids, 1 % glucose, 1 % sodium pyruvate, 1 % L-glutamine and 10 % FBS. Generation and maintenance of the chronic proliferative dermatitis mice (*cpdm)* mouse embryonic fibroblasts cells MEF cell line has been described previously (Rantala et al., 2011). All of the cell lines were tested regularly for contaminations. All cell lines with the exception of *cpdm* MEFs, were from American Type Culture Collection (ATCC). Lipofectamine 2000 (HEK-293 and HeLa cells) and Lipofectamine 3000 (U2Os and NCI-H460 cells) (Life Technologies) were used to perform plasmid transfections. Hiperfect (Qiagen) was used to perform siRNA transfections.

4.1.6 Sharpin-knockout cell lines using CRISPR

CRISPR genome engineering was used to create Sharpin-Knockout NCI-H460 cell lines. This was done using two guide RNAs to excise a distinct 488bp region in the Sharpin-encoding gene. MIT CRISPR Designer (http://crispr.mit.edu/) was used to design these guide RNAs. These guide RNAs were individually cloned in pSpCas9(BB)-2A-GFP (PX458) as described previously (Ran et al., 2013). Co-transfection of both plasmids was done in NCI-H460 cells followed by sorting of the GFP-positive cells with FACSaria IIu Cell Sorter (BD Biosciences) 4 days post transfection. This was followed by screening for clones lacking the intervening DNA sequence by PCR forward primer 5′-GTGTCCATTTGTGGGCAAAG-3′ and reverse primer 5′-GGCACTGACCATTCTGTCCT-3′) to verify the gene disruption. Western Blots of two cell lines with the said 488 bp deletion in the Sharpin gene validated a successful knockout. The WT cells were subjected to the same procedure but did not have the deletion and showed normal levels of Sharpin expression.

4.1.7 Accession numbers

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004734.

4.1.8 Immunoblottings, immunoprecipitations and pulldowns

Immunoprecipitations, GFP-bead (ChromoTek) pulldowns and immunoblottings were performed as previously described (Pouwels et al., 2013). To perform GST pulldown assays, glutathione–Sepharose 4B beads (GE Healthcare) were washed twice with binding buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5 mM EDTA and 2 mM $MgCl_2$). This was followed by incubation of the beads with either 10 µg GST or GST–Sharpin in binding buffer (1 h rotation at 4 °C). Following this, the beads were washed and incubated with 2 µM purified Arp2/3 complex and 0.1 mM ATP (1.5 h rotation at 4 °C) in binding buffer. The beads were then washed and suspended into the loading buffer. Samples were analysed using immunoblotting.

4.1.9 Immunofluorescence of cells

Paraformaldehyde (4 %) was used to fix HeLa or NCI-H460 cells followed by staining. 3i Marianas Spinning disk confocal microscope, equipped with Yokogawa CSU-W1 scanner (Intelligent Imaging Innovations), ORCA-Flash4.0 v2 sCMOS Camera (Hamamatsu Photonics), and a Plan-Apochromat 63×1.4 NA oil objective was used to obtain the immunofluorescence images in Fig. S4 of Publication I (except Fig. S4C, D). Super-resolution radial fluctuations (SRRF) ImageJ plugin (Gustafsson et al., 2016) was

used to process images in Fig. S4G of I. A Zeiss AxioVert 200 M inverted wide-field microscope equipped with a Plan-NEOFLUAR 63×1.25 NA oil objective (Zeiss) and Orca-ER camera (Hamamatsu Photonics) was used to obtain images in Figs 4 and 5, Figs S4C, D and S5 of I. Fiji image analysis software (Schindelin et al., 2012) was used to process all images.

4.1.10 Total internal reflection fluorescence microscopy

Freshly adherent HeLa cells on 3 cm glass-bottom dishes (MatTek Corporation) coated with 5 µg/ml fibronectin, were stained for active (12G10) or total (P5D2) integrins, along with paxillin, F-actin (Atto Phalloidin) and DAPI. A Carl Zeiss Laser-TIRF 3 Imaging System equipped with a 63×1.46 NA oil objective (alpha Plan-Apochromat, DIC) and Hamamatsu ImageEM C9100-13 emccd camera (Hamamatsu Photonics) was used to obtain images of samples. Fiji software was used to perform all image analysis.

4.1.11 Fluorescence recovery after photobleaching

NCI-H460 cells on glass-bottom dishes (MatTek Corporation) expressing GFP-Sharpin, in Ham's F12 nutrient mixture with 10 % FBS and 1 % HEPES, were used for FRAP imaging. The FRAP imaging was performed with 1 s intervals using the 3i Marianas Spinning disk confocal microscope, equipped as described above. After imaging for ∼10 s, bleaching of the cellular areas was performed followed by imaging for 90–120 s. SlideBook 6 (Intelligent Imaging Innovations) FRAP analysis module was used to analyse the FRAP data. Fiji software was used to prepare movies and still images.

4.1.12 Live-cell imaging

mEmerald-Lifeact expressing NCI-H460 cells were plated on fibronectin coated (5 µg/ml) glass-bottom dishes (MatTek Corporation). These cells were incubated at 37 °C for 4 h in Ham's F12 nutrient mixture with 10 % FBS and 1 % HEPES. This was followed by imaging the cells at 37 °C at 5 frames/min for 5–10 min with a Carl Zeiss Laser-TIRF 3 Imaging System equipped as described above. Fiji software was used to prepare live cell imaging movies.

To perform live cell imaging of *cpdm* MEFs overexpressing either GFP alone, or GFP-tagged WT or V240A/L242A Sharpin, cells were sparsely plated in regular medium supplemented with 5 % HEPES onto eight-well µ-Slides (Ibidi) coated with 5 µg/ml fibronectin. Following a 6h incubation, cells were imaged at intervals of 10 min for at least 8 h under phase contrast, with GFP images taken every 10 frames. 3i Marianas Spinning disk confocal microscope, equipped as described above was used for imaging. A 10× objective was used.

Wound healing assays were performed by using Hela cells, equal number of cells were seeded on IncuCyte ImageLock™ 96-well plate (Essen BioScience). Using an Essen BioScience WoundMaker™, a wound was made in the confluent monolayer of cells the following day. IncuCyte Zoom™ System (Essen BioScience) with a 10× objective was used to image the wound closure at intervals of 2 h.

4.1.13 Pyrene-actin polymerization assay

For a final concentration of 20 µM of the pyrene-actin mixture, 5 % of pyrene-labelled actin was mixed with non-labelled G-actin in G-buffer (5 mM Tris-HCl pH 7.5 with 0.2 mM DTT, 0.2 mM $CaCl_2$ and 0.2 mM ATP). 11 µM of GST–Sharpin was mixed with 40 nM Arp2/3 and 30 nM VCA (or equal volume of VCA-buffer) in 50 mM Tris-HCl pH 7.5 with 150 mM NaCl, 3 mM DTT and 10 % glycerol in the presence of 1× initiation mix (1 mM EGTA, 5 mM $MgCl_2$, 0.25 mM ATP and NaCl to the total 100 mM in the final sample). A temperature of 22 °C was used to measure the polymerization of actin filaments with excitation at 365nm (excitation slit=10 nm) and emission at 407 nm (emission slit=20 nm) following the addition of 4 µM pyrene-actin. An Agilent Cary Eclipse Fluorescence Spectrophotometer with BioMelt Bundle System (Agilent Technologies) was used to make measurements. To analyze the data Origin 7.5 software (OriginLab Corp.) was used.

4.1.14 FACS

HeLa cells, treated for 30 min with 100 µM CK666 (Sigma-Aldrich), 10 µM CytD (Sigma-Aldrich) or DMSO, were detached and fixed with 4 % paraformaldehyde. Cells were then stained for active β1-integrin (12G10) or total β1-integrin (P5D2). FACSCalibur with CellQuest software (BD Biosciences) and non-commercial Flowing Software ver. 2.5 (Perttu Terho; Turku Centre for Biotechnology, Finland; www.flowingsoftware.com) were used to analyze the samples. Integrin Activation Index was obtained by dividing background-corrected active cell-surface integrin levels by total cell-surface integrin levels.

