FUNCTIONAL REGULATION OF INTERLEUKIN-17 RECEPTOR SIGNALING

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An appropriate inflammatory immune response requires the effective orchestration of molecular and cellular effectors. Interleukin-17 (IL-17) is a cytokine, small signaling proteins, made by immune cells to communicate with mainly non-immune cells. IL-17 signaling engages its receptor (IL-17R) to induce inflammation by stabilizing transcripts or by inducing expression of target genes. IL-17R downstream intermediate proteins activate and downregulate intracellular signaling. IL-17R shares some, but not all, intracellular intermediate proteins with Toll-like receptor (TLR) and Tumor necrosis factor α receptor (TNFR) signaling. Previous research has shown that not all intermediates from TLR or TNFR1 pathways participate of IL-17R signaling. We aimed to identify novel intermediate proteins acting downstream in IL-17R by using molecular and cellular biology and biochemical techniques. After identifying five possible novel intermediates that either activate (caveolin-1 and AnapC3) or inhibit (ABIN-1, CYLD, Otulin) IL-17R signaling, we aimed to describe the mechanism of action of two of them, ABIN-1 and caveolin-1. We described the molecular mechanisms by which ubiquitin-binding protein ABIN-1 and membrane-bound scaffolding protein Caveolin-1 control IL-17 signaling. Interestingly, as previous research on other signaling intermediates of IL-17R signaling has shown, IL-17R signaling also regulates the expression of these two proteins in what we propose is a self-modulatory signaling mechanism. Specifically, we identified that ABIN-1 inhibits baseline and IL-17-activated signaling by binding polyubiquitin independently of A20. Moreover, we showed that IL-17 signaling induced degradation of ABIN-1. For caveolin-1, we observed a different

mechanism of regulation. Thus, Caveolin-1 interacted with a subunit of IL-17R and regulated trafficking of IL-17R to the cell surface. Interestingly, caveolin-1 regulatory functions correlated with an activating role of caveolin-1 in IL-17R signaling. Lastly, caveolin-1 was downregulated by IL-17 signaling. In this thesis we report novel functions for ABIN-1 and caveolin-1 downstream of IL-17R. These results provide insight into the mechanism by which ABIN-1 and caveolin-1 regulate IL-17R signaling, and how IL-17R signaling self-regulates by modulating the expression or degradation of these two proteins. The results from this thesis contribute to the understanding of IL-17-mediated inflammatory signaling, and set the groundwork for future research on therapies to modulate this inflammatory response.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 CHAPTER ONE: INTRODUCTION

1.1 INFLAMMATION IS MEDIATED BY INTERLEUKIN-17 AND ITS RECEPTOR

The immune system uses inflammation as an effective tool to protect the host against microbial pathogens and harmful foreign molecules. An optimal immune response requires the orchestration of molecular and cellular effectors to regulate inflammation. Since inflammation causes tissue damage, initiation and termination of the inflammatory response is tightly regulated by lymphocytes. A mechanism by which lymphocytes control inflammation is through their production of cytokines, master signaling proteins that regulate the inflammatory response. The Interleukin-17 (IL-17) family of cytokines induce inflammation to fight pathogens, and dysregulated IL-17-mediated inflammation is associated with the development of autoimmune disorders (1,2). IL-17-mediated inflammation is achieved by activating intracellular signaling of the IL-17 receptor (IL-17R). Thus, IL-17 signaling promotes expression of inflammatory genes either by inducing their *de novo* transcription or by stabilizing their mRNAs (3–7). This thesis focuses on understanding the intracellular regulation of the IL-17 response, and describing the mechanisms of action of two novel regulators downstream of IL-17R.

IL-17A and its receptor are the best studied members of the IL-17 cytokine and receptor sub-families, comprising six cytokines (IL-17A through IL-17F) and five receptors (IL-17RA through IL-17RE). IL-17A forms a homodimer or heterodimer with IL-17F to engage its receptor

(8–10). In this thesis, I studied the signaling of the IL-17A homodimer, which from hereon I refer to as IL-17. All IL-17 receptors contain extracellular domains formed by fibronectin type-III (FN) domains and cytoplasmic SEF/IL-17R (SEFIR) domains **(figure 1.1)** (11–13). IL-17RA and IL-17RC are the only two receptors in their family that express an extended C-terminal SEFIR (SEFEX) domain required for intracellular signaling (14,15). In addition to the SEFEX domain, IL-17RA is the only subunit to express an additional C-terminal domain, the distal CCAAT/enhancer binding protein β (C/EBPβ) activation domain (CBAD). Our group and others have shown that the CBAD is required for inhibitory signaling of IL-17. The CBAD regulates sequential phosphorylation and inactivation of C/EBPβ by regulating the activation of glycogen synthase kinase-3 beta (GSK-3β), and also binds A20 and TRAF3 to inhibit IL-17 signaling (16–19). IL-17 signals through a heterodimeric receptor formed by IL-17RA and IL-17RC. The intracellular regulation of signaling downstream of this heterodimeric IL-17RA/IL-17RC receptor (from now on referred as IL-17R) is the focus of this thesis.
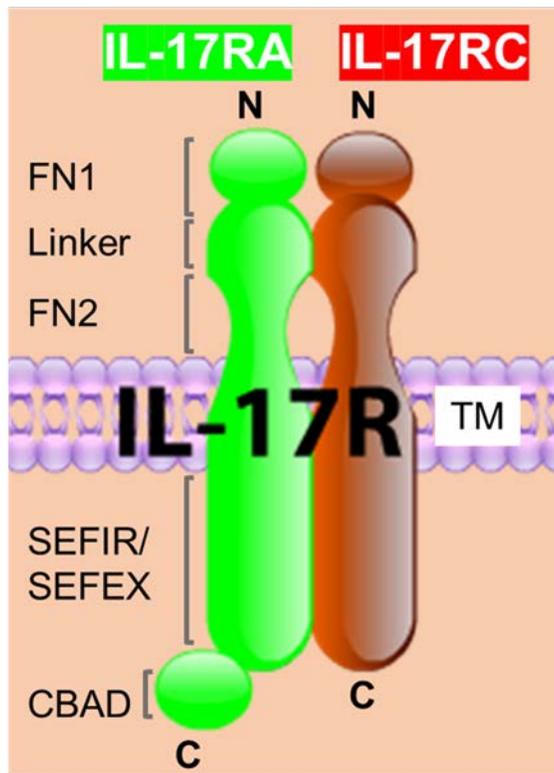
Figure 1.1: Domains and subunits of the IL-17R.

Schematic diagram of domains and subunits of the heterodimeric interleukin-17 receptor (IL-17R). This diagram depicts the heterodimer formed by IL-17RA (green) and IL-17RC (brown). Known domains of IL-17RA are annotated on the left side of the figure. Many of these domains have homology in the IL-17RC subunit. Acronyms: FN1/2: Fibronectin-III-like domain 1 or 2; TM: transmembrane region; SEFIR: SEF/IL-17R domain; SEFEX: SEFIR extension; CBAD: C/EBPβ activation domain.

1.2   THE TYPE 17 RESPONSE: IL-17-PRODUCING CELLS

Lymphocytes are subdivided into different lineages. T cells are typically described as adaptive immune cells that are activated by engaging a specific antigen through their T-cell receptor (TCR). Two important defining characteristics of adaptive T cells are their specificity in recognizing an antigen and their ability to form memory (20,21). T cells are further categorized based on their functional specialization and their expression of specific surface receptors or markers. T cells with direct cytotoxic activity and expressing CD8 are classified as cytotoxic T cells. On the other hand, T cells that produce cytokines, help other immune cells, and express CD4 on the cell surface are classified as T Helper (T_H) cells. In 1989, Mossman and Coffman hypothesized that two types of T_H cells could be differentiated based on their ability to make cytokines: interferon-γ (IFNγ)-producing T_H1 cells, and IL-4-producing T_H2 cells (22). More than twenty years later, the T_H1- T_H2 hypothesis was expanded to include T_H17 cells as an additional subset of CD4+ T cells (23,24). T_H17 cells are characterized by the IL-23-dependent activation of the Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathway, and by their production of IL-17 (25–28).

More recently, several types of innate immune cells have also been found to respond to IL-23 to produce IL-17 (29–33). Currently, IL-17-producing cells are characterized by their expression of the transcription factor retinoic acid receptor-related orphan receptor-γt (RORγt, encoded by Rorc). IL-17-producing cells can be either innate or adaptive immune cells, and include conventional T_H17 cells, mucosal CD8+ T cells or Tc17, "natural" T_H17 cells that are

innate as they do not require TCR activation, innate lymphoid cells Type 3 (ILC3), γδ T cells, natural killer T (NKT) cells, and invariant NKT (iNKT) cells (33–35). Together, these cells are termed "type 17" cells. The innate type 17 cells in non-lymphoid tissues quickly respond to tissue injury or pathogenic insults, producing type 17 signature cytokines like IL-17, IL-22, and granulocyte-macrophage colony stimulating factor (GM-CSF) (34,36–38).

Innate type 17 immunity depends on IL-1β and IL23 to activate the JAK/STAT3 pathway. JAK1 phosphorylates and activates STAT3 to induce its translocation to the nucleus to act together with RORγt (2,39–42). Furthermore, STAT3 controls the expression of *Rorc* and other T$_H$17-specific transcription factors like interferon-regulatory factor 4 (*Irf4*) and basic leucine zipper transcription factor ATF-like (*Batf*) (43–45). Therefore, STAT3, RORγt, IRF4, and BATF function coordinately to initiate and maintain the IL-17-producing phenotype. The differentiation, initiation and activation of T$_H$17 cells is the most well-studied of all IL-17-producing cells, and although I will not investigate T$_H$17 cells themselves in this thesis, I will briefly mention the knowledge accumulated from previous studies on T$_H$17 cells to provide a framework for appreciating the upstream events that ultimately lead to activation of IL-17-producing cells **(figure 1.2)**.

T$_H$17 differentiation is driven by additional factors such as transforming growth factor β (TGFβ) and IL-6, during initial T cell recognition of cognate antigen. The contribution of TGFβ signaling to differentiation is complicated by the fact that TGFβ could either control T$_H$17-inducing factors or indirectly restrict alternative cell fates. In contrast, the contribution of IL-6 to the T$_H$17-phenoytpe is better understood. IL-6 activates STAT3, which directly drives transcription of *Rorc*, *IL23r,* and *Il17*. Additionally, STAT3 inhibits TGFβ-induced forkhead box P3 (FOXP3) expression, and consequently suppresses the differentiation of undifferentiated T

cells to a regulatory T (T_{reg}) cell phenotype (46,47). Besides initiating T cell differentiation, IL-6 induces expression of the IL-1β receptor (IL-1R) in T_H17 cells. Activation of IL-1R signaling induces the phosphorylation of mammalian target of rapamycin (mTOR), activating IRF4 and enhancing the metabolic fitness of the dividing T_H17 cells (48). For maturation and maintenance of the IL-17-producing phenotype, IL-23R signaling is essential, and the pathogenicity of T_H17 cells is promoted by IL-23. Genetic studies that link *IL-23R* polymorphisms to autoimmune disorders like psoriasis, ankylosing spondylitis, multiple sclerosis, and Crohn's disease (described in more detail, below) support the pathologic role of IL-23R signaling in the type 17 immunity (49–51). In this pathologic context, dysregulated T_H17 cells make IL-17, which induces downstream signaling in responder cells to drive inflammation and tissue destruction.

1.3   THE TYPE 17 RESPONSE: IL-17-RESPONDING CELLS

The IL-17-responding cells are mainly non-immune cells. Their response to IL-17 is dependent on cell surface expression of the heterodimeric IL-17RA/IL-17RC receptor. The IL-17RA subunit is ubiquitously expressed across most cell types. However, the IL-17RC subunit is mostly expressed in non-immune cells, and provides specificity in terms of the ability to respond to IL-17 (52). Furthermore, IL-17RC contributes to intracellular signaling of IL-17 through its SEFIR/SEFEX domain, which is absolutely required for intracellular signal transduction *in vitro* and *in vivo* (15,53,54). Selective expression of IL-17RC restricts the IL-17-responding cells to include epithelial cells, stromal cells and osteoclasts. Indeed, IL-17-induces osteoclastogenesis, the differentiation of monocytic precursors into osteoclasts, which drives bone resorption. Osteoclasts, in turn, have a key role in rheumatoid arthritis by regulating IL-17-dependent matrix

metalloproteinases and RANKL production that modulates joint tissue remodeling. Additionally, recent evidence suggests that neutrophils and natural killer (NK) cells may also respond to IL-17 via an induced expression of IL-17RC (55,56) although these findings are controversial In sum, primarily non-immune cells express IL-17RC to form a functional IL-17R that responds to IL-17 and activates intracellular signaling.
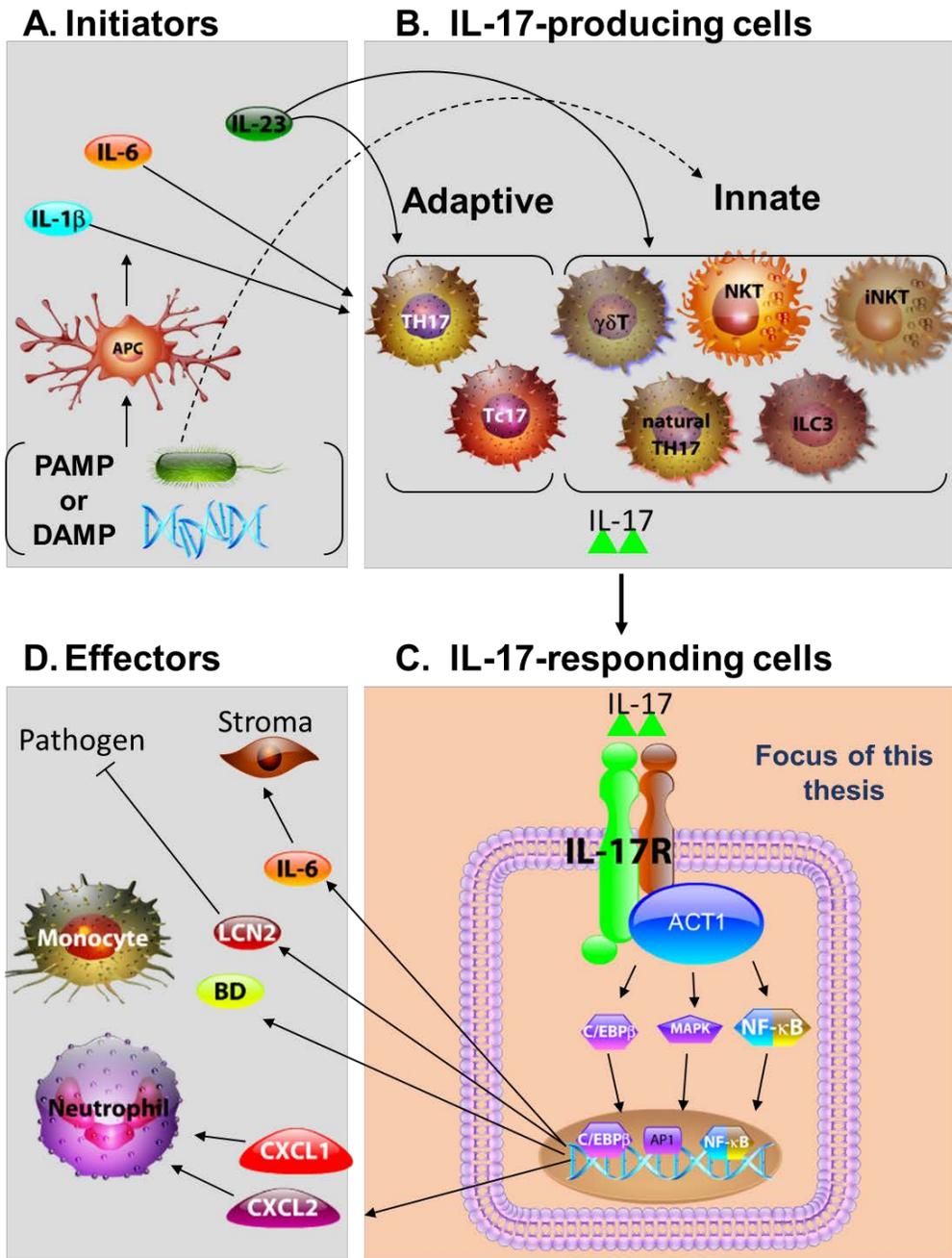
Figure 1.2: Type 17 immunity connects immune and non-immune cell compartments.