4.1.15 Proximity ligation assays

PLA was performed according to the protocol described previously (Söderberg et al., 2006). A Carl Zeiss LSM780 laser scanning confocal microscope equipped with 63×1.2 W Corr Apochromat objective (Zeiss) was used to obtain the images. Cell Profiler software (Carpenter et al., 2006) was used to determine the amount of PLA signal dots in one field of view. This was then divided by the number of cells, which were determined by counting the nuclei, to obtain PLA signals per well.

4.1.16 FRET measurements by FLIM

HeLa cells were transfected with either donor alone [GFP–Sharpin constructs (WT, fragments or point mutants) or Arp3–GFP] in control samples, or co-transfected with donor along with the acceptor (Arp3–TagRFP or mCherry–Sharpin). Cells were also transfected with Arp3–GFP and mCherry alone, as an additional control. Cells were then fixed and mounted using Mowiol 4-88 (Sigma-Aldrich) after 24 h. A fluorescence lifetime imaging attachment (Lambert Instruments) on a Zeiss AXIO Observer D1 inverted microscope (Zeiss) was used to measure the GFP fluorescence lifetime. A sinusoidally modulated 3W, 497 nm LED at 40 MHz under epi-illumination was used for excitation. The 63× NA 1.4 oil objective (excitation, BP470/40; beam splitter, FT495; emission, BP525/50) was used to image the cells. The phase and modulation were determined using the manufacturer's software from images acquired at 12 phase settings. As a reference standard, fluorescein at 0.01 mM, pH 9 was used. The apparent FRET efficiency was calculated using the measured lifetimes of each donor-acceptor pair (τ_{DA}) and the average lifetime of the donor-only (τ_D) samples, according to:

$$\text{FRET efficiency} = \left(1 - \left(\frac{\tau DA}{\tau D}\right)\right) \times 100\%$$

4.1.17 Micropatterns

As described previously (Azioune et al., 2009), linear micropatterns of 9 µm were printed on glass coverslips. These were then coated with 5 µg/ml fibrinogen (Alexa Fluor 488 conjugate; Thermo Scientific) and 50 µg/ml fibronectin followed by three washes with PBS. NCI-H460 cells in antibiotic free medium were seeded onto these coverslips and incubated for 7 h. This was followed by fixing the cells and staining as described above. A Zeiss AxioVert 200 M inverted wide-field microscope equipped with Plan-NEOFLUAR 100× 1.30 oil objective (Zeiss) and Hamamatsu Orca-ER camera (Hamamatsu Photonics) were used to image these cells. Fiji software was used to process the images.

4.1.18 Statistical analysis

GraphPad Prism version 5.03 for Windows (GraphPad Software) was used to perform all statistical analyses. For normally distributed data, the Student's *t*-test was used (Shapiro-Wilk normality test $\alpha=0.05$). All other data was analyzed using the Mann–Whitney test where $P<0.05$ was considered significant.

4.2 Sharpin regulates granulocytes rolling and adhesion *in vivo* (unpublished, hereafter called II)

4.2.1 Animals

Mouse strain (C57BL/KaLawRij-Sharpin knockout/RijSunJ (Stock No: 007599)) with the spontaneous mutation leading to complete loss of Sharpin protein (HogenEsch et al., 1993; Seymour et al., 2007) were obtained from The Jackson Laboratory (Bar Harbor, ME). The colony was maintained by heterozygote breeding and genotyping as previously described (Peuhu et al., 2017; Peuhu, Salomaa, et al., 2017). Mice used in the experiments were age-matched seven weeks old males. Standard housing conditions (12-hours day/night cycle) with food and water made available as desired. The mice were monitored daily for clinical signs and viability. Cervical dislocation in conjunction with CO_2 was selected as the method of euthanasia. Ethical assessment and authorisation of experiments was done by the National Animal Experiment Board and were in conformance with The Finnish Act on Animal Experimentation (Animal license numbers ESAVI-9339-04.10.07-2016 and 5587/04.10.07/2014).

4.2.2 Intravital microscopy

This method was adapted from Heit et al., (Heit et al., 2008) and performed as previously described by Stolen et al., (Stolen et al., 2005). The mice were briefly anaesthetised by administering ketamine (125 mg/kg body weight; Pfizer) and xylazine (12. 5 mg/kg body weight; Bayer) intraperitoneally. Dissection and exteriorization of the cremaster muscle were done on a special microscopic stage. Thermo-controlled bicarbonate buffered saline was continuously superfused in the exposed cremaster muscle. 0.1µm of fMLP (Heit et al., 2008; Khajah et al., 2013) was added to the superfusion buffer for granulocytes stimulation.

Endothelial interactions of granulocytes were analysed at 0 min (before addition) and 5, 20, 30, 45 and 60 min following the addition of fMLP. Microscopy was performed using the Olympus microscope (BX50WI) using the water immersion objective and a CCD camera (C5405, Hamamatsu). A dual photodiode and a digital online cross-correlation program (CircuSoft) was used to measure the velocities of centreline red blood cells. An empirical factor of 0.625 was multiplied with these velocities to calculate the mean blood flow velocities. The wall shear rates (s-1) were estimated to be 2.12 x 8 x Vb/d, where 2.12 is a median empirical correction factor, Vb is the mean blood flow velocity, and d is the diameter of the vessel (Stolen et al., 2005). VetScan HM5 (Abaxis) was used to measure the granulocyte concentration (granulocytes /ul) from systemic blood samples. Imaris 8.1.2 software was used to measure all other parameters as

previously described (Jung et al., 1998). Postcapillary venules with a diameter between 20 and 50 µm were analysed (number of analysed vessels; 20.0/mouse).

The rolling flux, expressed as granulocyteocyte/min, was ascertained by counting the number of rolling granulocytes passing a perpendicular line to the axis of the vessel. The rolling flux fraction reflects rolling efficiency, which was determined by dividing rolling flux by total granulocyte flux through the same vessel per unit time. The total granulocyte flux was estimated as [WBC] x Vb x π x $(d/2)^2$, where [WBC] is the systemic granulocyte count. The rolling velocity was measured by observing the time required for a granulocyte to roll through a 100µm vessel segment. This measurement was taken for ten rolling cells per vessel. The number of cells stuck to the endothelium and were immobile for more than 30 s in a 100µm cells segment was defined as granulocyte adhesion. Adhesion efficiency (adherent cells/mm^2 endothelial surface area per 100 rolling cells/min) is a measure for the capability of rolling granulocytes to firmly adhere. Transmigration is the number of extravasated cells which is perivenular tissue within 50 µm of the 100 µm vessel segment, and transmigration efficiency was calculated as the transmigration/adherent intravascular cells.

4.2.3 Intrascrotal staining of blood vessels for Immunofluorescence

In order to label blood vessels, mice under isoflurane anesthesia were injected (intrascrotally) with an Alexa488-conjugated antibody against mouse CD31 (2 µg; MEC13.3, BD Biosciences) in saline. Euthanasia was performed on the mice two hours later, followed by dissection of the cremaster muscle. The dissected cremaster muscle was then pinned flat on silicone coated plates (Sylgard 184 Silicone elastomer, Dow Corning). Fixation of the cremaster muscle using 4 % paraformaldehyde for 1 hour was done next. Samples were then washed followed by mounting with DAPI Vectashield (Vector Laboratories). Imaging of the samples was done using the 20x objective of the Carl Zeiss LSM780 laser scanning confocal microscope. The images were then analysed with ImageJ software to present Maximum intensity projections.

4.2.4 Neutrophil isolation from peripheral blood and bone marrow

Mice were euthanised by cardiac puncture method, and whole blood samples were collected in heparin, followed by lysis of red blood cells as described in the Flow Cytometry section. As an alternative, a femur from the euthanised mice was cut in half followed by flushing of the bone marrow with a syringe. This was done in order to obtain a larger and pure neutrophil population. A Neutrophil Isolation Kit (mouse, 130–097-658, Miltenyi Biotec) was used to isolate granulocytes from cell suspensions, as instructed by the manufacturer.