IL-17 is secreted by immune cells after differentiation and activation. IL-17 signals through the IL-17 receptor located on the surface of stromal, epithelial, and tissue-forming cells. (A) Antigen Presenting Cells (APC) sense PAMPs or DAMPS and make IL-6, IL-23, and IL-1β. (B) Adaptive IL-17-producing cells respond to IL-23, IL-6 and IL-1β to make IL-17. In addition, innate IL-17-producing cells respond to IL-23 and other environmental factors

(e.g., other cytokines and/or directly responding to PAMP or DAMP) and make IL-17. (C) IL-17-responding cells produce effector molecules to respond to the initial insult. Additionally, IL-17-producing cells secrete chemokines that attract effector cells like neutrophils or monocytes to amplify the response. (D) Effector molecules and cells. This thesis focusses on the intracellular response of IL-17-responding cells highlighted in the orange box. Acronyms: PAMP: Pathogen-associated molecular patterns; DAMP: Damage-associated molecular pattern; APC: antigen-presenting cell; IL-6: interleukin-6 T_H17: T helper-17; Tc17: T cytotoxic-17; NKT: natural killer T cells; iNKT: invariant NKT; ILC3: innate lymphoid cells-3; IL-17: interleukin-17; IL-17R: IL-17 receptor; ACT1: Nuclear factor-NF-kappaB activator 1; C/EBPβ: CCAAT/enhancer-binding protein beta; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor-kappaB; AP1: Activator protein 1; CXCL1/2: C-X-C motif chemokine 1 or 2; GM-CSF: Granulocyte-macrophage colony-stimulating factor. Adapted from Hernandez-Santos 2012.

1.4   IL-17R SIGNALING, UBIQUITINATION AND IL-17-TARGET GENES

Much effort has been dedicated to understanding the transcriptional regulation and cellular sources of IL-17. In this thesis, I aim to elucidate the regulation of IL-17R signaling occurring in IL-17-responding cells. Upon IL-17 secretion and binding to IL-17R, myriad intracellular events occur that induce gene expression and stabilize nascent transcripts, which ultimately leads to inflammation.

Of these intracellular events, the post-translational protein modifications of phosphorylation and ubiquitination are the most relevant for IL-17R signaling. Phosphorylation on tyrosine (Y), serine (S), and threonine (T) residues regulates enzymatic activities and interactions of proteins by acting mainly as a bimodal on/off system. In contrast, ubiquitination is a multi-modal signaling system in the sense that addition of different ubiquitin chains to the same target protein has different outcomes. Each of ubiquitin's seven lysine (K) residues can be joined to the N-terminal of a different ubiquitin molecule to form a wide array of ubiquitin chains. The functions of these covalently-bound ubiquitin chains on target proteins are diverse and depend on the type of K-linkage. For example, K48-pUb signals the proteasomal degradation of the target protein, whereas K63-pUb signals intracellular processes, such as interactions with other proteins, to activate or inhibit signaling pathways. This sophisticated coding system is mediated by E3-ligases (57). E3 ubiquitin ligases are important in IL-17R signaling due to their ubiquitination activity and concurrent function as scaffolding proteins. Ubiquitination is reversible and counter-regulated by deubiquitinases (DUBs). The human

genome is predicted to encode over 600 E3-ligases and nearly 100 DUBs. Thus, E3-ligases are thought to be more specific to their substrates than DUBs. The addition and removal of ubiquitin are key post-translational modifications of IL-17R (58) and its downstream proteins.

IL-17R-mediated inflammatory signaling is initiated by recruiting the proximal adaptor ACT1 to the SEFIR domain of IL-17R. The primary function of ACT1 is to act as an adaptor connecting IL-17R to other downstream intermediates from the tumor necrosis factor-alpha (TNFα) receptor-associated factor (TRAF) family of proteins. In addition to its scaffolding function, ACT1 also catalyzes the addition of a K63-pUb chain on K124 of TRAF6 (59,60). ACT1 and TRAF6 are both E3-ligases that positively regulate IL-17-dependent nuclear factor kappa-B (NF-κB) signaling by binding to and adding pUb chains to downstream intermediates (61). One published report indicates that TRAF6 ubiquitinates IL-17R (58) and activates TGFβ-activated kinase 1 (TAK1), which in turn interacts with ACT1. Next, TAK1 forms a complex with TAK1-binding protein-2 (TAB2) and -3 (TAB3) to interact with the SEFIR domain of IL-17RA (62). Subsequently, TAK1 phosphorylates and activates the inhibitor of the κB (IκBα) kinase (IKK) complex formed by IKKα, IKKβ, and two subunits of IKKγ (also called NEMO). The activated IKK complex phosphorylates IκBα, inducing IκBα K48-ubiquitination and degradation. This degradation releases NF-κB transcription factor proteins to translocate to the nucleus and activate NF-κB-dependent genes.

In addition to triggering NF-κB, IL-17R signaling also stabilizes mRNA transcripts, and initiates C/EBPβ/δ, IκBζ, and the mitogen-activate protein kinase (MAPK) pathways, including p38, JUN N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) (**figure 1.3**)(6,10,63,64). Upon IL-17 stimulation, p38 and JNK are activated by a mechanism involving the tumor progression locus 2 (TPL2) kinase. IL-17 activates the IKK complex, which in turns

phosphorylates p105 and releases TPL2 to activate p38 and JNK. TPL2 also binds, phosphorylates and activates TAK1, which subsequently activates the IKK complex, forming a positive-feedback loop (65). The mechanism of IL-17-dependent ERK activation is still unclear. In addition to MAPK proteins, C/EBPβ and C/EBPδ are also important effectors of IL-17 signaling. In fact, synergy between IL-17 and TNFα signaling is partially mediated by C/EBP proteins (66). IL-17 induces transcription of *Cebpd*, while C/EBPβ is regulated post-transcriptionally (via alternative splicing) and post-translationally (via phosphorylation) (17,67). C/EBPβ has three alternative start sites, and IL-17 induces the splicing and formation of the less dominant mRNAs. Additionally, C/EBPβ is sequentially phosphorylated on two serine residues to inhibit its transcriptional activity (17). Moreover, IκBζ is another effector of IL-17 signaling that contributes to IL-17/TNFα synergy. Karlsen *et al.* showed that TNFα induces *Nfkibz* (encoding IκBζ) and that IL-17 stabilizes the transcript, causing an increase of IκBζ. Then, IκBζ collaborates with NF-κB to enhance the expression of *Lcn2*, an iconic IL-17 target gene (68). Most of these signaling effector molecules converge to activate IL-17 target genes.

IL-17 signaling induces different subsets of target genes. For example, IL-17 induces the expression of *Cxcl1*, *Cxcl2*, and *Cxcl5* to recruit neutrophils to the target tissue. *Ccl20* is also induced to promote trafficking of mucosal-associated cells expressing CCR6, a typical receptor of IL-17-producing cells like T$_H$17 cells and ILC3s. IL-17 also induces *Csf2* (encoding GM-CSF) and *Il6* to control survival, proliferation and recruitment of myeloid cells, especially neutrophils. Moreover, IL-17 signaling induces β-defensins, small cationic microbicidal peptides that directly destroy pathogens and prevent microbial colonization of epithelial surfaces. *Lcn2* is another archetypal IL-17-target gene with antimicrobial activity encoding Lcn2 (also called 24p3 or Ngal). Lcn2 limits bacterial growth by restricting access to dietary iron. The diversity of these

genes indicates that IL-17R signaling regulates several intracellular functions, and the different ways in which these genes are controlled demonstrate the different mechanisms by which IL17R signaling is regulated. Binding sites for NF-κB, C/EBP, and the MAPK-dependent transcription factor AP-1 have been identified in the promoter regions of many of these IL-17 target genes (63), demonstrating that regulation of IL-17R signaling is mediated, in part, by activation and inhibition of C/EBP, MAPK and NF-κB. In this thesis, I focus mainly on the regulation of IL-17-dependent NF-κB signaling.

Some IL-17-dependent genes are fairly tissue-specific. For instance, IL-17 signaling in central nervous system (CNS) and gut epithelium induces expression of occludin proteins to maintain tight junctions and barrier integrity of these tissues (69,70). Another example of differentiated regulation and signaling of IL-17 is in oral and kidney epithelial cells, which express β-defensin and kallikrein proteins for antimicrobial activity and tissue protection (71,72). These examples demonstrate that in addition to driving inflammation, IL-17R signaling also drives specific gene expression in different tissues. Hence, the study of regulation of IL-17R signaling is vital to understanding the physiological consequences of tissue-specific type 17 responses.

Given that transcripts of many IL-17 target genes are highly unstable, IL-17 also regulates inflammation by stabilizing the mRNAs of these downstream target genes. The IL-17-induced mRNA stability pathway is dependent on adaptor proteins ACT1, TRAF2 and TRAF5, but is independent of TRAF6. In the specific example of IL-17-induced *Cxcl1* stability, for example, IL-17 signaling activates IKKε to phosphorylate ACT1 at S311. TRAF2 and TRAF5 are then recruited to ACT1, which sequester the mRNA splicing regulatory factor (ASF/SF2) away from the 3'-untranslated region (UTR) of *Cxcl1*, promoting its stability (6,73).

Furthermore, IL-17 also recruits the human antigen R (HuR) protein to the ACT1/TRAF2/TRAF5 complex for ubiquitination and activation by ACT1. Activated HuR then binds to the 3'UTR of *Cxcl1* and stabilizes the transcript by competing with ASF/SF2 to bind to similar sequences (74). However, studies of IL-17-mediated mRNA stability have mostly focused on understanding stability of chemokine transcripts such as *Cxcl1* and *Cxcl5*. It is well-known that IL-17 induces stability of other unstable inflammatory mRNAs, which suggests that IL-17 may activate other mRNA stability mechanisms. More details about this and other IL-17-mediated mRNA stability pathways has been recently reviewed in Amatya et al. (11), and further discussion of this topic is outside the scope of this thesis.
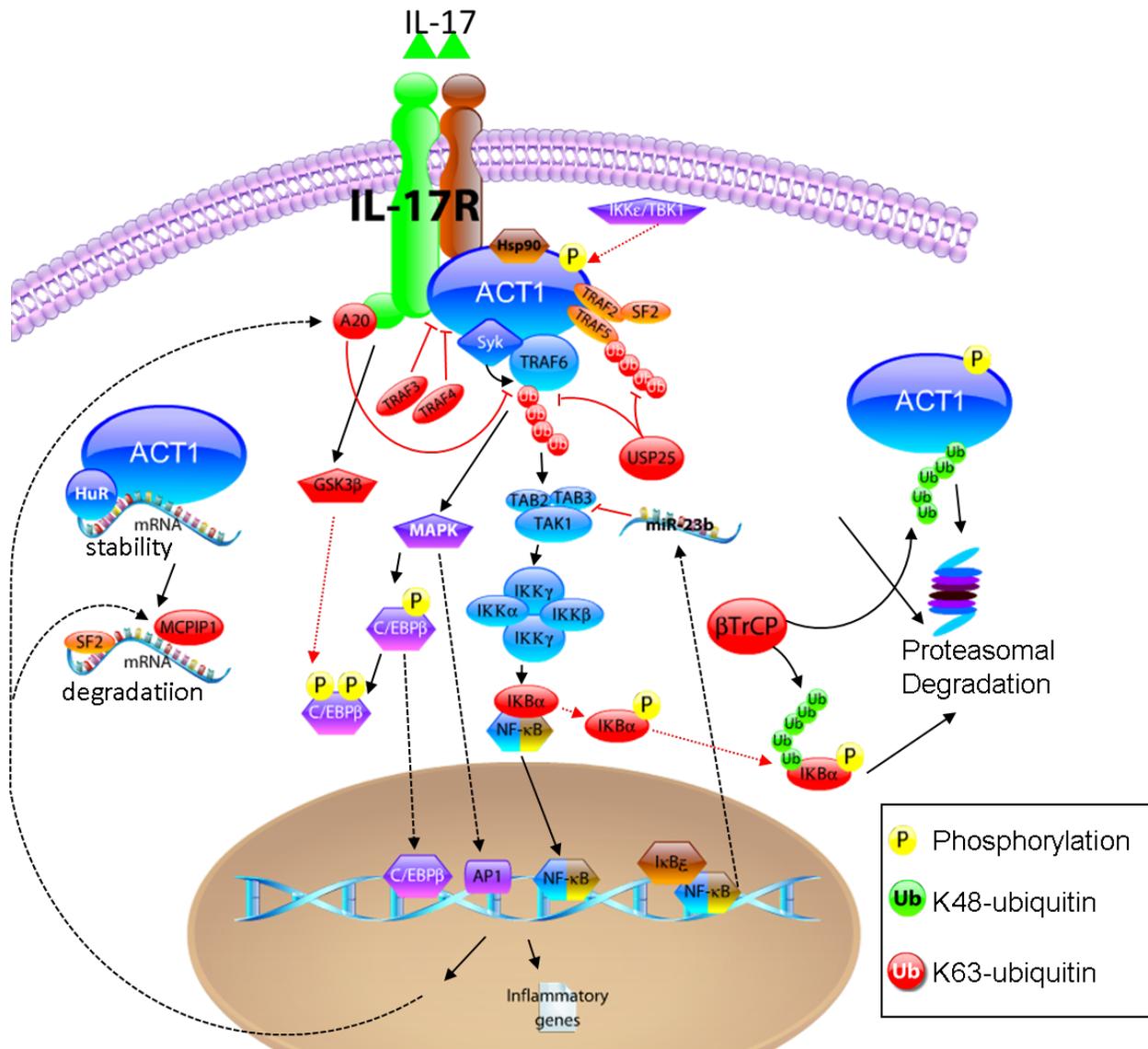
Figure 1.3: Schematic representation of IL-17R signaling.

Proposed model of IL-17R signaling including intermediate adaptor molecules, effectors and negative regulators. The red arrows with flat ends indicate inhibition and the black arrows with pointed ends indicate induction or activation. Dotted black arrows indicate feedback regulation, while dotted red arrows represent phosphorylation events.

1.5   REGULATION OF IL-17R SIGNALING

Regulation of IL-17R signaling requires the coordination of activating and inhibitory factors at key intermediate signaling steps. As aforementioned, IL-17R proximal adaptor proteins ACT1 and TRAF6 are crucial intermediates of IL-17 signaling, and both are regulated by post-translational modifications. Moreover, phosphorylation and ubiquitination of ACT1 are key mechanisms for regulating IL-17R signaling. ACT1 has multiple sites of phosphorylation, and various kinases can target different sites to induce a specific response in IL-17R signaling. For example, ACT1 is phosphorylated to promote its K48-ubiquitination and degradation. Specifically, the ubiquitination of ACT1 is mediated by the SCFβ-TrCP complex formed by Skp1, Cdc53 and F-box protein Cdc4, plus the β-TrCP1 and β-TrCP2 proteins. This complex also K48-ubiquitinates IκBα, leading to its degradation (75). Interestingly, although ACT1 and IκBα are degraded by similar mechanisms, the outcome of degradation of these proteins is different. These previously published results indicate that proteasomal degradation can coordinate activation of IL-17-dependent signaling by degrading intermediates at various levels in the signaling cascade.

In addition to post-translational modification of ACT1, IL-17R signaling is also regulated by proteins that bind to ACT1 and TRAF6. TRAF6 ubiquitinates both itself and downstream proteins to activate signaling, and the lack of response to IL-17 observed in TRAF6$^{-/-}$ fibroblasts demonstrated that TRAF6 is a crucial component in the IL-17R pathway (52,76). For instance, one report by Wu *et al.* showed that the Syk kinase binds ACT1 and TRAF6 to enhance TRAF6

ubiquitination and expression of IL-17-dependent CCL20 (77). One report by Zepp *et al.* showed that TRAF4 interacts with ACT1 to activate ERK5 in keratinocytes. Moreover, these authors observed that IL-17-dependent activation of ERK5 could promote proliferation of keratinocytes (78). Indeed, *Traf4* is overexpressed in skin cancer cells, suggesting that the overexpression of Traf4 in cancer cells causes IL-17R signaling to induce proliferation in a TRAF6-independent manner.

There are also several examples showing that blocking the interaction of ACT1 with downstream proteins inhibits IL-17R signaling. For instance, the tank-binding kinase-1 (TBK1), another IKK-related kinase, directly phosphorylates and binds ACT1 to block the interaction of ACT1 with TRAF6, thus inhibiting IL-17 signaling (79). Similarly, TRAF3 and TRAF4 bind to ACT1 to block the ACT1/TRAF6 interaction (18,78). These studies show that IL-17 signaling is regulated by proteins controlling the binding of ACT1 to downstream intermediates.