4.2.5 Assesement of expression levels of adhesion molecules using flow cytometry

In order to assess surface expression of key adhesion molecules on granulocytes, flow cytometry experiments were performed (II, Fig. 3), mice were first euthanised by cardiac puncture, and whole blood samples were collected in heparin. These samples were placed on ice and divided to 250ul per tube. Next, Fc-block was added, followed by directly conjugated antibodies on ice in recommended concentrations. (Ly6G-PE (551461), Ly6G-FITC (551460), ItgaL-FITC (CD11a, 553120), ItgaM-APC (CD11b, 553312), PSGL-1-PE (CD162, 555306) Itga4-PE (CD49d, 553157) CD11b-APC (553312), Gr-1-FITC all from BD Pharmingen, and L-selectin-Alexa647 (CD62L, 626-C100, EXBIO)). The samples were incubated at 37 ºC in a water bath for 5 min and then placed on ice. 500µl of cold PBS was added and samples were centrifuged at 3000 rpm for 3 min. The samples were then washed once with cold PBS followed by resuspension in 750ul of 1 % PFA in PBS. Samples were kept on ice for 10 min and then pelleted as before. In order to lyse the red blood cells, the cell pellet was briefly vortexed followed by addition of 1ml 0.2 % NaCl and then vortexed again for 18 sec. This was followed by addition of 1ml 1.6 % NaCl and vortexing again for 18 sec. The pelleted cells were then resuspended in PBS. FACS Calibur flow cytometer (BD Biosciences) was used to run these samples. Flowing Software was used for gating granulocytes based on FSC/SSC dot plot and Ly6G positivity. The geometric mean fluorescence (geoMFI) was measured for the gated population. To obtain the relative geoMFI, Sharpin-knockout samples were compared to the wt control in each independent experiment.

To obtain splenocytes for FACS analysis (II, Fig. S1), isolation of the spleen was done from adult mice and then homogenised mechanically. In order to obtain single cell suspension of splenocytes, this homogenised spleen was strained using a 70µm cell strainer (BD Biosciences). These samples were then processed as described above and labelled with CD11b-APC and Gr-1-FITC (A) or with CD11b-APC, Ly6G-PE and Ly6C-FITC and different cell populations were examined using flow cytometry. Different cell populations were defined based on the indicated SSC and expression profiles (II, Fig. S1).

4.2.6 ICAM-1 adhesion assay

A 96-well E-plate (Roche) was coated with 0.5 µg/ml ICAM-1 or 5 µg/ml ICAM-1 (796-IC-050; R&D systems) in PBS overnight at +4 °C. This was followed by blocking with 0.1 % BSA in PBS at 37 °C for one hour. fMLP (1µM for 5 minutes at 37 °C water bath) was used to stimulate granulocytes followed by addition of the stimulated granulocytes (200 000 granulocytes per well) in serum-free media. xCELLigence RTCA instrument (Roche) was used to measure impedance which is expressed as a cell index. Cell index

represents the impedance between the electrodes, present at the bottom of the plate. This impedance is used as a measure of cell adhesion.

4.2.7 Quantitative PCR

NucleoSpin RNA kit (Macherey-Nagel) was used to isolate total RNA from isolated granulocytes from the bone marrow of wt and Sharpin-knockout mouse. High-capacity cDNA reverse transcription kit (Applied Biosystems) was used according to the manufacturer's protocol to make cDNA from total RNA (1 ug). To quantify expression levels of the different proteins, a TaqMan qPCR reaction was performed using Real-Time PCR HT7900 (Applied Biosystems). Expression of beta-actin was used as an endogenous control. The TaqMan Universal Master Mix II incorporated the essential constituents required for a qRT-PCR reaction. The universal probes and primers used for this purpose are listed in table 2.

4.2.8 *In vitro* shear flow adhesion assay

The Neutrophil isolation kit (MACS Miltenyi Biotec) was used to isolate granulocytes from bone marrow using negative selection. Shear flow adhesion assays were performed as described by others (San Lek et al., 2013). Coating of 6 µg/ml ICAM-1, 5 µg/ml CXCL1, and 30 µg/ml E-selectin (R&D Systems, Minneapolis, MN) or only ICAM-1 was done on Ibidi VI0.4 µ-slides (Germany) overnight at 4 °C. Cells were filmed for 5-min period on ICAM-1/CXCL1/E-selectin-coated slides at a high (5 dynes/cm^2) shear flow rate, whereas for ICAM-1-coated wells a lower shear flow rate was used (0.3 dynes cm^{-2}). In one condition, cells on ICAM-1 coated slides were stimulated with 1µM fMLP, and the flow assay was performed at a low shear flow rate (0.3 dynes/ cm^2). Manual counting was employed to count the number of cells adhered in the field of view.

Table 2. Primers and probes used in Taqman PCR

Protein	Primer sequence		Universal probe used
	Forward primer	**Reverse primer**	
L-selectin	tggtcatctccagagccaat	gcagtccatggtacccaact	47
CD11a	ccccagacttttgctactgg	cgtgtgtccaggttgtagctc	78
PSGL-1	tctggcagtgtggactgg	caaggaagcttggggacat	13
CD11b	aaggatgctggggaggtc	gtcataagtgacagtgctctggat	16
ITGB2	cccagtgtgagtgtcagtgc	tcccaatgtagccagactca	32
Beta-actin	tggctcctagcaccatgaaga	gtggacagtgaggccaggat	5´caagatcattgctcctcctgagcgca (custom made probe)

5. RESULTS AND DISCUSSION

5.1.1 The Sharpin interactome reveals a role for Sharpin in lamellipodium formation via the Arp2/3 complex (Publication I, hereafter called I)

The multifunctional adaptor protein Sharpin regulates many signalling pathways (Chattopadhyay et al., 2016; Dubois et al., 2014; Rodgers et al., 2014; Tokunaga et al., 2009; Zak et al., 2011) as part of LUBAC (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). In addition, Sharpin binds and regulates a range of other proteins (He et al., 2003; Landgraf et al., 2010; Lim et al., 2001; Nastase et al., 2016; Park et al., 2016; Rantala et al., 2011). Significantly, Sharpin has oncogenic properties (Bii et al., 2015; De Melo and Tang 2015; He et al., 2010; Jung et al., 2010; Li et al., 2015; Zhang et al., 2014), and LUBAC has been linked to cancer progression (Yang et al., 2016, 2014), and immune-related diseases (Boisson et al., 2015; Lewis et al., 2015). However, despite these clear disease links and the rapid development of the linear ubiquitin and Sharpin research fields, a comprehensive overview of Sharpin and LUBAC functions has not been reported.

Therefore, to make a comprehensive study of Sharpin´s cellular interactors, HEK-293 human embryonic kidney cell line having a transient expression of GFP-Sharpin and GFP alone were used for a GFP pull-down and analysed by mass spectrometry. As Sharpin interacts with integrins and inactivates them (Rantala et al., 2011), this MS screen was intended to enrich novel interactors of integrin-bound Sharpin. Integrins are essentially active when cells are cultured on Fibronectin (GFP–Sharpin adherent dataset), and they are inactive when cells are in suspension (GFP–Sharpin Non-adherent dataset). Non-adherent cells should have more integrin-bound Sharpin as integrins are inactive in these cells (Rantala et al., 2011).

Three arbitrary thresholds were selected, which reflected the specificity as well as the quality of the putative Sharpin interactors in order to list and score them (I, Suppl. Table 1). 690 proteins were allocated in low confidence, these proteins were identified with at least 4 spectra and enriched two-fold in the GFP-Sharpin datasets (suspension or adherent) over control. 297 proteins were allocated in medium confidence, these proteins were identified with at least 5 spectra and enriched two-fold in the GFP-Sharpin datasets (suspension or adherent) over control. 48 proteins were allocated in high confidence, these proteins were identified with at least 10 spectra and enriched two-fold in the GFP-Sharpin datasets (suspension or adherent) over control (I, Suppl. Table 1).