Notwithstanding the crucial role of ACT1 in the IL-17R signaling cascade, inhibition of IL-17R signaling may also occur further downstream of ACT1. For instance, our group described that IL-17 signaling activates GSK-3β, which together with ERK1/2, sequentially phosphorylates C/EBPβ. This sequential phosphorylation inhibits C/EBPβ nuclear translocation and activation of C/EBPβ-dependent genes. The mechanism of this regulation is only partially understood, and requires CBAD of IL-17RA to activate GSK-3β after IL-17 stimulation (17). These studies suggest that there might be other adaptor proteins proximal to IL-17R that activate GSK3β and that remain to be discovered.

Inhibition of IL-17R signaling is also regulated at the mRNA level by a subset of IL-17-target genes. Recently our group showed that endoribonucleases Roquin1/2 and MCPIP1 degrade transcripts of genes such as *Il6*, *Il17ra*, *Il17rc*, and *Nfkbz* to decrease IL-17 signaling and

mediate inflammation in the setting of autoimmunity and lung inflammation (80,81). Interestingly, MCPIP1 recognizes loop structures in the 3'-UTR of these transcripts, destabilizing them and inducing their degradation. These results confirm that regulation of mRNA is another important arm of the regulatory mechanisms of IL-17R signaling. Moreover, microRNAs mIR30 and mIR23b also inhibit mRNA stability by directly binding to the 3'- UTR of the mRNA is encoding TAB2 and TAB3, destabilizing them and consequently decreasing activation of NF-κB signaling (82). Altogether, these reports indicate that there are many layers of complexity to the regulation of IL-17R signaling.

Another potential regulatory mechanism of IL-17R signaling is the control of receptor localization to the plasma membrane. However, the full extent by which the IL-17R surface localization is regulated remains poorly understood. To date, most studies on IL-17R signaling have focused on activation of downstream signaling, whereas studies of receptor trafficking and localization have been comparatively limited (13). Nonetheless, several key early studies have revealed the significance of IL-17R domains and subunits in the context of receptor localization. For instance, IL-17RA mutants with mutations in the intracellular SEFIR/SEFEX domain exhibit normal protein folding and transport to the cell surface, however, they exhibit different effects on intracellular signaling (14). One particular mutant, the IL-17RAdelta665 mutant lacking the inhibitor CBAD domain, expresses IL-17R more robustly than other receptors, which correlates with its rather stronger signaling capability (17). Furthermore, another study from our group using fluorescence resonance energy transfer (FRET) microscopy indicates that IL-17 stimulation induces disappearance of IL-17RA from the cell surface (83). Altogether, these observations demonstrate that intracellular signaling events and the capability of the receptor to signal could partially control IL-17R trafficking and localization. However, although the final

fate of IL-17RA after signaling is unknown, the fact that the receptor reappears on the cell surface ten to thirty minutes after removing IL-17 from the medium (Gaffen lab, unpublished), suggests that IL-17RA is recycled back to the plasma membrane. The mechanism of the possible receptor recycling is unknown, but may involve ubiquitination of IL-17RA, since and the IL-17RA is ubiquitinated (58) and ubiquitination mediates recycling of epidermal growth factor receptor (EGFR) (84). Further studies to identify the mechanism by which IL-17R is transported to and from the cell surface and recycled after signaling may reveal the involvement of plasma membrane proteins that regulate intracellular trafficking, such as scaffolding proteins or ubiquitin-related proteins. In sum, these reports indicate that there are many layers of complexity to the regulation of IL-17R signaling and subcellular localization.

1.6 INHIBITION OF IL-17R SIGNALING IS REGULATED BY UBIQUITINATION

As described above, ubiquitination of TRAF6 is an important step in the regulation of IL-17R signaling. Thus far, two DUBs, A20 and ubiquitin-specific protease 25 (USP25), have been described to remove K63-pUb chains from TRAF6 to inhibit IL-17R signaling via different mechanisms. Specifically, USP25 interacts with TRAF5 and TRAF6, and removes their ACT1-mediated K63-pUb chains. USP25 requires its DUB activity to inhibit both IL-17-dependent NF-κB activation and IL-17-mediated stabilization of *Cxcl1* and *Il6* transcripts. Consistently, USP25-/- mice exhibit elevated IL-17-mediated inflammation in a setting of lung and CNS inflammation (85). These findings demonstrate that the deubiquitination of K63-pUb is an important regulatory mechanism of IL-17R signaling, and suggest that other DUBs could potentially have roles in the regulation of IL-17-mediated inflammation.

19

Several reports indicate that A20 plays a role in IL-17R signaling, but this relationship appears to be different across cell types. In bone marrow stromal cells, our group showed that A20 inhibits IL-17R signaling by interacting with IL-17R to inhibit TRAF6 polyubiquitination and NF-κB activation. Additionally, IL-17 induces A20 expression via NF-κB (19). In astrocytes, although A20 also inhibits IL-17R signaling, IL-17 represses A20 expression by inducing A20-targeting microRNAs (miR-873 and miR-136-5p) (86–89). Lastly, in enterocytes, IL-17 does not regulate A20 expression, and A20 regulates TLR4 signaling without evidence of controlling IL-17R signaling in these cells (90). Together, these reports show that A20 functions downstream of different receptors, and that distinct cell types have different mechanisms to regulate A20. A possible explanation for these observations could be found by analyzing the differences in exposures among cell types. Enterocytes are constantly exposed to TLR ligands from gut microorganisms. Thus, there must be internal regulatory mechanisms that shift the inhibitory activity of A20 to control TLR signaling instead of IL-17R signaling. In contrast, the central nervous system (CNS) is rarely exposed to microbes or TLR4 ligands, so in homeostasis, inflammatory signaling in cells such as astrocytes is minimal and tightly regulated. However, if microbes enter the CNS, inflammation must be quickly invoked to control the microbial invasion. Thus, the expression of microRNAs that downregulate A20 is a mechanism to quickly increase type IL-17 immunity and eliminate microbes. Following this logic, stromal cells and fibroblasts have less exposure to the TLR4 ligand than enterocytes, so, additional regulatory mechanisms would make fibroblasts more responsive to IL-17 compared to enterocytes. Therefore, A20 inhibits IL-17R signaling because the IL-17 pathway is among the main inflammatory pathways to which fibroblasts and stromal cells respond. In summary, A20 is an anti-inflammatory molecule that inhibits, among other pathways, IL-17R signaling. Additionally,

regulation of A20 is diverse across different tissues, and A20-mediated inhibition of IL-17R signaling is coordinated by microRNAs and A20-related proteins specific for each tissue.

ABIN-1 and CYLD are two examples of A20-related proteins. ABIN-1 binds A20 and contributes to the A20-mediated inhibition of TNF signaling, at least in part, by bridging the interaction of A20 with NEMO (91,92). Additionally, A20 is a DUB with overlapping functions to CYLD. Both DUBs remove K63-pUb chains from NEMO and RIP1 to act as negative regulators of TNF-dependent NF-κB signaling (93–96). Therefore, it is possible that ABIN-1 and CYLD may also contribute to A20-mediated inhibition of IL-17R signaling.

Additionally, work by Draber *et al.* showed that the linear ubiquitin chain assembly complex (LUBAC) recruits CYLD and A20 to inhibit TNF signaling (97). Linear ubiquitination is an important process to regulate NF-κB signaling downstream of TNFα, via activation of the IKK complex (98). Interestingly, there is only one known DUB that removes linear ubiquitin, Otulin, which a report has shown can bind LUBAC to prevent activation of the IKK complex downstream of TNFα signaling (99). Moreover, another recent report has shown that in immune cells Otulin has cell-type-specific effects, including over-production of inflammatory cytokines, accumulation of linear-polyubiquitin chains and spontaneous NF-κB activation in myeloid cells (100). Based on these data, Otulin may be another inhibitor of IL-17R signaling.

Despite all these lines of evidence indicating that ABIN-1, CYLD and Otulin inhibit inflammatory pathways that are used by IL-17, no mechanisms involving these proteins downstream of IL-17-mediated inflammation have been described. These findings raise the question of whether these, and maybe other ubiquitin-related proteins, inhibit IL-17R signaling. Understanding the role of ubiquitination, deubiquitination and the proteins that regulate these processes will lead to a more complete understanding of regulation of IL-17R signaling.

1.7   IL-17R SIGNALING IN CONTEXT WITH OTHER INFLAMMATORY

PATHWAYS

Ligands like LPS, IL-1β or TNFα strongly activate inflammatory signaling by binding to TLR4, IL-1βR1 and TNFR1, respectively. IL-17 is a modest activator of signaling compared to these and other inflammatory ligands. Nevertheless, IL-17 cooperates with LPS, IL-1β, IFNγ, IL-22, lymphotoxin-beta (LT-β) and TNFα to induce inflammatory signaling. The molecular mechanisms of the cooperation of IL-17 with these inflammatory molecules are diverse and poorly understood. However, cooperativity occurs because these pathways share some, but not all, intermediate adaptors that converge on the activation transcription factors to enhance signaling. An additional mechanism of synergy involves stabilization of gene transcripts. For instance, IL-17 and TNFα signaling synergize to activate the promoters of *Lcn2* and *Il6*, but also to stabilize *Il6* transcript.

The differences and similarities among IL-17R, TLR4 and TNFR1 provide the basis to understand cooperation among these signaling pathways. Both TNFR1 and TLR4 have the potential to signal on both the cell surface and inside the cell, but there are no studies indicating that the IL-17R signals on another subcellular compartment besides the cell surface. Additionally, both TNFR1 and TLR4 use different receptor-proximal adaptor proteins to activate their signaling. TNFR1 uses TNFR-associated death domain (TRADD), TRAF2, Receptor-interacting protein (RIP) and Fas-associated death domain (FADD), while TLR4 uses MyD88 (myeloid differentiation primary response protein 88) and Tirap (toll/interleukin-1 receptor domain-containing adapter protein), TRIF and TRAM. Except for TRAF2, none of the TNFR1- or TLR4-proximal adaptor proteins have been reported to function downstream of IL-17R. In terms of differences between signaling effects, TNFR1 induces cell death, while TLR4 activates

several downstream pathways like IFN-responding factor 5 (IRF5) and TBK1/IKKε-dependent IRF3. The similarities among IL-17R, TNFR1 and TLR4 lie in their many shared signaling intermediates. IL-17R and TNFR1 activate the MAPK pathway and the IKK complex to induce NF-κB signaling. Both IL-17R and TLR4 activate TRAF6 to induce MAPK and NF-κB signaling. As aforementioned, A20 and USP25 inhibit IL-17R signaling in addition to TLR4 signaling. The cross-talk of these inhibitors suggest that other regulators from TLR4 and TNFR1 may also play a role downstream of IL-17R signaling. Based on our current knowledge about TLR4 and TNFR1 signaling, I selected genes coding for some of their intermediate adaptors and tested the role of these genes in IL-17R signaling. I describe those results in chapter 3. In summary, insight from TLR4 and TNFR1 signaling can be used to guide research on IL-17R signaling.

1.8   THE ROLE OF IL-17 IN INFECTIONS

A functional IL-17-dependent inflammatory response is required for controlling a wide range of pathogenic organisms including parasites, extracellular and intracellular bacteria, and fungi. Elimination of these pathogens is organized by IL-17-induced antimicrobial peptides that directly kill pathogens, or by IL-17-induced chemokines that indirectly destroy pathogens through the recruitment of neutrophils and monocytes to the site of the infection. For example, IL-17 induces S100A8 and S100A9, which together form the heterodimeric protein calprotectin. Similar to Lcn2, which sequesters iron (as explained above in section 1.4), calprotectin limits microbial growth by restricting access to manganese and zinc.

IL-17-producing cells have been examined in the context of mucosal infection by fungi such as *Candida albicans, Pneumocystis, and Cryptococcus neoformans*, and bacteria such as *Klebsiella pneumoniae, Staphylococcus aureus* and *Mycobacterium tuberculosis* (101–103). The role of IL-17 signaling is less clear for the regulation of viral infections.

Regarding fungal pathogens, humans with defects at various points along the Th17 pathway are susceptible to mucosal, but usually not disseminated, candidiasis. Furthermore, human studies have shown that patients with complete deficiency in IL-17RA or IL-17RC, or partial deficiency in IL-17F (and consequently in IL-17F/F and IL-17A/F), have susceptibility to chronic mucocutaneous candidiasis infection (104,105). This not only ties IL-17 to the anti-*Candida* response but also indicates that the dominant activity of IL-17 in humans is antifungal. Additionally, multiple studies have shown that a STAT1 gain-of-function (GOF) mutation and caspase recruitment domain-containing protein 9 (CARD9) deficiency increase susceptibility to *Candida* infections, and are associated with decreased IL-17 production. STAT1 GOF competes with and overcomes the binding of STAT3 to the *Rorc* promoter, decreasing expression of *Rorc*, *Il17* and IL-17 target genes (106). Furthermore, patients with Job's Syndrome/Hyper-IgE Syndrome (HIES) have autosomal dominant-negative mutations in STAT3 associated with reduction of T_H17 cell frequencies. These patients are susceptible to chronic mucocutaneous candidiasis (CMC) (107). Similarly, mutations in the autoimmune regulator (AIRE) increase the susceptibility to CMC. This is thought to be because AIRE-deficiency leads to production of autoantibodies against IFNs and T_H17-related cytokines like IL-22, IL-17F and IL-17A, resulting in blockade of the type 17 response (108). Altogether, these finding with patients confirm the key role of IL-17 signaling in clearance of *Candida albicans* infection. Indeed, the robust

association of IL-17 signaling with *Candida albicans* infection allows murine models of candidiasis to be used as physiological read-outs of the type 17 immunity (28,81).

In *Pneumocystis* infections, a recent study showed that while IL-17 production was increased in the lungs of infected mice, IL-17A-/- mice cleared *Pneumocystis* infection with kinetics similar to wild-type mice, suggesting that IL-17 is not required in all situations to control *Pneumocystis* infection (109). Despite these conflicting murine studies, *Pneumocystis* remains an important pathogen in immunosuppressed patients deficient in TH17 cells, which indicates that IL-17 signaling plays a role in the clearing of this infection.

In both immunocompromised and immunocompetent individuals, *Cryptococcus neoform*ans causes significant morbidity and mortality. *Cryptococcus neoformans* uses a PGE2- and IRF4-dependent mechanism to specifically inhibit induction of IL-17 during TH17 differentiation (110). Experiments in IL-23 deficient mice have shown a reduction in IL-17 increases susceptibility to infection by *Cryptococcus neoformans* (111). Thus, altogether, these findings suggest that *Cryptococcus neoformans* infection is regulated by type 17 immunity.

IL-17 signaling is also involved in the inflammatory response to extracellular bacterial infections. For example, deficiency in IL-17 signaling decreases pulmonary clearance of *Klebsiella pneumon*iae in mice. Extensive work performed by the Kolls group has shown that the regulation of *Klebsiella pneumoniae* is dependent on IL-17 receptor signaling in the lung epithelium, and that IL-17 signaling is crucial for inducing Lcn2 production and for mediating mucosal defense (112–114). As described above, Lcn2 restricts *Klebsiella pneumoniae* from accessing free iron, thereby limiting bacterial growth (113,115,116). These studies show the importance of the IL-17-dependent production of antimicrobial peptides in controlling bacterial burden. *Staphylococcus aureus* infection is another bacterial infection that requires an IL-17-

mediated immune response, as human and murine studies suggest. Patients with HIES have increased susceptibility to *Staphylococcus aureus* infection. In a *Staphylococcus aureus* infection murine model, the IL-17 response induces neutrophil recruitment and bacterial clearance, reducing mortality (40). In sum, gram-positive and gram-negative extracellular bacterial infections are regulated by IL-17 signaling.

The IL-17 response also exhibits a protective role for intracellular bacteria in human studies and murine models. Notably, there is an apparent role for IL-17 in protective immunity against hypervirulent *Mycobacterium tuberculosis* infection. The Khader group showed that IL-17 has a protective role against *Mycobacterium tuberculosis* infection by limiting hypoxia around bacterial granulomas, through inhibition of hypoxia-induced factor-1alpha (HIF1α). *In vivo* neutralization of IL-17 increased *Mycobacterium tuberculosis* susceptibility. This was consistent with results showing that a human polymorphism upstream of the *IL17* promoter correlates with decreased IL-17 production and increased risk of tuberculosis (117). Additionally, the Khader group has found that IL-17 also mediates an unexpected protective immunity against *Mycobacterium tuberculosis* via the IL-17-dependent induction of CXCL13, which is required for localization of T cells within lung lymphoid follicles. Appropriate T cell localization within lung lymphoid follicles is, in turn, necessary for macrophage activation and control of Mycobacterium tuberculosis (118).