All the proteins recruited to Sharpin were defined using functional annotation clustering based on gene ontology (GO) using DAVID (Huang et al., 2009) and presented as network-based enrichment maps (Merico et al., 2010) (I, Fig. 1A, Table S2). Overall, the MS screen identified many protein hits, which are involved in known processes to be regulated by Sharpin or LUBAC, for instance, cell death (Gerlach et al., 2011; Kumari et al., 2014; Nastase et al., 2016), ubiquitin-dependent processes and cell signalling. The last one includes NF-κB signalling (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011), providing important validation of the MS screen. DNA/RNA-based processes, regulation of the cell cycle, metabolic processes, and protein transport are examples of those cellular processes, which have never been linked to Sharpin or LUBAC.

The focus have been on Arp2/3, but MS analysis provided us with a list of E2 and E3 ubiquitin ligases as potential Sharpin interactors as well, such as UBE2V1, UBE2D3, STUB1 and RNF114. It would be interesting to investigate the interplay of these ubiquitin ligases with Sharpin. Does Sharpin have specific function with ubiquitin ligases? Perhaps Sharpin releases these ligases from their auto-inhibitory state (if they exist in any such auto-inhibition state) and regulates their functions. Sharpin could also be a substrate for the E3 ligases.

5.1.2 Validation of interaction between Sharpin and the Arp2/3 complex

Two members of the Arp2/3 complex were present in the Sharpin interactome, as well as many other proteins which are associated with the Arp2/3 complex (I, fig 1C). We validated the Sharpin-Arp2/3 interaction by various molecular biology techniques and confirmed the direct interaction of Sharpin and the Arp2/3 complex (I, Fig. 2A-F). Further experiments provided us with an insight that this interaction requires an intact cytoskeleton and active Arp2/3 complex (I, Suppl. Fig. S3A, C).

These findings prompted us to investigate the role of Sharpin in the Arp2/3-dependent *in vitro* actin polymerization assay (Prehoda et al., 2000) using commercially available Arp2/3 complex and recombinant GST-Sharpin (Rantala et al., 2011) (I, Fig. 2G,). We saw no change in the Arp2/3 activity in the absence or presence of VCA (the WASP verprolin-cofilin-acidic homology domain), which activates the Arp2/3 complex (Prehoda et al., 2000). Interestingly, unpublished data from our lab suggest that Sharpin phosphorylation is required to bind the Arp2/3 complex (Butt et al., unpublished data), which could explain why Sharpin is not able to make any change in the Arp2/3 activity *in vitro* actin polymerization assay. This suggests that Sharpin-mediated Arp2/3 complex activation requires another, yet to be identified protein. It is also possible that post-translational modifications of Sharpin might be obligatory for Sharpin-mediated Arp2/3 activation.

In addition to the Arp2/3 complex, the Sharpin interactome contains several other regulators of the cytoskeleton (I, Fig. 2B), such as Destrin (DSTN), Twinfilin 1 (TWF1) and TWF2. Twinfilin is ADP-actin-monomer-binding protein enriched at the cortical actin cytoskeleton and at the ends of actin filaments bundles (Palmgren et al., 2002; Vartiainen et al., 2000), and DSTN depolymerises actin filaments and enhances the turnover rate of actin *in vivo* (Carlier and Shekhar, 2017). Dynamic polymerization and depolymerization of actin filaments are required for lamellipodia formation and its retraction. Whether Sharpin regulates, any other cytoskeletal proteins and how this affects lamellipodia formation requires further studies.

5.1.3 The Arp2/3 specific binding deficient Sharpin mutant

Sharpin consists of three domains; an N-terminal PH domain, (Lim et al., 2001; Stieglitz et al., 2012a) a central UBL domain, which is the hub of protein interaction (De Franceschi et al., 2015) and a C-terminal NPL4 zinc finger domain (NZF), which is responsible for the interaction with the T cell receptor (Park et al., 2016). Using individual GFP-tagged Sharpin domains (De Franceschi et al., 2015) in GFP pulldown and FRET/FLIM interaction experiments we showed that the Arp2/3 complex interacts with the UBL domain of Sharpin (I, Fig. 3A, B).

Due to the lack of structural data on Sharpin UBL domain, a model of the Sharpin UBL domain was created using the Swiss Model Server (Arnold et al., 2006). Surface hydrophobicity analysis (Fig. 12) was performed in order to identify putative interaction sites (De Franceschi et al., 2014), which were mutated into alanines, resulting in the creation of three single and three double Sharpin mutants; L276A, I272A, V267A, L261A/F263A, E260A/L261A and V240A/L242A. It has been previously shown that Sharpin-I272A abolishes HOIP binding (Ikeda et al., 2011).

We used these mutants in our FRET-FLIM experiments, and showed that three mutations (V240A/L242A, V267A and L276A) in the Sharpin UBL domain (De Franceschi et al., 2015) abolish the binding between GFP–Sharpin and Arp3–TagRFP (I, Fig. 3C). V240A/L242A mutant appears to be specifically unable to interact with the Arp2/3 (I, Fig. 3C) as this mutation does not affect Sharpin-dependent integrin inhibition and NF-κB activation (De Franceschi et al., 2015). The other mutants (E260A/L261A, L261A/F263A and I272A) did interact with the Arp2/3 complex, although their binding was reduced (I, Fig. 3C). Using GFP-trap beads pull-down, we confirmed that V240A/L242A mutant of Sharpin had reduced ability to interact with the Arp2/3 complex (I, Fig. 3D).

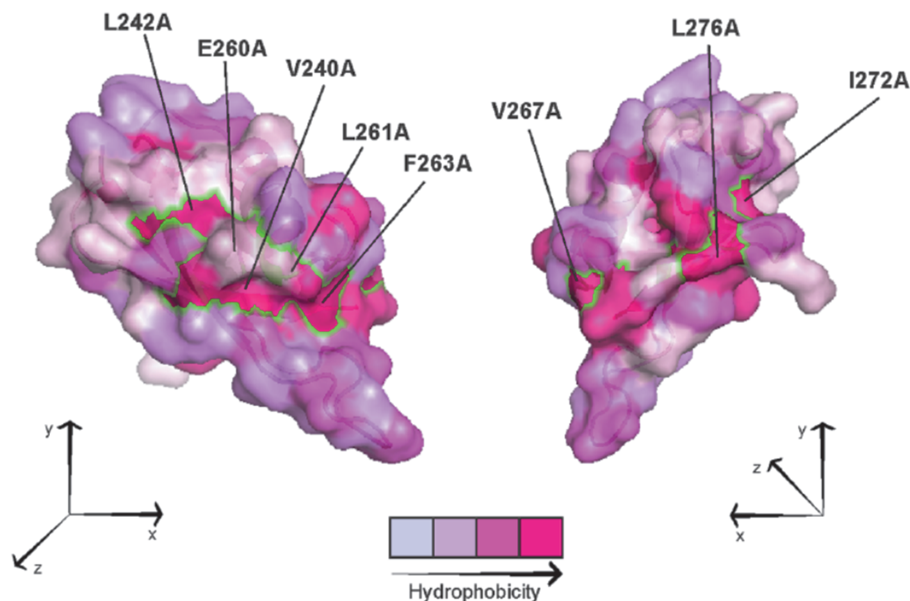

Fig 12. Sharpin UBL domain surface model, residue hydrophilicity is shown from light to deep purple. Residues mutated are indicated as green outlines. [Copied from (De Franceschi et al., 2015)].

In summary, with the help of these experiments, we mapped the Arp2/3 interaction site in the Sharpin UBL domain and, essentially, detected a Sharpin mutant (V240A/L242A) that is specifically not capable to interact with the Arp2/3 complex.

5.1.3.1 The UBL domain of Sharpin is a protein interaction hub

It is evident from our study (I, Fig. 3C,D, 5A, B) and from De Franceschi et al., 2015 that Sharpin´s UBL domain plays a critical role in the interaction between Sharpin and most of its interactors (De Franceschi et al., 2015). A full length protein´s crystal structure would be extremely useful to define which other amino acids play a decisive role for the interaction to take place successfully. A very recent study by Jianping Liu et al., reported a crystal structure of Sharpin UBL in complex with HOIP UBA domain (Liu et al., 2017). Another report by Stieglitz et al., solved the crystal structure of PH domain of Sharpin and suggested that this domain is responsible for the dimerisation of Sharpin (Stieglitz et al., 2012). It seems that the crystal structure of a full-length protein is quite challenging to obtain, as it is not yet reported. Sharpin has diverse functions in many cellular processes. Generally, proteins attain diversity via post-translational modifications. Recombinant proteins produced in prokaryotic organisms lack post-translational modifications. To attain any specific stable protein confirmation, Sharpin

might need these post-translational modifications. This might be a reason that recombinant Sharpin produced in bacterial expression system lacks proper protein folding, and this in turn might be the reason why a full-length Sharpin crystal structure is still missing. Altogether, the link of these mutations to the diseases is still missing, and it falls into the category of research which needs further investigation.

5.1.4 Sharpin-Arp2/3 interaction promotes lamellipodia formation

Previous studies showed a role for Sharpin and the Arp2/3 complex in lamellipodia formation (Rogers et al., 2003; Rotty et al., 2012; Wu et al., 2012; Rantala et al., 2011). We confirmed this, by observing fewer lamellipodia in Sharpin and Arp3-silenced NCI-H460 lung cancer cells (I, Fig. 4A). However, silencing of HOIP did not interrupt the lamellipodium formation, which implies that this function of Sharpin is independent of LUBAC (I, Fig. 4A). These findings were also confirmed using two monoclonal Sharpin knock out NCI-H460 cell lines created using CRISPR (I, Suppl. Fig. S5C), which also showed reduced lamellipodia formation (I, Fig. 4B).

We clearly showed that cells have a reduced number of lamellipodia in the absence of Sharpin (I, FIG. 4). However, these experiments did not reveal whether Sharpin promotes the formation of new lamellipodia, or whether Sharpin is needed to stabilize existing lamellipodia. To address this, we plated Sharpin and control silenced cells on line-shaped micro-patterns (Azioune et al., 2009), and treated them with CK666 (a known inhibitor of the Arp2/3 complex (Nolen et al., 2009)) (I, Fig. 4D, S5B). CK666 treatment inactivates the Arp2/3 complex and abolishes lamellipodia (Brayford et al., 2016). Upon wash-out of the inhibitor, Arp2/3 activity is reinstated, and new lamellipodia are formed within minutes (Liu et al., 2013; Nolen et al., 2009), allowing us to tease out the role of Sharpin in the very early stages of lamellipodium formation (I, Fig. 4D, S5B). It was observed that while control silenced cells effectively formed lamellipodia within minutes after CK666 washout, Sharpin-silenced cells showed significantly reduced number of lamellipodia in this time frame (I, Fig. 4D, S5), showing that Sharpin plays a role in the formation of new lamellipodia.

Serum contains growth factors and cytokines, such as EGF and PDGF, which, upon binding to their respective receptors (such as EGFR and PDGFR) can trigger and promote lamellipodium formation (Zimmermann et al., 2006). To determine whether Sharpin promotes receptor-induced lamellipodium formation, control or Sharpin silenced U2OS cells were serum starved overnight followed by serum stimulation for 120 min to induce lamellipodia formation. Serum starvation abrogated lamellipodia in control silenced cells. Subsequent replacement of the medium with serum supplemented medium induced a burst of lamellipodia (I, Fig. 4C). However, in Sharpin silenced U2OS cells a decreased induction of lamellipodium formation was observed

after serum stimulation (I, Fig. 4C). This suggests that Sharpin regulates the lamellipodia formation induced by receptor signalling.

To investigate whether Sharpin has a role in lamellipodium formation driven by Rac1, NCI-H460 cells were expressed with either constitutively active GFP-Rac1 (Q61L) or GFP alone in WT and Sharpin CRISPR knockout NCI-H460 cell lines (KO1 and KO2). Upon overexpression of Rac1 (Q61L), large, flat and round WT cells accompanied by the presence of profound lamellipodia were observed. The same effect was observed in *Sharpin^{cpdm}* cells overexpressing GFP–RacC1 (Q61L) (Fig. S6A). This indicates that Sharpin is not essential for lamellipodia formation driven by Rac1. Validation of these results was done using control or Sharpin silenced HeLa cells; where overexpression of GFP-Rac1 (Q61L) brought about similar changes in cell shape (Fig. S6B; circularity of cells was also quantified for these cells).

These experiments confirmed the role of Sharpin in lamellipodia formation and also that this function of Sharpin is independent of LUBAC. *In vitro*, Sharpin does not directly affect Arp2/3 activity, although this could be different in cells, for example due to the lack of additional proteins or posttranslational modifications. Thus, we have not pinpointed the exact molecular mechanism behind the Sharpin-Arp2/3 interplay. However, we did show that Sharpin is needed for receptor-induced, but not Rac-induced lamellipodium formation, although one could argue that the constitutively active Rac used for the latter was not physiologically relevant as the Arp2/3 inhibitor CK666 was also unable to prevent formation of these Rac-induced lamellipodia. Future experiments could be aimed at finding out which signaling pathway Sharpin is involved in to control the Arp2/3 complex and lamellipodia formation.

5.1.5 The Role of Sharpin and the Arp2/3 interaction in cell migration

Using a wound healing assay, previous studies have highlighted that the Arp2/3 complex plays a role in cell migration (Liu et al., 2013; Suraneni et al., 2012). In line with these reports, we observed decreased wound healing upon Arp3 silencing in HeLa cells (I, Fig. S6C). On the contrary, Sharpin silencing did not affect wound healing under these conditions (I, Fig. S6C). This is consistent with the previous work by Rantala et al., where they reported that Sharpin silencing in several cell lines increases integrin activity, which affects cell migration (Rantala et al., 2011). These results suggest that Sharpin dependent cell migration is regulated by various interactors, for instance, it inhibits cell migration by inactivating integrins, and in the same cell, it promotes lamellipodia formation by interacting with the Arp2/3.

To tease out the role of the Sharpin-Arp2/3 interaction in cell migration, a random migration assay was performed using live cell imaging. Sharpin deficient immortalised

mouse embryonic fibroblasts (MEFs) (Rantala et al., 2011) were used in this assay. We overexpressed different GFP constructs (GFP alone, GFP–Sharpin WT or GFP–Sharpin V240A/L242A). MEFs overexpressing GFP-Sharpin WT exhibited increased cell migration, compared to cells overexpressing GFP only (I, Fig. 6). However, MEFs having GFP–Sharpin V240A/L242A mutant, which is unable to interact with the Arp2/3 complex but is capable of inactivating integrins, migrated slower as compared to GFP-Sharpin WT alone cells. The probable reason for this observation is that this construct (GFP–Sharpin V240A/L242A) is a fully active integrin binding Sharpin protein, but it is not able to interact with the Arp2/3 complex leading to the slow migration (I, Fig. 6). This observation suggests that Sharpin-Arp2/3 interaction plays a role in cell migration.

5.1.6 Sharpin, a novel player in Arp2/3 or actin dependent cellular processes

Taken together, these results suggest that the Sharpin-Arp2/3 interaction is physiologically significant as it stimulates lamellipodium formation and cell migration. The Arp2/3 has many other significant roles inside the cell as well (Rotty et al., 2012), e.g. membrane trafficking. The Sharpin interactome comprises proteins involved in endocytosis, such as RAB13 (I, Table S2). RAB13 plays an important role in regulating membrane trafficking between the trans-Golgi network (TGN) and recycling endosomes (RE) (Morimoto et al., 2005). Further analysis is required to establish how Sharpin affects RAB13 functions.

Other fascinating hits in the MS analysis were RhoA and Rac1. Both are important for focal adhesion maturation and actin filaments organization (Nobes and Hall 1995; Chrzanowska-Wodnicka and Burridge 1996; Rottner et al., 1999; Kuo 2013). A recent study from Peuhu et al., 2016 reported larger focal adhesions and faster assembly and disassembly rates of focal adhesion in Sharpin-deficient mammary gland fibroblasts (Peuhu et al., 2017), signifying Sharpin´s role in focal adhesion dynamics. This could potentially lead to a novel mechanism governed by Sharpin via RhoA or Rac1 in dynamics of focal adhesion assembly and disassembly.