Finally, a dual protective and pathogenic role has been associated with IL-17 in the clearance of Influenza virus and rhinovirus infections. Importantly, Influenza A co-infection has been shown to decrease IL-17, IL-22, and IL-23 production after *Staphylococcus aureus* infection (119), suggesting that the virus regulates type 17 immunity. In addition to a possible role in controlling viral infections, IL-17A is also related to viruses by homology with the

herpesvirus saimiri (HVS). Sequence alignment of mouse IL-17 molecule contains 147 amino acids and shares 57% identity to HSV13, an open reading frame (ORF) from HVS (120).

In summary, IL-17 signaling is crucial for fighting certain fungal and bacterial pathogens, and might also play at least a limited role in response to some viral infections. The control of these pathogens requires the expression of IL-17-target genes to induce inflammation. Depending on the pathogen, the organization of different IL-17 target genes contributes to the clearing of the infection. Altogether, these studies suggest that type 17 immunity is key for clearing or controlling some pathogens, and that understanding how to enhance IL-17R signaling could contribute to the development of future anti-microbial therapies.

1.9   THE ROLE OF IL-17 SIGNALING IN AUTOIMMUNE DISEASES

In autoimmune diseases, tolerance to self-antigens is compromised, triggering inflammation and tissue destruction, and the specific disease manifestation depends on the organ where this excessive inflammation is found. Dysregulation of IL-17 expression or signaling in different tissues is associated with the onset of different autoimmune diseases. A large body of evidence indicates that IL-17-mediated inflammation contributes to the pathology of autoimmune diseases such as psoriasis, multiple sclerosis, Crohn's disease, rheumatoid arthritis, ankylosing spondylitis and chronic noninfectious uveitis. In this section, I will discuss the roles of IL-17 in the

pathogenesis of a few autoimmune diseases, focusing on those that have animal models that could be used in future studies, and those that might benefit from IL-17-related therapies.

1.9.1 Psoriasis

Psoriasis is a chronic skin disease characterized by dermal hyperplasia that results from the dysregulation of inflammatory signaling between keratinocytes and infiltrating immune cells. In psoriasis, skin-resident IL-17-producing cells respond to inflammatory molecules producing IL-17. Keratinocytes respond to IL-17-inducing inflammatory and chemoattracting molecules that recruit neutrophils to the local area (121). The IL-17-mediated inflammation induces rapid recycling of epidermal cells, leading to the formation of red, scaly lesions and chronic skin inflammation. A mechanism underlying the role of IL-17 in psoriasis may involve its propensity to synergize with other factors. For example, IL-17 synergizes with other cytokines like IFNγ, TNFα, and IL-22 to induce inflammatory cytokines and chemokines. IL-17, in combination with TNFα, induces inflammatory genes that are characteristic of psoriasis from human keratinocytes, such as IL-6, IL-8 and ICAM-1 (122,123). In addition, IL-17, together with IL-22, synergistically increases the expression of skin antimicrobial peptides, including β-defensin-2 (BD-2) and the S100 proteins, S100A7, -8, and -9 (124). Consistent with the elevated production of antimicrobial peptides, psoriasis patients are more resistant to skin infections than healthy people (125). Furthermore, keratinocytes stimulated with IL-17, IL-22 and TNFα produce CCL20 (a ligand for CCR6), which is probably required for the continuous recruitment of CCR6-positive T_H17 cells in the psoriasis lesions (126).

The success of inhibiting IL-17 as a therapy to reduce skin disease in patients with plaque psoriasis highlights the role of IL-17 in this disease. Therapeutically, blocking of IL-17 was found to be more effective than blocking TNFα for the treatment of psoriasis (127). Despite the clear association of IL-17 signaling with the onset of psoriasis, human genetic studies have found no link between psoriasis and polymorphisms in genes coding for IL-17 or its receptor. However, several polymorphisms in genes that regulate NF-κB signaling have been found, suggesting that the role of IL-17 on the onset of psoriasis might be to activate NF-κB-dependent inflammatory genes.

Additional studies have shown that polymorphisms in NF-κB-inhibitory genes such as *TNFAIP3* and *TNIP1* are linked to the onset of psoriasis (128–131). Consistent with these findings from human studies, the mouse homologues Tnfaip3 and Tnip1 have been shown to regulate IL-17-mediated inflammation in murine models of psoriasis. Mechanistically, A20 inhibits IL-17R signaling (19), which explains why the lack of A20 increases IL-17-mediated inflammation; however, the molecular mechanism of ABIN-1 (coded by *Tnip1*) function downstream of IL-17R signaling has not been solved.

In addition to human genetic studies, skin biopsies from psoriatic patients provide evidence of the proteins involved in psoriasis. For example, patient biopsies have elevated levels of TGFα compared to controls (132,133), suggesting a role for TGFα in this disease. Additionally, a study comparing psoriatic with non-affected skin biopsies from the same patients showed that the psoriatic areas have decreased ABIN-1 protein (134), consistent with genetic associations mentioned above. Moreover, several studies have shown that caveolin-1 protein is dramatically downregulated in psoriatic skin lesions (135–139), suggesting that caveolin-1 has a role in the pathogenesis of psoriasis. However, no clear connections between caveolin-1

functions and the molecular pathways involved in the onset of psoriasis have been established. Thus, further mechanistic studies are required to understand the role of Caveolin-1 protein in the molecular pathways involved in the development of psoriasis.

1.9.2   Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS. Patients with MS have more IL-17-producing cells in blood and cerebrospinal fluid (CSF) than healthy controls (140), and necropsy samples taken from the brains of patients with multiple sclerosis show increased IL-17 expression (141). Although these findings suggest a role for IL-17 in the CNS, more solid evidence of this comes from the murine experimental autoimmune encephalitis (EAE) model of MS.

In EAE, T_H17 cells accumulate in the CNS, and the production of IL-17 induces inflammation mediated by glial cells (142). Furthermore, lack of IL-23 signaling arrests these T_H17 cells in an early activation stage in which they do not cause EAE (143). An important pathogenic aspect of T_H17 cells is the IL-23-driven production of GM-CSF, as evidenced by the observation that IL-23R-deficient T_H17 cells do not cause disease (144,145). IL-23 signaling is also pathogenic because it induces IFNγ expression in T_H17 cells, and these double IL-17/IFNγ-producing cells have been shown to transfer EAE susceptibility (146–148).

As described in section 1.2, IL-6 and IL-1β signaling are important for the development of T_H17 cells, which is exemplified by results showing that mice deficient in IL-6 signaling or IL-1β signaling have fewer T_H17 cells, and consequently, are protected from developing EAE

(149,150). Moreover, plasticity between Treg and T$_H$17 cells has been observed in EAE and in patients with MS (147,151).

Additionally, in EAE, IL-17 induces p38-MAPK activation, which in turn activates dual specificity protein phosphatase 1 (DUSP1) in CNS-resident cells to induce expression of chemokines that recruit T$_H$17, T$_H$1, and macrophages to the CNS. This mechanism maybe important for initiation of EAE (152), and possibly important for the initiation of MS. The relevance of IL-17 in the development of EAE makes it a commonly-used model for testing IL-17 signaling under physiological conditions (81,153). As with other autoimmune diseases, MS is a multifactorial disease, and the use of the EAE model has contributed greatly to understanding the role of type 17 immunity in this disease.

1.9.3   Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a heterogeneous and chronic disease that also has an IL-17R signaling component in its etiology. The role of IL-17 signaling in this disease has not been clearly established, in part due to challenges arising from disease heterogeneity. RA is characterized by the proliferation of synovial fibroblasts, infiltration of CD4+ T-cells, and autoantibody-producing plasma cells into joint spaces, leading to joint and cartilage erosion. RA patients are diagnosed based on joint inflammation and autoantibody production, symptoms that can be caused by different etiologies. This heterogeneity is exemplified by the fact that there is no single therapy that works for all patients (something that is common to most autoimmune diseases). Instead, therapies work for subsets of patients, leading to the classification of them as responders or non-responders. Different pools of patients with RA respond differently to treatments such as the small molecule methotrexate (MTX), anti-TNF blocking antibody, anti-

CD20 antibody or anti-IL-17 blocking antibody. Interestingly, in clinical trials, the beneficial effect of blocking IL-17 was not significantly greater than the effect of blocking TNF, a currently approved therapy (154,155). Thus, results from clinical studies show that although IL-17 may play a pathologic role in the development of RA, this effect is probably restricted to a subset of patients. Additionally, this suggests that the role of IL-17 signaling in RA is part of multiple underlying mechanisms for disease, but not all patients manifest disease due to the same mechanisms. The mechanisms of IL-17 in RA pathogenesis require cooperation between IL-17 and TNFα to induce expression of chemokines, cytokines, and MMPs to destroy cartilage and remodel joint tissue (156,157). Specifically, osteoclasts respond to IL-17 stimulation by inducing expression of mediators like MMPs and RANKL. The expression of these inflammatory genes leads to cartilage and bone destruction, which destroys and remodels the joint tissue (158). Altogether, results from human studies of RA reveal that IL-17 signaling is one component of the multifactorial onset of disease.

Additional evidence for a role of IL-17 in RA comes from experimental murine models. Collagen-induced arthritis (CIA), a mouse model of RA, is much more severe in mice lacking IL-12 or IFNγ, arguing against the importance of T_H1 cells in this model. A key insight into this disease came from the discovery that IL-23 is more important for the development of CIA than IL-12 (159), suggesting an essential role for T_H17 cells. Indeed, neutralizing IL-17 or its receptor in CIA mouse models reduces joint inflammation, cartilage destruction and bone erosion (160), whereas ectopic expression of IL-17 promotes collagen arthritis and aggravates joint destruction (161). IL-17-deficient mice were resistant to CIA, confirming the critical role of IL-17 in CIA (162). However, given differences between murine CIA and human RA, these findings do not

discredit the role of other mechanisms in human RA, but they do support a contributing role for IL-17 signaling in RA.

1.9.4   Ankylosing Spondylitis

Ankylosing spondylitis (AS) is a chronic immune-mediated inflammatory disease characterized by progressive spinal rigidity and chronic spinal inflammation initiating in the sacroiliac joints, presenting as chronic inflammation at the sites of ligamentous and tendinous insertions into bone (163). A successful treatment for AS is the use of anti-TNF blocking antibodies, which confirms that this disease is highly driven by TNFα-mediated inflammation. Furthermore, IL-17-mediated inflammation is also an important component for the development of AS. Recent clinical trials with anti-IL-17 antibodies have shown significant clinical efficacy in AS (164), superior to the effect of blocking TNFα. Measured by clinically assessing disease severity and changes in baseline in the high-sensitivity C-reactive protein (CRP) level in serum, they showed that blocking IL-17 is not only is effective in patients who have not received TNF agents previously but also may be effective in patients in whom previous anti-TNF treatment failed (165). Consistent with a model in which IL-17 contributes to the pathology of AS, genetic studies have shown a strong genetic association with a series of polymorphisms in the *IL23R* gene, including R381Q. This polymorphism confers protection from AS (148), and it was experimentally corroborated that the R381Q polymorphism impairs IL-23–dependent IL-17 production by T_H17 cells *in vitro*. (166). These results suggest that genetically determined down-modulation of type 17 immunity provides protection against AS. Additional evidence indicating that IL-17 is

pathologic in AS comes from the observations that serum levels of IL-17 and IL-23, and circulating T_H-17 and $\gamma\delta$ T cells, are increased in AS patients compared to controls (167,168). Additionally, in mice, IL-23 overexpression induces an AS-like disease mediated by increased IL-17 production (39), providing supportive evidence that type 17 immunity is pathologic in AS. Further understanding of IL-17R signaling may contribute to the development of better therapies for this and other autoimmune diseases.

1.9.5 Noninfectious Uveitis

Noninfectious uveitis is a form of intraocular inflammation that remains a major cause of vision loss. Uveitis is an organ-specific T cell–driven autoimmune disease of an immune-privileged site. Both T_H17 and T_H1 cells are thought to contribute to tissue damage in the intraocular tissue (169). Moreover, upregulation of IL-23 and IL-17 occurs in patients with active uveitis (170), suggesting that IL-17 signaling plays an inflammatory role in the pathogenesis of uveitis. Furthermore, the use of anti-IL-17 blocking therapy is approved as second-line treatment for AS, suggesting that excessive IL-17 signaling is pathologic in AS.

1.9.6 Crohn's Disease

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by increased inflammation in the gastrointestinal track, decreased nutrient absorption and significant disruption of the intestinal epithelial barriers. Associations between polymorphisms in the *IL23R*

gene and the risk of adult and pediatric CD suggested a link between type 17 immunity and CD (171). Furthermore, biopsy samples from patients with CD showed an increased expression of IL-17, IL-23, IL-22, and IL-6 compared to control patients, suggesting that these cytokines are involved in the pathogenesis of this disease (172). Mechanistically, IL-17 has both pro- and anti-inflammatory roles as described in animal models of IBD. In some models, IL-23 administration exacerbates disease, and inhibition of IL-23 is and subsequent decrease in IL-17 is protective. In contrast to its inflammatory role, IL-17 has a protective role in a model of T cell-mediated intestinal inflammation (69). This dichotomy between IL-23 and IL-17 in mediating pathogenesis of IBD is because in colitis, the intestinal epithelial barrier is preserved by IL-17-dependent expression of occludin and claudin-1, two major tight junction proteins (69). The IL-17-dependent induction of tight junctions preserves the epithelium integrity and limits IBD. Consistent with the protective role of IL-17 in colitis, clinical trials of anti-IL-17 and anti-IL-17R showed no improvement, and even exacerbated disease (173). These findings emphasize that IL-17R signaling is an important inflammatory mediator, but that it also contributes to tissue-specific functions such as maintenance of intestinal epithelial integrity.

1.10   STATEMENT OF THE PROBLEM

Our understanding of IL-17-mediated functions in the immune response is driving several advances in the treatment of human disease. Infectious and autoimmune diseases are regulated by IL-17 signaling. For autoimmune diseases, therapies have already been employed to block IL-17 cytokine, and some studies have investigated blocking the IL-17 receptor directly as a more

specific therapeutic. However, IL-17R downstream signaling is incompletely understood. The identification of novel downstream intermediates of the IL-17 receptor can contribute to our understanding of the effects of blocking IL-17R and inform the development of more specific therapies to control IL-17-mediated inflammation. In this thesis, I aimed to identify novel signaling intermediates of IL-17R, and to describe the molecular mechanisms by which two of these intermediates regulate IL-17 signaling.

1.11   SUMMARY

In this thesis, I discovered several genes that potentially regulate IL-17 signaling. I describe the screening and general characterization of these genes in Chapter 3. Among the candidates, I decided to focus on ABIN-1 (*Tnip1*) and caveolin-1 (*Cav1*) in an attempt to describe their mechanisms of action within the IL-17 signal transduction setting.

ABIN-1 is an A20-binding protein, and both human genetic studies and experimental animal models demonstrated the relevance of ABIN-1 in inhibiting the inflammatory pathways involved in the onset of psoriasis (129,134,174,175). Since IL-17 signaling is associated with psoriasis and inhibited by A20, I asked whether ABIN-1 was also a downstream inhibitor of the IL-17 signaling pathway. Chapter 4 provides data to demonstrate that ABIN-1 inhibits tonic and IL-17-induced signaling, and in turn, IL-17 signaling induces degradation of ABIN-1. These data suggest a novel regulatory mechanism by which ABIN-1 functions in the IL-17 signaling pathway.

Among its numerous intracellular functions, caveolin-1 regulates inflammation by interacting with TNFR1 and TLR4, thereby regulating some of their downstream effectors (176,177). Since proteins that regulate and interact with TNFR1 and TLR4 often also regulate IL-17R signaling, I explored the hypothesis that Cav1 regulates IL-17R signaling. Chapter 5 describes preliminary findings that suggest caveolin-1 may indeed be a positive regulator of IL-17 signaling.

In summary, this thesis describes my studies of the different molecular mechanisms and proteins that regulate IL-17R signaling. My results contribute to the characterization of downstream intermediate molecules of IL-17R signaling, and provide insight into novel molecular targets for developing future therapies to control IL-17-mediated inflammation.

2.0   CHAPTER TWO: MATERIAL AND METHODS

2.1   ANTIBODIES

The antibodies used for western blot and immunoprecipitation analysis are listed on table 2.1 along with the sources, the dilution used to detect proteins and notes regarding the incubation.

2.2   CELL LINES

HEK293T cells, ST2 cells and MEFs were cultured in α-MEM (Sigma, St Louis MO) containing 10% fetal bovine serum (FBS) with 1% L-glutamine and 1% antibiotics (Invitrogen).

2.3   DERIVATION OF TAIL FIBROBLASTS

Mice were sacrificed following all the regulations established by the Institutional Animal Care and Use Committee (IACUC) from the University of Pittsburgh. Tails from 5- 15 weeks old mice were cut and stored in α-MEM (Sigma, St Louis MO) containing 10% fetal bovine serum (FBS) with 1% L-glutamine and 2% antibiotics (Invitrogen) on ice or at $4^{O}C$ until the tails were processed.