A very recent paper by Kammen et al., shows that depletion of the Arp2/3 complex in mouse epidermis causes a severe psoriasis-like skin disease (Kammen et al., 2017). Similar skin phenotype is observed in Sharpin-deficient mice, which exhibit chronic proliferative dermatitis and psoriasis (Gijbels et al., 1996; Ikeda et al., 2011; Gerlach et al., 2011; Tokunaga et al., 2011), which might indicate that both Sharpin and the Arp2/3 complex are either involved in a common signalling pathway or regulate a common protein, which leads to psoriasis-like disease in their absence. As the Arp2/3 knockout mouse is embryonically lethal, the other phenotypes of Sharpin mouse, such as multi-

organ inflammation and chronic eosinophilia, can further be studied only using conditional knockouts of the Arp2/3 complex.

A recent review by Nicolas & Gautreau nicely correlated the Arp2/3 complex and its associated proteins to cancer (Molinie and Gautreau, 2018). In this report, they mentioned that the Arp2/3 is hyper-activated in several types of cancer along with many Nucleation Promoting Factors (NPFs) (Molinie and Gautreau, 2018). NPFs, such as WAVE and N-WASP, activate the Arp2/3 complex. Arpin, which inhibits the Arp2/3 complex and keeps the cell migration controlled (Fetics et al., 2016) is downregulated in many different cancer types (Molinie and Gautreau, 2018). Interestingly, Arpin was also in the MS analysis as a putative interactor of Sharpin (I, Fig. 1A). Sharpin is overexpressed in several different types of cancer (Bii et al., 2015; De Melo and Tang 2015; Jung et al., 2010; Li et al., 2015; Zhang et al., 2014; He et al., 2010). If shown that Sharpin downregulates Arpin and keeps the Arp2/3 complex in the hyper-activated state to accomplish an increased invasion and metastasis, it would be a novel mechanism. The GO analyses suggest Sharpin regulate many cytoskeleton proteins (I, Fig. 1B). These cytoskeleton proteins, such as RhoA, Cdc42 and Rac1 directly influence cell invasion and metastasis (Ridley, 2015). Perhaps Sharpin make conducive environments for cancerous cells to invade surrounding tissues by regulating theses putative interactors. Our finding, where Sharpin interacts with cytoplasmic Arp2/3 and promotes its function in cell migration, and lamellipodia formation can be investigated further to confirm the potential role in cancer progression.

5.2 Sharpin regulates slow rolling and adhesion in granulocytes (unpublished, hereafter called II)

5.2.1 Under resting conditions, granulocytes from both *Sharpin*^{cpdm} and wild-type mice show similar transmigration

Inflammation or infection causes transmigration of granulocytes towards the infected site via the endothelial layer of vessels. This transmigration of granulocytes is a complex process of innate immunity, which is impaired in several inflammatory diseases or cancer. Transmigration of these granulocytes comprises four significant steps; rolling, activation, arrest and transmigration. These successive and coinciding steps (Nourshargh and Alon, 2014) are regulated by the expression and activity of different receptors present on both granulocytes and endothelial cells. Pouwels et al., reported that Sharpin causes inactivation of integrin αLβ2 (LFA-1) (Pouwels et al., 2013) and this inactivation is essential for the release of the trailing edge of the granulocyte after crossing the endothelial layer, the last step of granulocyte transmigration.

We investigated the role of Sharpin in the early steps of granulocyte transmigration in this study. Post-capillary veins in the cremaster muscles of wild-type (wt) and Sharpin deficient (Sharpin deficient hereafter will be called as *Sharpin^{cpdm}* or Sharpin lacking) mice were observed under the intravital microscope, although other hemodynamic parameters [blood vessel size (vessel diameter), blood flow (centerline velocity) and wall shear rate)] were normal in the cremaster muscles of *Sharpin^{cpdm}*. The vasculature in *Sharpin^{cpdm}* cremaster muscle appeared normal (II, Fig. 1A); unlike the previously reported increased angiogenesis in *Sharpin^{cpdm}* mice skin (HogenEsch et al., 2016).

Under resting conditions, at t=0 (II, Fig 1B-H), increased rolling flux was detected in *Sharpin^{cpdm}* mice compared to wt using real-time intravital microscopy (II, Fig1B). Rolling flux represents the total number of rolling granulocytes per minute. Because the total number of leucocytes was increased in *Sharpin^{cpdm}* mice compared to wt mice, we needed to normalise quantifications to the total number of granulocyte and relate each step to the previous one (II, Table 1) (Gurung et al., 2016; Liang 2011; Potter et al., 2014). Therefore, we determined the 'Rolling flux fraction', which is the number of rolling granulocytes per minute divided by the total number of granulocytes that pass the vessel in the same time frame. Rolling flux fraction for both *Sharpin^{cpdm}* and wt mice shows no significant difference (II, Fig. 1C), which shows that initial selectin-dependent adhesion to the endothelium is independent of Sharpin. While we observed a modest increase in rolling velocity in *Sharpin^{cpdm}* mice in resting conditions at t=0 (II, Fig 1D), other steps of granulocyte transmigration were unaffected (II, Fig 1E-H). Those steps are adhesion (II,number of adherent granulocytes per mm^2; Fig. 1E), adhesion efficiency (II, adherent granulocytes per mm^2 divided by the number of rolling granulocytes /min; Fig. 1F), transmigration efficiency (II, transmigrated granulocytes per mm^2 divided by adherent granulocytes per mm^2; Fig. 1G) and overall transmigration efficiency (total number of extravasated granulocytes per 100 µm of vessel (II, Fig. 1H)). Together, under resting conditions, these results demonstrate that, apart from a modest reduction in rolling velocity, transmigration proceeds normally in Sharpin-deficient mice.

5.2.2 Impaired transmigration observed in *Sharpin^{cpdm}* granulocytes upon stimulation

In addition to the un-induced state, we studied chemoattractant-induced granulocyte transmigration in wt and *Sharpin^{cpdm}* mice. Granulocyte transmigration was induced using a synthetic peptide (Trp-Lys-Tyr-Met-Val-D-Met-NH2 (WKYMVdM)) in the cremaster muscle by infusion. This peptide mimics N-Formylmethionine-leucyl-phenylalanine (fMLP) (Marasco et al., 1984) oligopeptides released by bacteria (Heit et al., 2008; Khajah et al., 2013), and stimulates blood granulocytes via specific formyl-

peptide receptors (Marasco et al., 1984). TNFα or IL-1 stimuli, which are normally used to stimulate granulocytes, cannot be used due to the fact that they stimulate granulocyte via an NF-κB dependent pathway, which is impaired in *Sharpin^cpdm^* mice (Ikeda et al., 2011; Gerlach et al., 2011; Tokunaga et al., 2011). fMLP, on the other hand, causes chemotaxis independent of the NF-κB pathway.

To study the role of Sharpin in inflammation-induced transmigration, all the transmigration steps were quantified at 5, 20, 30, 45 and 60 minutes after fMLP treatment (II, Fig. 1B-H). Major defects in transmigration steps of stimulated granulocytes in *Sharpin^{cpdm}* mice were observed from t=5 minutes to t=60 minutes (II, Fig. 1B-H), suggesting that Sharpin-deficient granulocytes have defective inflammation-induced transmigration. Granulocyte rolling was largely unaffected in *Sharpin^{cpdm}* mice as shown by the rolling flux (II, Fig. 1B) and rolling flux fraction (II, Fig. 1C). Though, unlike wt granulocytes, *Sharpin^{cpdm}* granulocytes demonstrated mild decrease in rolling velocity after fMLP treatment (II, Fig. 1D), indicating defective conversion from fast to slow rolling. Moreover, fMLP-induced granulocyte adhesion was strongly reduced in *Sharpin^{cpdm}* mice (II, Fig. 1E, and F). Transmigration efficiency, (II, Fig. 1G) in *Sharpin^{cpdm}* and wt granulocytes were similar, suggesting that the final step in these mice are not dependent on Sharpin.

All-in-all, our intravital microscopy data show that while under resting condition granulocyte migration is similar in wt and *Sharpin^{cpdm}*, granulocytes demonstrate strongly reduced fMLP-induced slow rolling and firm adhesion in *Sharpin^{cpdm}* mice. This reduced capacity of *Sharpin^{cpdm}* granulocytes to slow down and adhere, might impose risk of infection or inflammation in these *Sharpin^{cpdm}* mice.