All the post-collection processing of the tails was performed in a tissue culture hood under sterile condition. Tails were cut with a razor blade into the smallest pieces attainable in a tissue culture plate. Next, the pieces of tail were resuspended in 2 mL PBS with PenStrep and collagenase (total concentration = 1000U/mL). Tails were digested in collagenase for 30 minutes at 37°C, and tubes containing the tails were mixed every 5 minutes to ensure correct collagenase treatment. When incubation was over, the tubes were spun at 1200 RPM for 5 minutes and washed twice with HBSS. After washing, 3 mL of trypsin was added, and tails were incubated for additional 20 minutes at 37°C. After incubation, trypsin was neutralized with 3 mL of media (20% FBS) and centrifugated for 5 minutes. The supernatant was removed, the pellet resuspended in 5 mL of complete media (α-MEM containing 20% fetal bovine serum (FBS) with 1% L-glutamine and 2% antibiotics) and transferred into T25 tissue culture flasks. The tissue pieces were kept until layers of fibroblasts were visible at the bottom of the flask. After 3 days, more media was added on top of the culture, and 2-3 days later the media with the unattached cells and tissue was discarded, and replaced with fresh media. After fibroblast were confluent on the original flask, they were split into larger areas and cultured. To immortalize cells, fibroblasts were transfected with SV40 T antigen (24-well plate) and cultured for few passages until growth advantage was visible. Aliquots of all cells were frozen and stored in liquid nitrogen.

2.4   PLASMIDS

Plasmids expressing E-tag ABIN-1 and the human ABIN-1 promoter were provided by Dr. Rudi Beayert (University of Ghent, Belgium) and Dr. Brian Aneskievich (University of Connecticut).

These plasmids and the Lcn2 promoter were previously described (Heyninck 2003, Gurevich 2012, Shen 2006}. Other plasmids in this study were cloned using primers from table 2.2.

2.5   SMALL INTERFERING RNA

All small interfering RNAs (siRNAs) used in this project were purchased from Dharmacon (GE, Lafayette CO) and they are listed in tables 2.3 and 2.4.

2.6   TRANSFECTION

Transfections to knockdown specific genes were performed using siRNAs purchased from Dharmacon. Cells were plated in either 12-, 24- or 6-well plates in αMEM medium with 10% FBS and 1% L-glutamine. No antibiotic was added to the medium. Next day in the morning, cells were siRNA Transfected with 5μM siRNA prepared in 1X siRNA transfection buffer (Dhamacon), Dharmafect reagent (Dhamacon) and OptiMEM medium. Mix was prepared following vendor's instructions. Twenty-four hours later, the medium was changed for fresh medium. Next day, cells were stimulated and harvested as indicated for each specific experiment. In all cases efficiency of knock-down was confirmed by qPCR and western blotting.

Transient transfections to overexpress a specific gene were performed using Fugene HD (Promega, Madison WO) or Lipojet (SignaGen, Rockville MD). Cells were plated the day before the transfection in antibiotic free αMEM (Fugene HD transfection) or complete αMEM (Lipojet transfection). Mixes were prepared following vendor's instructions. Briefly, plasmids coding for

40

the gene of interest were mix with the appropriate reagent and incubated for up to 10 minutes at room temperature. Mix was added drop-by-drop to cells and left there for 24h before stimulation and harvesting of cells.

2.7 LUCIFERASE ASSAY

Luciferase assays were performed as described (Shen 2006). ST2 (0.05×10^6) or fibroblasts (0.05×10^6) cells were plated in a 12-well plate using antibiotic free αMEM the day before the transfection. Transfections were performed as described above, mixing the plasmid of interest or empty vector, 100-500 ng promoter reporter-luciferase and 5ng renilla-luciferase. Next day after transfection, cells were stimulated for 6-8h with IL-17 and harvested. Cells trypsinized for 5 minutes, trypsin was neutralized, and cells were transferred into a 1.5mL tube. Tubes were spun for 5 min at 3000g and the supernatant removed. The cell pellet was resuspended in 50μL lysis buffer (1% NP40, 150mM NaCl, 50mM Tris pH 8.0, 2mM NaF, supplemented with fresh 1mM Na2VO4, 50mM PMSF, 10mM β-glycerophosphate disodium salt and a protease inhibitor cocktail (Sigma-Millipore)) and incubated for 20 minutes in ice. After lysis, tubes were spun for 20 minutes at 20000g at 4°C. After spinning, 15μL of supernatant was added to each well of a

white 96-well plate for luciferase(Fisher) assay, and the plate was inserted in the Veritas microplate Luminometer. The luminometer automatically mixed the cell lysated with Dual-Glo® Luciferase Assay System (PROMEGA) reagents, and measured luciferase activity.

2.8   INHIBITORS

ST2 cells were plated 24-48h before treatment in complete αMEM medium. Inhibitors and conditions used are described in table 2.5. All these drugs were purchased from EMD Millipore.

2.9   RNA ISOLATION AND QUANTIATIVE REVERSE TRANSCRIPTASE PCR

RNA was extracted using RNAeasy Kits (74106, Qiagen) and cDNA was made to perform qPCR. Primers used for qPCR analysis were purchases from Qiagen. The lists of primers are in tables 2.6 and 2.7. Relative gene expression was normalized to *Gapdh* and to the relevant control. All primers were purchased from Qiagen.

2.10   ELISA

ELISA analyses were performed using kits were from eBioscience (IL-6; 88-7064-88) or from R&D systems (Lcn2; MLCN20, & CXCL1; DY453).

2.11   IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Cells were lysed on ice for 20 minutes in lysis buffer (1% NP40, 150mM NaCl, 50mM Tris pH 8.0, 2mM NaF) supplemented with 1mM Na2VO4, 50mM PMSF, 10mM β-glycerophosphate disodium salt and a protease inhibitor cocktail (Sigma-Millipore). Samples were boiled (5-15 minutes) in running buffer and immunoblotted. Band intensity was analyzed using AlphaView SA Version:3.4.0 (Protein Simple, San Jose CA).

2.12   INTRAPERITONEAL INJECTION OF MICE AND CARDIAC PUNCTURE

All mice were handled according to the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) guidelines. To study the IL-17-induced cytokine response in serum, age- and sex-matched mice were intraperitoneally injected with PBS alone or with a combination of IL-17 (1μg per mouse) and TNFα (200ng per mouse) in PBS. All mice were awake during the injection, and remained awake in their cage for 4 hours. After the end of 4 hours mice were sacrificed using CO_2. Immediately after each mouse stopped breathing and moving, the rear paw was pinched to confirm that the animal was dead. If the mouse did not react to pinching, blood was collected using a 1mL syringe with a 26G needle by inserting the needle in the area immediately below the sternum, and slightly to the left of the mouse. A volume of 300μL to 900μL of blood was collected per mouse and gently transferred into a 1.7mL Eppendorf tube. Tubes were rested on horizontal position at 4ºC during overnight. Next day, the tubes were spin at 4000g for 10 minutes at 4ºC. 80μL to 300μL of serum (supernatant) per sample were collected and transferred to new tubes. Samples were stored at -20ºC until they were used for IL-6,

CXCL1 and Lcn2 ELISA analysis. For IL-6 and CXCL1 analysis, the samples required no dilution and were reused to measured different cytokines. For Lcn2, the samples required a large (> 1:1000) dilution since there seems to be a high background.

2.13   STATISTICS

To assess significance, we used Student's t test, ANOVA with post hoc Tukey's analysis, or linear regression. $P < 0.05$ was considered significant. Error bars reflect the means $\pm$ SEM of biological replicates within individual experiments or pooled data, as indicated in legends. All experiments were repeated a minimum of twice. We use R (R Foundations for Statistical Computing) to perform linear regression to model the effect of duration of IL-17-stimulation on ABIN-1 mRNA or protein.

Table 2.1: List of antibodies used in this thesis.

Antibody	company	Catalog#	Dilution	Species	Notes
4G10	Millipore	05-1050	1:1000	Mouse	ON/4C
A20	Cell Sig.	5630	1:1000	Rabbit	
ABIN1	Invitrogen	37-6100	1:1000	Mouse	1h/RT – Unreliable
ABIN1	Sta. Cruz	SC-134660	1:1000	Rabbit	ON/4C – Works well
ABIN1	Cell Sig.	4664S	IP	Rabbit	2h antibody/4C -> 2h Protein A agarose/4C
Act1	Sta. Cruz	SC-11444	1:1000	Rabbit	ON/4C
anti-E-tag	Cell Sig.	13419	1:2000	Rabbit	Overnight/4C or 1h/RT
Anti-GFP	Sta. Cruz	SC-8334	1:1000	Rabbit	ON/4C
Anti-goat HRP	Pierce	431402	1:5000	Rabbit	1h/RT
Anti-IL-17RA	RD		1:500	Goat	1h/RT
B-TrCP	Cell Sig.	11984	1:1000	Rabbit	ON/4C
Caveolin1	BD	610406	1:1000	Mouse	ON/4C
CYLD	Sta. Cruz	SC-25779	1:1000	Rabbit	It does not work
CYLD	Cell Sig.	8462T	1:1500	Rabbit	ON/4C – works great
E-FITC	Abcam	Ab19399			
ERK-Phospho	Cell Sig.	9106S	1:1000	Mouse	ON/4C
FLAG M2	Sigma	F1804	1:1000	mouse	Overnight/4C
GSK3B	Cell Sig.	9315	1:1000	Rabbit	
HA	Sigma	H3663-200UL (H-274)	1:2000	mouse	Overnight/4C
IkBa(C-21)	Sta. Cruz	sc371	1:1000	Rabbit	1h/RT
IKBa-Phospho	Cell Sig.	9246L	1:1000	Mouse	Block ON/4C
IKKalpha	Cell Sig.	11930	1:1000	Mouse	ON/4C – Do not reuse
IKKbeta	Cell Sig.	2678	1:1000	Rabbit	Block in Milk ON/4C
IKKepsilon	Cell Sig.	3416P	1:1000	Rabbit	ON/4C
IL-17RC	R&D	AF2270	1:500	Goat	ON/4C
JNK-Phospho	Cell Sig.	9251S	1:1000	Rabbit	ON/4C
MYC	Cell Sig.		1:1000	Mouse	Overnight/4C
Otulin	Sta. Cruz	sc-162789	1:200	Goat	
p38-phopho	Cell Sig.	9211S	1:1000	Rabbit	ON/4C
P-GSK3B	Cell Sig.	9336S	1:1000	Rabbit	Block in milk (1h) - ON/4C
P-IKKα/β	Cell Sig.	2697	1:1500	Rabbit	1h/RT – TBST, block ON
Poly-ubiquitin	ENZO	BML-PW8805	1:200	Mouse	Overnight/4C
Syk	Cell Sig.	13198	1:1000	Rabbit	ON/4C
TAK1	Sta. Cruz	sc-7162	1:1000	Rabbit	Overnight/4C or 1h/RT

TAK1-Phospho	Cell Sig.	4531	1:1000	Rabbit	Overnight/4C
total ERK	Cell Sig.	9102	1:2000	rabbit	ON/4C
total JNK	Cell Sig.	9252	1:1000	rabbit	ON/4C
total p38	Cell Sig.	9212	1:1000	Rabbit	ON/4C
TRAF3	Sta. Cruz	sc-949	1:1000	Rabbit	ON/4C
TRAF6	Sta. Cruz	sc-7221	1:1000	Rabbit	It works for IP and WB
Ubiquitin (P4D1)	Sta. Cruz	sc8017	1:200	Mouse	Antibody in BSA
α-Tubulin-HRP	Abcam	Ab40742	1:20000		ON/4C or RT/1h
Anti-Goat	Sta. Cruz	SC-2020	1:5000	Donkey	
IKBζ	Cell Sig.	92445	1:1000	Rabbit	
β-actin - HRP	Abcam	ab49900	1:100000	Mouse	Overnight/4C or 1h/RT

Table 2.2 : Primers used to clone plasmids used in this thesis.

Gene	Forward Primer	Reverse Primer
ABIN-1	GCAAGCTTATGGA AGGGAGAGGACCC	GCTCTAGATGCCT GGGGCCCATCACA
Caveolin-1 WT	GCAAGCTTATGTC TGGGGGCAAATAC	GCTCTAGATGCTA TCTCTTTCTGCGT
Caveolin-1 ΔY14F	AAGCTTATGTCTGGGGG CAAATACGTAGACTCCGAGG GACATCTCTTCACTGTTC	Same as WT
Caveolin-1 ΔY14A	ATAAGCTTATGTCTGGGGGC AAATACGTAGACTCCGAG GGACATCTCGCCACTGTTC	Same as WT
IL-17RA-HAΔcav1-binding site	TGGGATGGCTGC TTCTGCTG	GTTTGGGTCCCCATCAGGGATGGGCTT CGGCAGGTGGTGAACAGTCACTTCATACTCC TGGCCAGGATCTACCACAGCGTGGCTGA AGGAAGCCCGCGCCCG

48

Table 2.3: Murine siRNA used in this project.

	Gene	Catalog #
1	Tnip1 (ABIN-1)	L-047652-01
2	Tnfaip3 (A20)	L-058907-02
3	Non-targeting mock	D-001810-10-20
4	Traf3ip2(ACT1)	L-059885-01
5	Fam105b(Otulin)	L-067300-01
6	Cyld	L-055575-01
7	Caveolin-1	L-058415-00
8	Cdc27 (AnaPC3)	L-167064-00
9	USP15	L-040417-01
10	Sykb	L-041084-00
11	Otub7b (Cezanne)	L-050018-01
12	IRAK2	L-060091-00
13	Parp10	L-164453-00
14	Btrc (Btrcp1)	L-044048-00
15	Fbwx11 (Btrcp2)	L-058886-00

Table 2.4: Human siRNA used in this project.

	Gene	Catalog #
1	**TNIP1 (ABIN-1)**	L-016358-00
2	**Tnfaip3 (A20)**	L-009919-00
3	**Traf3ip2(ACT1)**	L-004311-01
4	**FAM105B (OTULIN)**	L-018991-02
5	**CYLD**	L-004609-00
6	**CAV1**	L-003467-00
7	**CDC27 (AnaPC3)**	L-003229-00

Table 2.5: Small molecule inhibitors used in this project.

Inhibitor	Catalog #	Working concentration	Time of treatment	Reference
MG132	474790-100UG	20μM	30 min pt & various with IL-17	(75)
Staurosporine	569396	100nM	20 min pt & 2h with IL-17	(75)
IKK inhibitor VII	401486	10μM	30 min pt & 2h with IL-17	(19)
Bay 11-7082	196870-10MG	100μM	1h pt & various with IL-17	(178,179)

Abbreviations: pt: pre-treatment.

Table 2.6: Murine primers used for quantitative RT-PCR.

Gene	Catalog #
Tnip1	QT00149009
Tnfaip3	QT00134064
Gapdh	QT01658692
Il6	QT00098875
Lcn2	QT00113407
Cxcl1	QT00115647
Ccl20	QT02326394
Nfκbiz	QT00143934
Btrc	QT00129087
Fbxw11	QT01078168
Cxcl2	QT00113253
Irak2	QT0013785
Otulin	QT00302981
Cyld	QT00103768
Traf3ip2	QT00107422
Cdc27	QT00262444
Otud7b	QT00293853
Parp10	QT01284829
Sykb	QT02527896

Table 2.7: Human primers used for quantitative RT-PCR.

Gene	Catalog #
GAPHD	QT02504278
CDC27	QT01680819
CAV1	QT02408112
OTULIN	QT01028405
CYLD	QT02395827
TNFAIP3	QT00041853
TNIP1	QT00044072
IL6	QT00083720

3.0   CHAPTER THREE: SCREENING OF IL-17 RECEPTOR SIGNALING INTERMEDIATES

3.1   BACKGROUND

Inflammation is a process initiated by inflammatory molecules like interleukin-17 (IL-17, IL-17A), lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNFα) and mediated by their respective receptors: IL-17R, TLR4 or TNFR1. During mucosal infections, these receptors promote inflammation as a necessary process to protect the host. Upon stimulation with TNFα, LPS or IL-17, receptor signaling cascades are activated, ultimately resulting in activation of the Nuclear factor-kappa B (NF-κB) pathway (16,66,180–182). IL-17 promotes expansion and recruitment of neutrophils and monocytes, and cooperates with LPS and TNFα to drive expression of β-defensins and anti-microbial peptides. Inappropriate activation of the NF-κB signaling pathway is implicated in the pathogenesis of chronic inflammation and autoimmunity. Mechanistically, IL-17R and TLR4 signaling converge on the ubiquitination and activation of TRAF6, which in turns recruits TAK1 to subsequently activate the IKK complex, comprising two catalytic kinase subunits (IKKα/β) and a homodimer of the scaffolding regulatory subunit (IKKγ/NEMO). TNFR1 and IL-17R signaling converge at the level of the IKK complex downstream in the NF-κB pathway.