5.2.3 *Sharpin^{cpdm}* granulocytes adhere better in ICAM-1 adhesion assay

Sharpin is a known inhibitor of integrins (Pouwels et al., 2013; Rantala et al., 2011) including granulocyte-specific αLβ2 integrins (Pouwels et al., 2013). This inactivation of αLβ2 causes increased adhesion of human peripheral blood mononuclear cells on the ICAM-1 substrate. The steps of transmigration, such as slow rolling and firm adhesion heavily depend on αLβ2 integrin activity in granulocytes. Therefore, our intravital microscopy data that *Sharpin*^{cpdm} mice granulocytes have reduced ability to convert to slow rolling and to establish firm adhesion was counterintuitive.

Granulocytes from wt and *Sharpin^{cpdm}* mice were used in an ICAM-1-based cell adhesion assay using the xCELLigence cell analyser RTCA. RTCA (real-time cell analyzer) xCELLigence was used to measure impedance between the electrodes, present at the bottom of the plate, which is expressed as a cell index. This impedance is used as a measure of cell adhesion. This assay demonstrates that granulocytes from *Sharpin^{cpdm}*

mice display increased adhesion on ICAM-1 substrate (II, Fig. 2), due to the increased integrin αLβ2 activity, which is in line with the previous findings (Pouwels et al., 2013). Conversion from fast to slow rolling and firm adhesion of granulocytes are reduced in Sharpin deficient mice (II, Fig. 1), which is contrary to the expected increase seen in this experiment. This difference can be explained by the previous reports, which suggest that the activation switch of granulocyte transmigration, rather than integrin activity itself, regulates granulocyte transmigration (Imai et al., 2008; Lefort et al., 2012). Successful granulocyte migration depends on precise integrin activity regulation with balanced cycles of adhesion and de-adhesion (Pouwels et al., 2013; Damgaard et al., 2016). The binding of kindlin 3 to the intracellular β2 tail of the integrin changes the affinity conformation of αLβ2 to high infinity, resulting in a change from slow rolling of granulocytes to their firm adhesion. This leads to the speculation that the increased activity of integrins in Sharpin-deficient granulocytes may cause interference in the dynamic regulation of the activation state of integrins resulting in interference with the slow rolling and adhesion of granulocytes *in vivo*.

Altogether, the real-time adhesion data generated in this experiment (II, Fig. 2) was contrary to our *in vivo* data (II, Fig. 1B-H). This counterintuitive result can be explained by the fact that this experiment was performed without any flow, and the more active integrins of Sharpin deficient granulocytes (Pouwels et al., 2013) are mainly responsible for more adhesion observed here.

5.2.4 *Sharpin^{cpdm}* granulocytes have reduced expression of several critical adhesion receptors

Compromised transmigration observed in *Sharpin^{cpdm}* (II, Fig. 1) could be explained by the fact that the granulocytes of these mice have reduced expression of several essential trafficking receptors. Differential expression of surface adhesion receptors in granulocytes (SSC^{hi}Ly6G^{+}; II, Fig. S1C) was observed using flow cytometry; L-selectin (II, Fig. 3A), integrin αL (II,CD11a; Fig. 3B), and P-selectin glycoprotein ligand 1 (PSGL-1, CD162; II, Fig. 3C) had reduced expression, whereas integrin α4 (CD49d) levels were unaffected (II, Fig. 3D) and αM-integrin (CD11b; II, Fig. 3E) was upregulated.

We further checked the mRNA levels of these surface adhesion receptors using qRT-PCR, to explore if the differential expression of these adhesion molecules is due to reduced mRNA levels. Granulocytes isolated from the bone marrow of *Sharpin^{cpdm}* and wt mice were used in these experiments. Interestingly, L-selectin mRNA levels were reduced in *Sharpin^{cpdm}* mice granulocytes (II, Fig. 4A), while mRNA levels of the other adhesion receptors shown in Fig. 3, were unaffected (II, Fig. 4B-D). Additionally, *Itgb2* mRNA levels were not changed (II, Fig. 4E), implying that the decreased integrin αL surface expression levels were not due to reduced *Itgb2* mRNA levels. All in all, our

FACS data show that surface expression levels of several key adhesion molecules are reduced in *Sharpin^{cpdm}* granulocytes (II, Fig. 3), which could be the reason behind the defective transmigration steps in granulocytes migration upon induction *in vivo* (II, Fig. 1).

Our flow cytometry and qRT-PCR data show that L-selectin is indeed downregulated in these Sharpin deficient granulocytes. The differential expression of other receptors on the cell surface of granulocytes could be explained as differential translation or subcellular localisation of the receptors. Altogether, we have seen here that the surface expression of many significant surface receptors is downregulated on the Sharpin deficient granulocytes (II, Fig. 3), which could potentially impair the conversion of granulocytes from fast rolling to slow rolling and to firmly adhere *in vivo* (Fig. 1B-H).

5.2.5 *Sharpin^{cpdm}* granulocyte roll and adhere normally in *in vitro* shear flow assays

The Adhesion assay using ICAM-1 as a substrate shows an increased adhesion of *Sharpin^{cpdm}* granulocytes (II, Fig 2). As there was no flow used in these adhesion assays, and ICAM-1 was the only substrate for the cells to adhere to, this condition does not closely mimic physiological conditions of granulocyte transmigration. Consequently, we simulated more physiological conditions and performed an *in vitro* shear flow experiment with wt and *Sharpin^{cpdm}* granulocytes (II, Fig. 5) (San Lek et al., 2013).

Similar to our *in vivo* phenotype (II, Fig. 1), *Sharpin^{cpdm}* and wt granulocytes do not show any difference in adhesion on ICAM-1 under baseline conditions (II, Fig5A (t=0)) in a shear flow assay. Migration speed analysis showed that these *Sharpin^{cpdm}* granulocytes rolled slower (II, Fig. 5B), which fits with the increased αLβ2-integrins activity in these cells. Intravital microscopy *in vivo* data (II, Fig. 1) is counterintuitive of the phenotype observed in *in vitro Sharpin^{cpdm}* granulocytes (II, Fig 2 & Fig. 5).

As expected, when granulocytes were stimulated using fMLP we observed an increase in adhesion to ICAM-1 (II, Fig. 5A & Fig. 5C). However, no difference in migration speed and adhesion efficiency was detected between wt and *Sharpin^{cpdm}* upon stimulation by fMLP. This suggests that increased integrin activity in Sharpin^{cpdm} granulocytes does not affect adhesion of granulocytes to ICAM-1 in the presence of flow (II, Fig. 5C & D).

In the shear flow assays described above (II, Fig 5A-D) ICAM-1, a substrate for integrin αLβ2, is the only substrate. However, *in vivo* initial adhesion and fast rolling are dependent on selectins, and the later steps (slow rolling and firm adhesion) require adhesion to ICAM-1 (Nourshargh and Alon, 2014). We simulated similar physiological condition using ICAM-1 and E-selectin as a substrate for cells under high flow rate, and

we stimulated the cells using cxcl1. Also under these conditions granulocytes from wt and *Sharpin*^{cpdm} mice do not show any differences in rolling and adhesion (II, Fig. 5E). In the shear flow experiments, we can only simulate conditions similar to *in vivo* experiments, it is difficult to create exactly same conditions in these experiments. Several proteins are required for a granulocyte to successfully transmigrate (Ley et al., 2007). If the observed phenotype in our intravital microscopy experiment is due to the cumulative role of different proteins involved in transmigration, than it is very challenging to reproduce similar results using *in vitro* system. All these results obtained from shear flow assay imply that the phenotype observed in *Sharpin*^{cpdm} mice, where the granulocytes are unable to switch from fast rolling to slow rolling and to firm adhesion, is not just because of the defective granulocytes alone but that other defects also contribute to this phenotype of *Sharpin*^{cpdm} mice.