As described in chapter 1, IL-17R signaling activates primarily three effector pathways, NF-κB, CAAT enhancer binding protein beta (C/EBPβ) and mitogen-activated protein kinase (MAPK). In contrast, TLR4 and TNFR1 signaling each involve other downstream effector pathways (figure 3.1). Specifically, the TLR4 receptor binds several ligands, the most studied being LPS from gram-negative bacteria. Moreover, TLR4 signaling involves two proximal adaptors: MYD88 and TRIF (183). On the cell surface, MYD88-dependent signaling recruits the IRAK-TRAF6-TRAF3 complex, leading to NF-κB or AP-1 activation (184). Once TLR4 is internalized in vesicles, TRIF-signaling is initiated. TRIF-dependent signaling activates TBK1, in turn activating transcription factors interferon-responder factors (IRFs). TRIF signaling also mobilizes TRAF6 to activate MAPK and NF-κB signaling (185).

Like TLR4, TNFR1 signals differently depending on the localization of the ligand-receptor complex. When TNFα binds to the trimeric TNFR1 complex on the cell surface, it forms complex I that activates MAP3Ks and JNK, which subsequently activate the transcription factor AP-1. Next, after linear ubiquitination of adaptor proteins, complex I activates the IKK complex and NF-κB signaling to induce cell survival. The internalization of the whole signaling complex forms complex II or the Death-Inducing Signaling Complex (DISC). DISC-mediated signaling induces cell death. These events are coordinated by different signaling intermediates including TRADD, TRAF2 or FADD (186). Despite the differences among these signaling pathways, some TLR4- and TNFR1-activated genes overlap with IL-17 target genes, and all of these pathways have the ability to individually activate NF-κB signaling. Furthermore, several intermediates from TLR4 and TNFR1 signaling also participate in IL-17R signaling. Therefore, their signaling intermediates partially overlap, with some intermediates from TLR4 and TNFR1 pathways playing roles in IL-17R signaling.

Comparing the IL-17R signaling pathway to TLR4 and TNFR1 signaling pathways provides potential insight into potentially novel intermediates of IL-17 signaling. Therefore, since not all intermediates of TLR4 or TNFR signaling are necessarily used by the IL-17R, I screened candidate genes that I hypothesized have a role in IL-17 signaling. I selected the candidate genes based on the following criteria: involvement in the (i) regulation of signaling downstream of the TLR or TNFR families of cytokine receptors, (ii) regulation of NF-κB signaling, and (iii) regulation of ubiquitination of signaling proteins by having a E3-ligase or deubiquitinase (DUB) activity. Details of these candidate genes are provided in Table 3.1.

Table 3.1 : List of candidate genes and properties considered relevant for this study.

Gene	Regulatory role in			Ub	Relevant mechanism	Ref.
	Receptor signaling		NF-κB signaling			
	TLR/ TNFR	Other	Inhibit /Activate			
Tnip1 (ABIN-1)	Both	EGFR	Inhibits	Binds pUb.	Blocks NEMO	(187)
Irak2	TLR4	N.D.	Activates	P-TRAF6	Activates TRAF6 pUb	(188)
Otud7a	TNFR1	N.D.	Inhibits	DUB	RIP1's DUB	(189)
Parp10	TNFR1	IL-1R	Inhibits	Binds pUb.	Block pUb of NEMO	(190)
Cdc27	N.D.	N.D.	N.D.	E3-ligase	Part of AnapC complex with AnapC5/7	(191)(192)
Cyld	Both	TCR	Inhibits	DUB	TRAF6's DUB; Binds TAK1 & NEMO	(193)
Otulin	TNFR1	N.D.	Inhibits	Linear DUB	DUB linear Ub.	(194)
Usp15	N.D.	TGFβ / BMPR	N.D.	DUB	DUB mono-Ub	(195)
Cav1	Both	N.D.	Inhibits / activates	No	Binds TLR4 / TNFR1	(196)
Syk	Both	N.D.	Activates	N.D.	N.D.	(197)(77)

Abbreviations: DUB: deubiquitinases; TLR: Toll-Like Receptor; Ub: ubiquitin/ubiquitination;

pUb: poly-Ub; P: phosphorylation; N.D: no described.
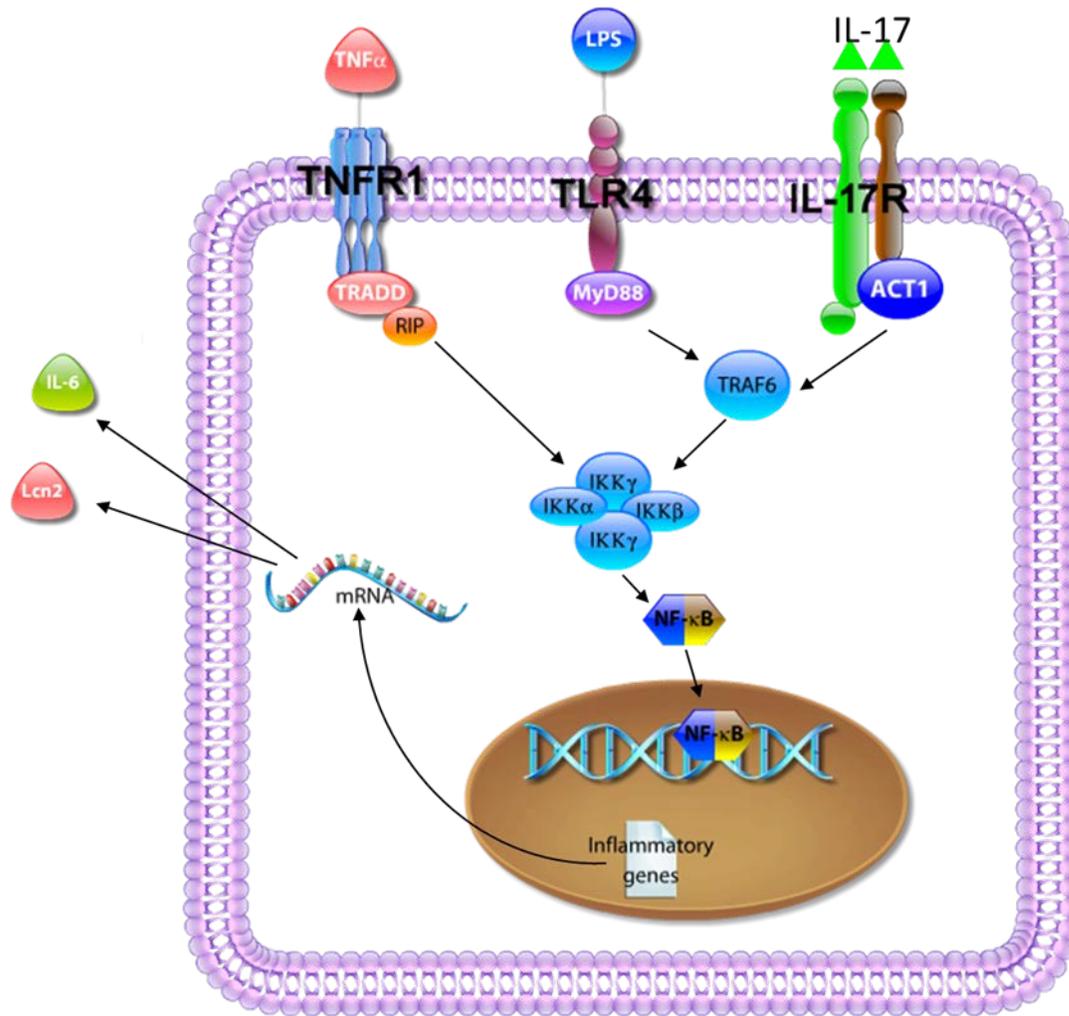
Figure 3.1: Candidate genes regulate other inflammatory pathways.

TNFα and TLR signaling shared some intermediates with IL-17R signaling. Upon ligand binding, these inflammatory signals converge in their activation of NF-κB. I investigated the role of some intermediates from these pathways on IL-17R signaling. TNFα: Tumor Necrosis Factor-α; TNFR1: TNF-Receptor 1; TRADD: TNFR-Associated Death Domain; TLR4: Toll-Like Receptor-4; MyD88: Myeloid differentiation primary response 88; IL-17R: Interleukin-17 receptor; ACT1: NF-κB Activator 1; Lcn2: Lipocalin 2; IL-6: Interleukin-6.

3.2 RESULTS

3.2.1 Screening of candidate genes that may regulate IL-17R signaling

To identify new intermediates in the IL-17R signaling cascade, I silenced ten candidate genes using an siRNA knockdown approach **(Figure 3.2-A-B)**. Controls were *Traf3ip2* (ACT1), *Tnfaip3* (A20), and mock or non-targeting siRNA. *Traf3ip2* is a positive regulator of IL-17 signaling, and its knockdown decreases the production of IL-17-dependent IL-6. On the other hand, knockdown of the negative regulator of IL-17 signaling, *Tnfaip3*, increases IL-6 production at baseline and after IL-17 stimulation. The ten candidates fell into three groups: (i) possible negative regulators or inhibitors of IL-17 signaling (*Tnip1*, *Cyld*, and *Otulin*), (ii) possible positive regulators or activators (*Cdc27*, *Cav1*, and *Syk*), and (iii) genes with no evident regulatory activity in IL-17 signaling (*Otud7b*, *Irak2*, *Usp15* and *Parp10*) **(Figure 3.2-A-B)**. Knockdown of each gene was considered successful if gene silencing was above 20%, as assessed by qPCR **(Figure 3.2-C)**. Thus, seven out of twelve candidate genes were successfully silenced.
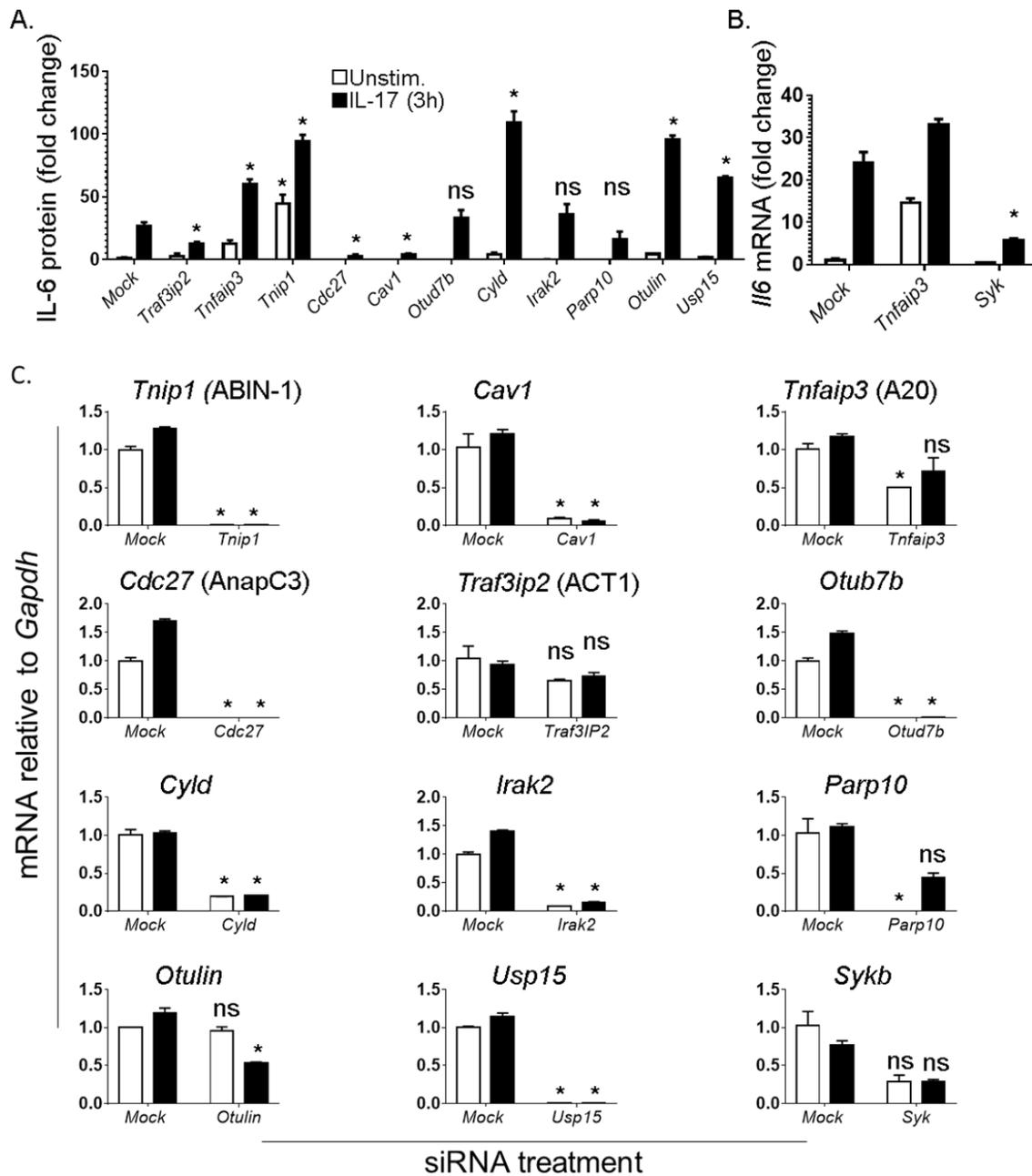
Figure 3.2: Screening of candidate genes that may regulate IL-17R signaling.

(A-B) Knockdown of some, but not all, candidates impacted IL-17R signaling. ST2 cells were transfected with the indicated siRNAs and stimulated with IL-17 for 3h. IL-17-dependent IL-6/*Il6* expression was quantified by (A) ELISA or by (B) qPCR. (A-B) Absolute values of IL-6/*Il6* expression were transformed into fold induction by comparing the values from each treatment with the values from mock-unstimulated cells. Knockdown of candidate genes was repeated at least twice. * p < 0.05 – Two-way ANOVA. (C) Degree of knock-down of candidate genes.

Knock-down was confirmed by measuring gene expression of targeted genes in ST2 cells after siRNA treatments. Most knock-downs resulting in at least a 20% decrease of gene expression.

3.2.2 Inflammatory stimuli control expression of *Tnip1* and *Cav1* genes

Because signaling pathway can regulate the expression of their own by downstream intermediates (e.g. *Tnfaip3*/A20 is a feedback negative regulator of IL-17R signaling (19)), I tested whether the genes from the siRNA screen that impacted IL-17R were also downstream of IL-17, TNFa, or LPS signaling. Specifically, I stimulated ST2 cells with these ligands and measured gene expression by qPCR. IL-17 stimulation did not regulate the expression of *Cdc27*, *Cyld* or *Otulin*. However, IL-17 stimulation increased expression of *Tnip1* 1.5-2.5-fold, and decreased *Cav1* expression to 0.5-0.8-fold of baseline **(figure 3.3-A)**. Stimulation with TNFα (figure 3.3-B) or TNFα combined with IL-17 **(figure 3.3-C)** had similar effects as IL-17 alone (figure 3.3-A). TLR4 stimulation with LPS downregulated *Il-17rd* expression but no other genes **(figure 3.3-D).** Thus, these data show that IL-17R or TNFR signaling regulates *Tnip1* (ABIN-1) and *Cav1* expression.
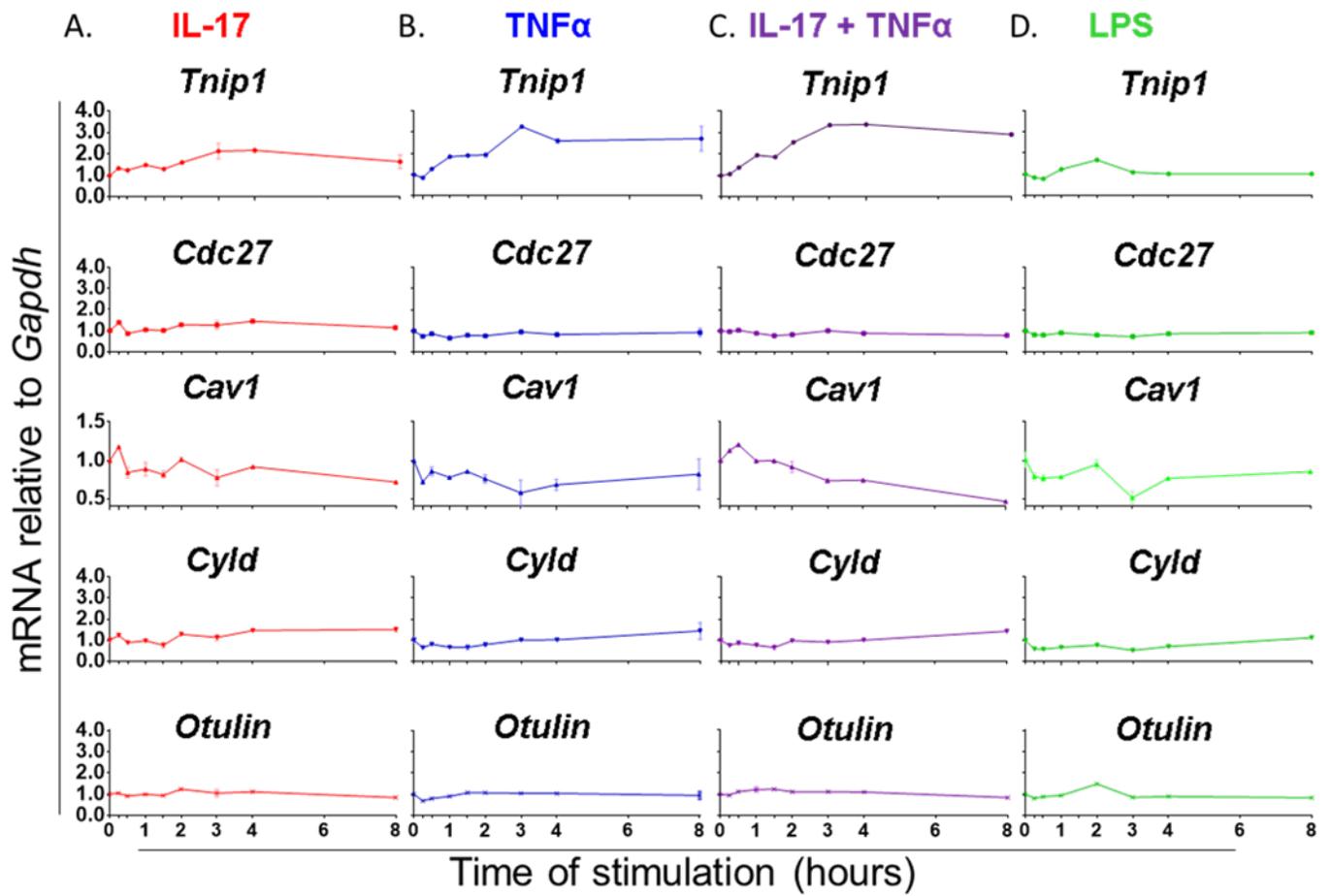
Figure 3.3: Inflammatory stimuli control expression of *Tnip1* and *Cav1* genes.

ST2 cells were plated and 24 hours later stimulated with (A) 200ng/mL IL-17, (B) 10ng/mL TNFα, (C) IL-17+TNFα or (D) 100ng/mL LPS at the indicated times. Expression of selected candidate genes was measured by qPCR. This experiment was repeated twice, and this representative data is from one experiment.

3.2.3 *Cyld* regulates IL-17 receptor signaling.

Cyld siRNA increased IL-17-dependent production of IL-6 without affecting its baseline expression **(figure 3.2-A)**, suggesting that *Cyld* may inhibit IL-17 signaling. To test whether *Cyld* regulates the expression of other IL-17-target genes, I knocked-down *Cyld* using siRNA in ST2 cells and measured gene expression by qPCR. The knockdown of *Cyld* enhanced production of *Lcn2, Ccl20,* and *Cxcl1* both at baseline and under IL-17-stimulated conditions, but had no detectable effect on the expression of *Nfkbiz* (encoding IκBζ) **(figure 3.4-A)**. To determine whether *CYLD* also inhibits IL-17 signaling in primary human cells, I knocked-down *CYLD* in primary human fibroblast-like synoviocytes (FLS) from a rheumatoid arthritis patient. Compared to other candidate genes that inhibit IL-17 signaling in murine cells, *CYLD* and *TNFAIP3* were the only genes to affect IL-17-dependent IL-6 production **(figure 3.4-B)**. Lastly, to confirm the inhibitory role of *Cyld* in IL-17 signaling, I stimulated murine embryonic fibroblasts (MEF) derived from *Cyld*$^{+/+}$ or *Cyld*$^{-/-}$ mice and measured IL-6 production by ELISA. Although these fibroblasts responded to IL-17, there was no significant difference between the *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ cells **(figure 3.4-C)**. In summary, the knockdown of *Cyld* in murine ST2 and human FLS cells increased IL-17-dependent IL-6 production, suggesting that *Cyld* inhibits IL-17 signaling. However, MEFs from *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ mice had no difference in their response to IL-17, which suggests that if *Cyld* inhibits IL-17 signaling, this function may be a tissue-specific. Overall, these results show that *Cyld* regulates a subset of IL-17-target genes in stromal cells.
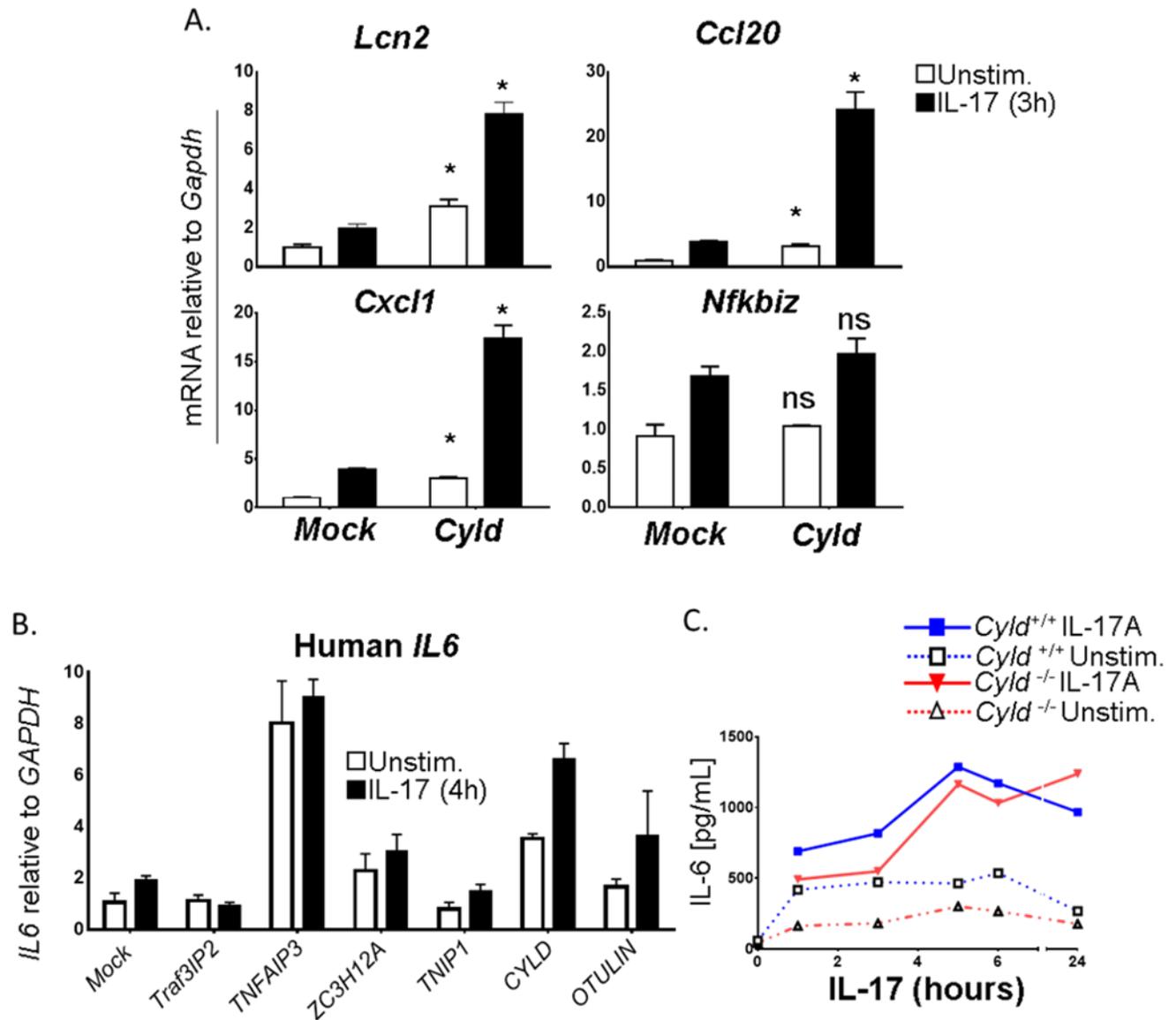
Figure 3.4: *Cyld* regulates IL-17 receptor signaling.

(A) *Lcn2*, *Cxcl1,* and *Ccl20* are inhibited by *Cyld* downstream of IL-17. ST2 cells were transfected with siRNA targeting *Cyld* or non-targeting Mock siRNA, and 48 hours later cells were stimulated with 200 ng/mL IL-17 for 3 hours. Expression of IL-17-dependent genes was assessed by qPCR. This experiment was performed three times, and these data are from one representative experiment. P < 0.05, two-way ANOVA test. (B) *Cyld* regulates the IL-17 response in human fibroblast-like synoviocytes (FLS). Human FLS obtained from the joints of a patient with rheumatoid arthritis were cultured. After three passages, FLS were transfected with human siRNAs targeting the indicated human genes (x-axis). Forty-eight hours after transfection, FLS were stimulated with human 200ng/mL

IL-17A for 4 hours, and *IL6* mRNA was determined by qPCR. This experiment was performed once. (C) *Cyld*$^{-/-}$ MEFs respond to IL-17A. MEFs derived from *Cyld*$^{+/+}$ or *Cyld*$^{-/-}$ mice were incubated with 200ng/mL IL-17A or culture medium only at the indicated times. IL-6 production was measured by ELISA, and this experiment was performed once.

3.2.4 Otulin regulates IL-17-dependent *Ccl20* expression.

Because OTULIN removes linear poly-ubiquitin chains from NEMO in TNF-dependent NF-κB signaling, I further explored whether Otulin regulates the expression of other IL-17-target genes. I knocked down Otulin in ST2 cells and measured gene expression by qPCR. In contrast to *Cyld*, Otulin only regulated *Ccl20* expression under IL-17-stimulated conditions without affecting *Lcn2*, *Cxcl1* or *Nfkbiz* expression **(figure 3.5-A)**. These data suggest that Otulin may function downstream of IL-17 signaling in regulating *Il6* and *Ccl20* expression.
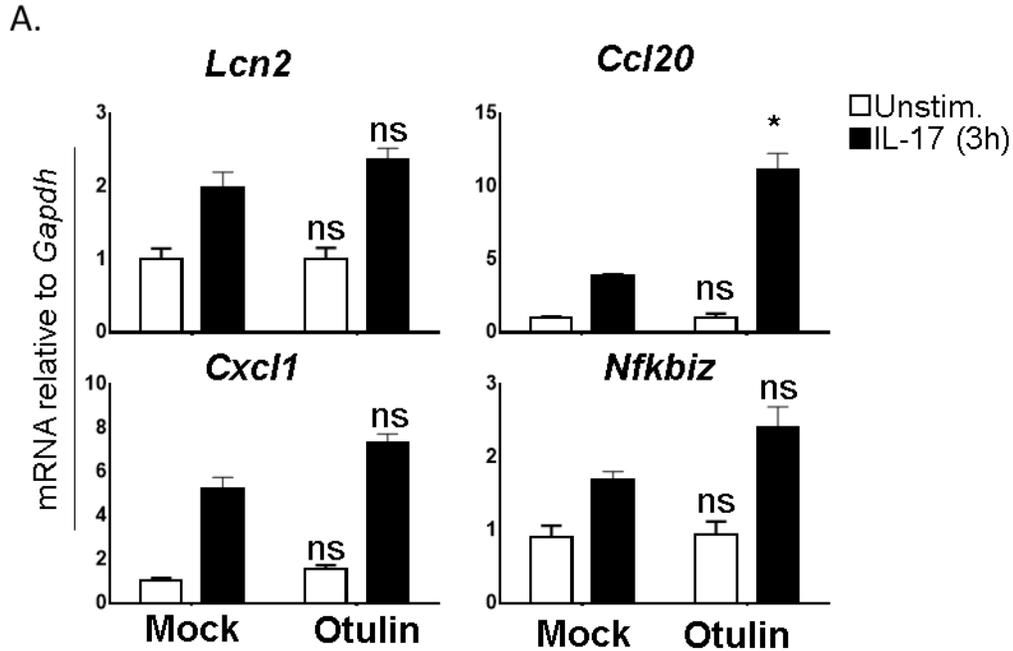
Figure 3.5: *Otulin* **regulates IL-17-dependent** *Ccl20* **expression.**

(A) Otulin regulates IL-17-dependent *Ccl20* expression. ST2 cells were treated with siRNA targeting Otulin or scrambled/non-targeting siRNA, and forty-eight hours later cells were stimulated with 200ng/mL IL-17A (3h). Expression of IL-17-dependent genes was assessed by qPCR. These data are from one representative experiment of three different experiments. $P < 0.05$, Two-way ANOVA test.

3.3 DISCUSSION

Binding of IL-17 to IL-17R triggers a cascade of events depicted in figure 1.4. Briefly, ACT1 and TRAF6 are recruited to activate the IKK complex and to induce NF-κB signaling. Here, I tested ten candidate genes that are known regulators of TNFR- or TLR-dependent NF-κB activation, and that control ubiquitination of downstream intermediates of TNFR or TLR signaling. My results showed that *Tnip1*, *Cyld*, and *Otulin* inhibited IL-17 signaling, while *Cav1*, *Cdc27* and *Syk* activated it. The molecular mechanism of action of these novel intermediates is yet to be described, but a preliminary model could be proposed based the current literature and these results.

Prior work has demonstrated that A20 (encoded by *Tnfaip3*) inhibits TRAF6-mediated signaling downstream of TLR-, TNFR- and IL-17R by removing ubiquitin (19,198,199). Taking the A20 deubiquitinase activity into consideration, I decided to study *Cyld* and *Otulin*, genes that encode for two other deubiquitinases. CYLD binds NEMO (200) and TRAF6 to inhibit TNFR1 signaling by regulating deubiquitination of K63-linkages (94,96) (97). *In vivo* murine models have shown that *Cyld* has a major anti-inflammatory role in the hematopoietic compartment. *Cyld*$^{-/-}$ macrophages are hyper-responsive to TLR4 stimulation (201) and monocytic osteoclast-progenitors have increased RANK-mediated signaling, which decreases osteoclastogenesis. Additionally, CYLD deubiquitinates and subsequently inactivates TRAF6 downstream of RANK signaling (202). In addition to macrophages and monocytes, *Cyld*$^{-/-}$ T cells are also defective because of dysregulated TCR signaling. Thus, immature T cell numbers are increased in thymus (203), while mature cells are hyperreactive to TCR signaling by decreased deubiquitination of

66

TAK1 and increased NF-κB signaling (204). Altogether, these defects cause excessive inflammation and the development of autoimmune-like disease resembling inflammatory bowel disease (201). Far fewer functions of CYLD have been studied in non-hematopoeitic cells, and my results suggest that *Cyld* inhibits IL-17R signaling in a murine cell line (ST2 cells) and in primary human fibroblast. Further characterization of the molecular mechanism by which CYLD regulates IL-17R signaling should consider deubiquitination of TRAF6 and NEMO as the regulatory step mediated by CYLD.

OTULIN removes linear ubiquitination, opposing the function of linear-ubiquitin assembly complex (LUBAC) formed by HOIL, sharpin and the catalytic subunit HOIL-1-interacting protein (HOIP). Mechanistically, after TNFα stimulation, LUBAC and OTULIN are recruited to the TNFR1-signaling complex. The recruitment of OTULIN is mediated by its binding to HOIP, which depends on the phosphorylation of OTULIN at tyrosine-56 (205). Once in this complex, Otulin deubiquitinates NEMO and RIPK2 to inhibit NF-κB activation (98); however, no studies have yet indicated whether linear ubiquitination plays a role downstream of IL-17R signaling. Thus, my finding that Otulin has specific inhibitory roles regulating *Il6* and *Ccl20*, but not other IL-17-target genes, needs to be taken with caution. Perhaps linear ubiquitination has a role downstream of IL-17R signaling, but this needs to be tested, and while OTULIN could mediate linear deubiquitination of IL-17 signaling intermediates, *Otulin* may also have other roles that are yet to be discovered. Thus far, mutations in *Otulin*, in human and mice, have been shown to cause autoimmunity and fatal inflammation, which was associated with defective anti-inflammatory function of Otulin in myeloid cells (100).