All the steps involved in the transmigration of an immune cell towards the infected site are essential to maintain homeostasis. Previously, Pouwels et al., identified a novel role for Sharpin in the last step of transmigration (Pouwels et al., 2013). Sharpin inactivates the αLβ2-integrins and lets the trailing end of granulocyte free, so that these granulocytes can be released from the endothelial layer toward the infection or inflammation site (Pouwels et al., 2013). The initial steps of transmigration play a significant role during the inflammatory response, all these steps depend on the frequently formed transient interactions among the cell surface receptors of granulocytes and the endothelium. Our work on inflammation-induced transmigration of granulocytes in post-capillary veins of the cremaster muscle also shows that earlier steps of granulocyte transmigration, i.e. slow rolling and firm adhesion, are severely compromised in the absence of Sharpin.

Moreover, integrin activity is higher (II, Fig. 5) in Sharpin-deficient granulocytes, which is counter-intuitive to the phenotypes observed in *in vivo* experiments (intravital imaging experiment); this suggests that the unusual *in vivo* phenotype of defective rolling and adhesion does not entirely depend on *Sharpin*^{cpdm} mice neutrophils. We cannot rule out the possible contribution of the receptors present on the endothelial cells, such as ICAM-1, E-selectin, P-selectin and VCAM-1. These receptors play a crucial role in this process, and interestingly, all these proteins are direct targets of NF-κB signalling pathway (Bunting et al., 2007; Iademarco et al., 1992; Pan and McEver, 1995; Schindler and Baichwal, 1994; Stolpe et al., 1994; Whelan et al., 1991), which is impaired in *Sharpin*^{cpdm} mice (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). Another possible reason for this defective immune cell transmigration of *Sharpin*^{cpdm} granulocyte is that these mice have severe systemic inflammation (Peuhu, Salomaa, et al., 2017; HogenEsch et al., 1993), which leads to the adaptation of the immune system where granulocytes become less reactive to the stimulation of fMLP

in our *in vitro* assay. An interesting experiment that can be performed is to make bone-marrow chimaeras that is to irradiate a wild-type mouse and replace its bone-marrow with *Sharpin*^{cpdm} bone-marrow. Primarily, it would provide us with a wild-type mouse with *Sharpin*^{cpdm}-neutrophils. Intravital microscopy using this chimaera mouse with *Sharpin*^{cpdm} granulocytes will suggest us about other factors contributing to this defective phenotype.

Previous studies contributed an extensive amount of knowledge about the transmigration process, but still, there are some unanswered questions left to answer. Our finding lacks a detailed mechanism, but in this study we identified a novel immunological role for Sharpin, which could contribute to the complicated cpdm phenotype of mice and its absence can severely affect the early and late steps of transmigration of immune cell.

6. CONCLUSION

Sharpin is an adaptor protein that interacts with other proteins and regulates their functions. Most of the known interactors of Sharpin, such as integrins, PTEN, and Caspase 1, are all strongly linked to cancer progression, suggesting that Sharpin could also play a decisive role in tumor progression. Importantly, Sharpin is amplified and overexpressed in many human cancers and promotes cancer cell proliferation, tumor formation and metastasis. Sharpin, as a critical component of LUBAC, also regulates various signaling cascades downstream of several receptors, for instance, TNFR1, TLR and IL1R.

Although different labs have recently confirmed the involvement of Sharpin in several significant diseases, the underlying molecular mechanisms behind this involvement remain poorly described. In addition, efforts to systematically identify all the signalling pathways where Sharpin is involved have been lacking. Therefore, a more profound and better understanding of Sharpin´s function is essential.

Using mass spectrometry, we identified the first interactome of Sharpin. This novel set of Sharpin interactors suggests that Sharpin is either involved in, or regulates several different signalling pathways, which are not so far reported, such as endosomal trafficking, RNA processing and metabolism. Importantly, the MS data suggest that Sharpin plays a role in regulations of the cytoskeleton, as more than ten cytoskeleton regulators appeared in the Sharpin interactome, including the extensively studied RhoA, Rac1 and Cdc42. Interestingly, the Arp2/3 complex was also identified as a putative interactor of Sharpin. We validated the interaction between Sharpin and the Arp2/3 complex using various molecular biology techniques. In addition, we demonstrated that Sharpin binds the Arp2/3 complex via its UBL domain, which most of the Sharpin interactors bind to, and identified an Arp2/3-specific Sharpin mutant that is fully capable of interacting with integrins and HOIP. Previously, different studies showed that Sharpin and Arp2/3 complex both play a role in lamellipodia formation. We showed that the Sharpin-Arp2/3 interacts, and this interaction promotes lamellipodia formation. In addition, we also identified a role for the interaction in cell migration, consistent with the role for lamellipodia in this process.

Sharpin interacts with Arp2/3 complex, but does not affect Arp2/3-dependent actin polymerization *in vitro*. Therefore, we lack mechanistic details here and many questions remain. Whether Sharpin play any role in *in vitro* actin polymerization and if it needs to be post-translationally modified to perform such function, can be studied in the future. It would be interesting to study the role of Sharpin-Arp2/3 interaction in

disease. Arp2/3 complex is associated with cancer, and whether this role of Sharpin has any link with cancer metastasis can be studied further using 3D migration assays, such as trans-well 3D invasion assays. In our 2D random migration assay, we observed a significant role of Sharpin. It is important to note that 2D adhesion and migration does not always reflect the *in vivo* adhesion and migration. Therefore, the role of Sharpin and Sharpin-Arp2/3 interaction should be investigated in 3D environment.

The second study in this thesis suggests the role of Sharpin in the early steps of granulocyte transmigration. Granulocytes transmigrate from the circulatory system through the endothelial layer towards the infected tissue to clear the infection, which is often deregulated in the disease state. Pouwels et al., 2013 reported that Sharpin causes inactivation of integrin $\alpha L\beta 2$ (LFA-1) and this inactivation is essential for the release of the trailing edge of the granulocyte after crossing the endothelial layer; the last step of granulocyte transmigration. The role of Sharpin in the early steps of granulocyte transmigration (rolling, activation and arrest) remained unknown. However, in this study, our data showed that Sharpin plays an important role in the early steps of transmigration as well. In *Sharpin*^{cpdm} mice, granulocytes show normal fast rolling. However, when inflammation is induced in *Sharpin*^{cpdm} and wt granulocytes , *Sharpin*^{cpdm} granulocytes showed an impaired reduction in rolling velocities, which implies they are unable to switch from fast rolling to slow rolling. Eventually, *Sharpin*^{cpdm} granulocytes show decreased affinity to adhere firmly to the endothelial cells. Our *in vitro* results suggest that the reduced slow rolling and firm adhesion in *Sharpin*^{cpdm} mice is not merely due to the defects in granulocytes. We cannot rule out that this phenotype is a cumulative effect of several other proteins involved in this process, such as adhesion receptors present on the surface of endothelial cells. Although the precise mechanism behind this striking phenotype of *Sharpin*^{cpdm} mice remains elusive, here we identify an important role for Sharpin in innate immunity, which could contribute to the complex immunological phenotype of the Sharpin knock out mouse.

As mentioned previously, these defective phenotypes observed in *Sharpin*^{cpdm} granulocytes is not solely due to the granulocytes, but the receptors present on the endothelial layer might also contribute to this phenotype. It would be interesting to study the role of these endothelial receptor proteins in transmigration of granulocytes. Some experiments that can be performed are staining of cremaster muscle using immunohistochemistry and check their expression levels using imaging-based techniques. It would be interesting to isolate granulocytes from wt and Sharpin deficient mice and differentially labelled them, co-inject these cells in a wild type mice and perform intravital microscopy *in vivo* experiment. This experiment will confirm the role of endothelial layer in this defective phenotype observed in Sharpin deficient mice.

In summary, the work compiled in this thesis showed that Sharpin regulates two significant biological processes; cell migration (via lamellipodia formation) and granulocyte extravasation. Both cellular processes are regulated by very different sets of proteins, but they have one common player, that is Actin. Actin plays important roles in cell migration and granulocytes transmigration, role of Sharpin-Arp2/3 interaction can be studied in granulocyte transmigration. If we look at the bigger picture, both the processes are deregulated in cancer, and these two studies further strengthen the notion that Sharpin is an essential player in cancer development.

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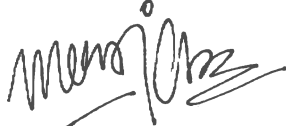

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