Altogether, my data suggest that *Cyld* and *Otulin* have inhibitory roles downstream of IL-17R signaling in stromal cells. However, a growing body of evidence suggests that the main anti-

inflammatory functions of these two genes lay in the non-hematopoietic compartment. Thus, their functions in IL-17R signaling may be a secondary protective anti-inflammatory mechanism or a novel system of differentially regulating IL-17 target gene expression. More details about the mechanism of inhibition mediated by *Tnip1* (ABIN-1) downstream of IL-17R signaling are explained in the next chapter.

Regarding the activators of IL-17R signaling discovered in this screening, *Cav1*, *Syk* and *Cdc27* (encoding for anaphase-promoting complex 3 /AnapC3), only *Cav1* expression was itself regulated by IL-17R signaling. Although a promising finding, the *Cdc27*-mediated activation of IL-17R is hard to reconcile with the current knowledge of *Cdc27*. The anaphase-promoting complex is a multi-protein structure of E3-ligases and scaffolding proteins that mediates progression of the cell cycle through mitosis and the G1 phase of the cell cycle. AnapC3 promotes ubiquitination of K11-, K48-, and K63-pUb chains in different target proteins such as the cell-division cycle protein 20 (CDC20) to regulate cell cycle signaling (191). Although AnapC3 has not been linked to inflammatory signaling, AnapC5 and AnapC7 from the same complex contribute to A20-mediated inhibition IL-17R signaling (192). In a yeast two-hybrid screen, Anapc7 was a frequent interacting partner of IL-17RC, which led to the discovery that AnapC5 also interacts with IL-17RC and IL-17RA. Furthermore, AnapC5 interacts with A20 and contributes to the inhibition of IL-17R signaling (192). Since AnapC3 is part of the same complex with AnapC5 and AnapC7, I included it in my siRNA screening of candidate genes. Thus, *Cdc27* knockdown decreased IL-17 signaling, suggesting that *Cdc27* is an activator of signaling. However, since the main function of *Cdc27* is controlling the cell cycle (206), a possible explanation is that dysregulation of the cell cycle affected IL-17 signaling as a secondary mechanism. Syk activates IL-17R signaling, by facilitating ubiquitination of TRAF6

(77), confirming the findings from my screening, and providing a molecular mechanism. Thus, I decided to further explore the link between *Cav1* and IL-17R signaling because this relationship has not been studied and presents opportunities to discover new functions for *Cav1*. More data about regulation of IL-17R signaling by *Cav1* is presented in chapter 5 of this thesis.

Although the data from this chapter are insufficient to derive a detailed molecular mechanism of action integrating these intermediates, I hypothesize based on published findings that CYLD and Otulin inhibit IL-17R signaling by deubiquitinating TRAF6, TAK1, or NEMO. Meanwhile, ABIN-1 binds NEMO (92), an interaction that can contribute to the inhibitory signaling of DUBs. In regard to the positive acting molecules, AnapC3 may indirectly regulate IL-17R signaling by controlling cell cycle progression, whereas Syk binds TRAF6 (77) to activate IL-17 signaling. Caveolin-1 activates signaling by a mechanism that is partially described in chapter 5. Altogether, findings from this chapter provide the basis for the work described in the following chapters, and lay the foundation for future research projects.

4.0 CHAPTER FOUR: IL-17R SIGNALING TRIGGERS DEGRADATION OF THE CONSTITUTIVE NF-κB INHIBITOR ABIN-1

4.1 BACKGROUND

Interleukin-17A (IL-17) is produced by lymphocytes and acts on tissue fibroblasts and epithelial cells to activate inflammatory gene expression. IL-17 is required to clear extracellular pathogens, particularly fungi such as *Candida albicans* (207). However, dysregulated or excessive IL-17 signaling is associated with the onset of autoimmune diseases such as psoriasis and ankylosing spondylitis, among others. Recently, IL-17 blocking therapies have shown considerable efficacy in treating psoriatic patients, and are now being evaluated for many other conditions (34).

The mechanisms of IL-17 signaling are still incompletely defined. Produced by Th17 cells as well as a variety of innate lymphocyte subsets, IL-17 and its receptor belong to a subclass of cytokines with distinct structural properties, compared to other cytokine superfamilies such as TNF and IL-1. Upon IL-17 binding to its receptor, numerous signaling intermediates are engaged to transduce downstream events. A key early event is activation of the Act1 and TRAF6 E3 ubiquitin ligases. These in turn activate a sequence of downstream signaling intermediates, culminating in degradation of the IκB inhibitor and nuclear translocation of the NF-κB transcription factor (11,208)(209). Consequently, genes with promoters containing

NF-κB-binding elements are upregulated by IL-17 stimulation, with IL-6 (Il6) and Lipocalin-2 (Lcn2) being prototypical IL-17 target genes (210).

To date, the mechanisms that constrain the IL-17 pathway are not well understood, which is an important clinical issue in light of the autoimmune potential of this cytokine. Recently, we showed that the ubiquitin editing enzyme A20 inhibits IL-17 signaling by de-ubiquitinating TRAF6 and thereby limiting NF-κB and MAPK activation (19). Moreover, IL-17 upregulates A20 expression, establishing a negative feedback loop that restricts IL-17-driven inflammation. A20 is encoded by *TNFAIP3*, a genetic locus frequently associated with human autoimmune diseases.

In order to understand how IL-17 controls autoimmunity, we evaluated other genetic loci connected to IL-17-driven autoimmune conditions. The A20-binding inhibitor of NF-κB activation 1 (ABIN-1) is an A20-binding protein encoded by the *TNIP1* gene. ABIN-1 deficiency is associated with psoriasis in human genome wide association analyses (GWAS) (128,129,134,174). Additionally, mice with keratinocytes lacking ABIN-1 are more sensitive to imiquimod-induced dermatitis, a common model of psoriasis (175). Since IL-17 signaling is associated with psoriasis and inhibited by A20, we hypothesized that ABIN-1 might also serve as a downstream inhibitor of the IL-17 signaling pathway.

Here, we demonstrate that *Tnip1* restricts the basal expression of IL-17 target genes such as *Il6* in fibroblasts and stromal cells. *Tnip1* also negatively regulates IL-17-induced production of *Il6*, *Lcn2* and other NF-κB-dependent genes, but not all IL-17-inducible genes are affected. Although ABIN-1 is known to interact with A20, we found that A20 is fully dispensable for ABIN-1-mediated inhibition of IL-17 signaling. Additionally, IL-17 induced expression of *Tnip1* mRNA by activating in its proximal promoter. Surprisingly, however, ABIN-1 protein levels are

71

reduced following IL-17 signaling, an event that is mediated by proteasomal degradation. Additionally, we found that phosphorylation protects ABIN-1 from degradation. Thus, IL-17 signaling serves to release the ABIN-1-controlled brake on inflammation, revealing a new regulatory circuit in the IL-17 inflammatory pathway.

4.2   RESULTS

4.2.1   *Tnip1* inhibits both basal and IL-17-induced gene expression

To delineate the role of *Tnip1* in IL-17-mediated signaling, we assessed IL-17 signaling in immortalized MEFs lacking *Tnip1* (211). Cells were treated with IL-17 over a 24 h period, and IL-6 in culture supernatants was assessed by ELISA. At baseline, *Tnip1*$^{-/-}$ fibroblasts secreted significantly more IL-6 than *Tnip1*$^{+/+}$ cells **(figure 4.1-A)**. Similarly, *Tnip1*$^{-/-}$ cells treated with IL-17 expressed more IL-6 than IL-17-stimulated *Tnip1*$^{+/+}$ cells. Consistent with these findings, elevated expression of *Il6* mRNA was observed in *Tnip1*$^{-/-}$ MEFs, both at baseline and after IL-17 stimulation **(figure 4.1-B)**. These results indicate that *Tnip1*restricts both tonic and IL-17-dependent IL-6 expression. To confirm that *Tnip1* limits IL-6 expression levels and to rule out the possibility that this effect was restricted to a particular MEF line, *Tnip1*$^{-/-}$ fibroblasts were reconstituted with ABIN-1 by transfection. Cells were then stimulated with IL-17 for 3 h and *Il6* mRNA was measured by qPCR. Consistent with its role as an inhibitor of inflammatory signaling, reconstitution with ABIN-1 decreased *Il6* production compared to cells transfected with a control vector **(figure 4.1-C)**.

To verify the inhibitory activity of *Tnip1 i*n another setting, we silenced *Tnip1* with siRNA in ST2 cells, a mouse bone marrow stromal cell line that responds robustly to IL-17 **(figure 4.1-D)** (212). Indeed, knockdown of *Tnip1* led to increased expression of IL-6 both at baseline and following IL-17 stimulation **(figure 4.1-E)**. Levels of *Il6* mRNA were similarly enhanced upon *Tnip1* knockdown, as was expression of other IL-17-target genes including *Lcn2*

and *Cxcl1* **(figure 4.1-F)**. However, not all IL-17-induced genes were impacted by *Tnip1* *d*eficiency; for example, *Ccl20* and *Nfκbiz* (encoding IκBζ) are IL-17-target genes that are restricted by A20 but not by *Tnip1* **(figure 4.2.1-F)** (19). Together, these results demonstrate that *Tnip1* is a negative regulator of tonic and IL-17-induced signaling, selectively controlling expression of a subset of IL-17-target genes.
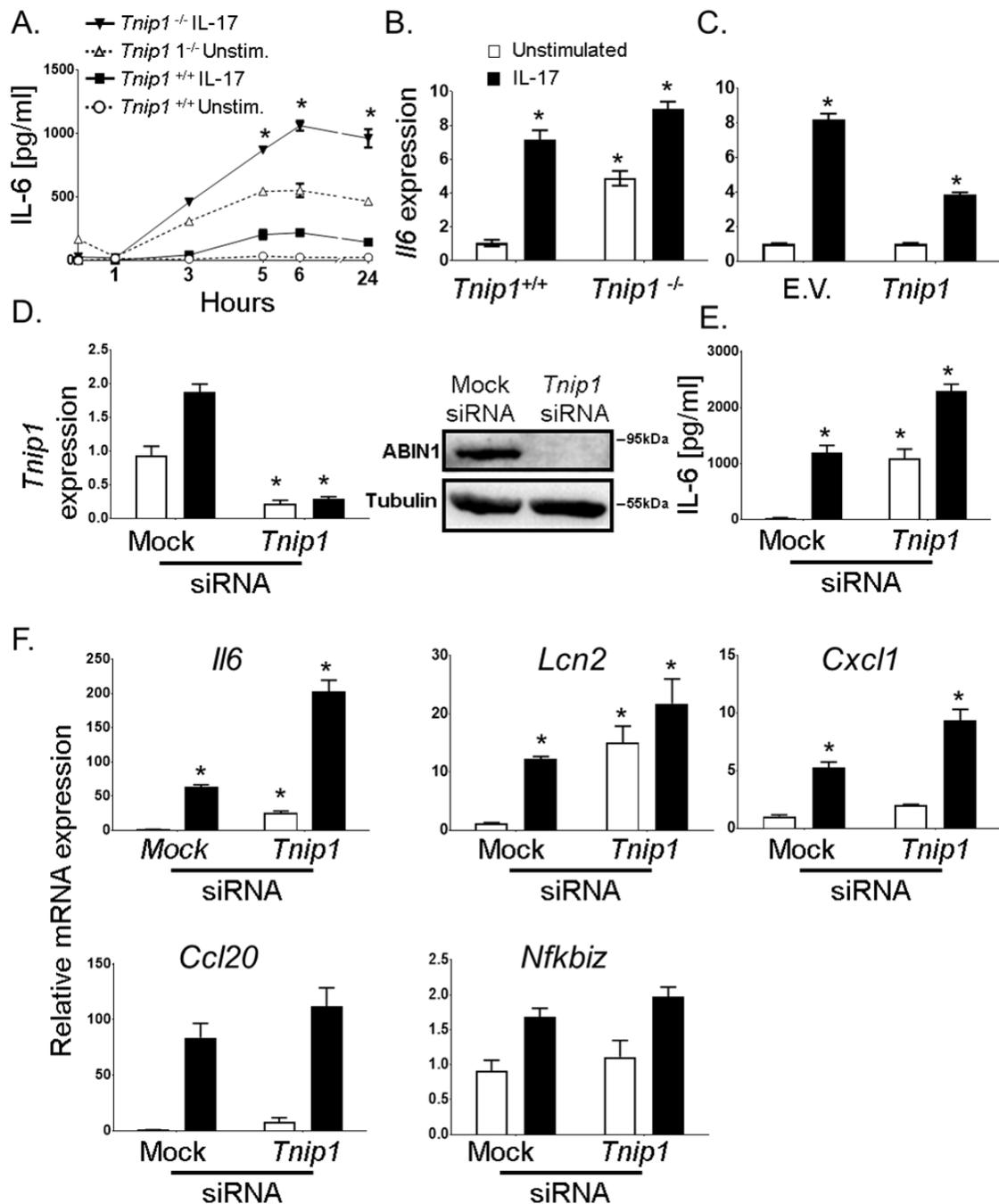
Figure 4.1: Tnip1 inhibits tonic and IL-17-dependent gene expression in mesenchymal cells.

(A) Kinetics of *Tnip1*-mediated inhibition of IL-6 expression. *Tnip1*$^{+/+}$ or *Tnip1*$^{-/-}$ fibroblasts were treated with IL-17 (200ng/ml) for the indicated times, and IL-6 in conditioned supernatants was measured by ELISA. Data are presented as mean values; *P<0.05, 2 way ANOVA with Sidak's multiple comparisons test analyzing all groups. Data are representative from 3 independent experiments. (B) *Tnip1* restricts *Il6* basal expression. *Tnip1*$^{+/+}$ or *Tnip1*$^{-/-}$

fibroblasts were treated with IL-17 for 3 h, and *Il6* mRNA was assessed by qPCR and normalized to *Gapdh*. *P<0.05, 2 way ANOVA with Sidak's multiple comparisons test compared to untreated $Tnip1^{+/+}$ sample. (C) *Tnip1* reconstitution inhibits *Il6* expression. $Tnip1^{-/-}$ fibroblasts were transiently transfected with a *Tnip1*-expressing plasmid or empty vector (E.V.). After 24 h, cells were treated with IL-17 for 3 h and Il6 was assessed by qPCR. Data are presented as the fold-induction of Il6 in IL-17-stimulated cells compared to unstimulated. Data are normalized to untreated cells transfected with the same plasmid. *P<0.05, 2 way ANOVA with Sidak's multiple comparisons test compared to $Tnip1^{-/-}$ untreated. (D) *Tnip1* knockdown efficiency. ST2 cells were transfected with siRNA targeting *Tnip1* or a scrambled siRNA (mock) control. *Tnip1* (ABIN1) expression was assessed by qPCR (left) and *Tnip1* protein by immunoblotting (right). *P<0.05, 2 way ANOVA with Sidak's multiple comparisons test compared to mock, unstimulated cells. (E) *Tnip1* silencing enhances tonic and IL-17-induced IL-6 production. ST2 cells were transfected with the indicated siRNAs. 48 h later cells were stimulated with IL-17 for 3 h, and secreted IL-6 was assessed by ELISA. *P<0.05, 2 way ANOVA with Sidak's multiple comparisons test compared to mock unstimulated cells. (F) *Tnip1* silencing enhances tonic and IL-17-induced expression of some but not all IL-17 target genes. ST2 cells from panel (E) were analyzed for the expression of the indicated genes by qPCR. *P<0.05, 2 way ANOVA with Sidak's multiple comparisons test compared to mock unstimulated cells. Panels B-F, data are representative of 3 independent experiments.

4.2.2   ABIN-1 inhibits IL-17R signaling independently of A20

Since ABIN-1 was identified based on its ability to bind A20, we asked whether ABIN-1-mediated inhibition of IL-17R signaling is dependent on A20 (213). To determine whether A20 was required for the inhibitory activity of ABIN-1, we knocked down *Tnip1* in $Tnfaip3^{-/-}$ fibroblasts by RNA silencing. As shown, reduced expression of *Tnip1* led to enhanced *Il6* and *Lcn2* expression, both at baseline and after IL-17 stimulation, similar to wild-type MEFs and ST2 cells **(figure 4.2-A)**. In an independent approach to address the role of A20 in ABIN-1

76

activity, we co-transfected *Tnfaip3*^{-/-} MEFs with ABIN-1, together with a luciferase reporter driven by the *Lcn2* promoter (63). Cells were stimulated with IL-17 for six hours and luciferase activity was assessed. As we previously reported (19), *Tnfaip3*^{-/-} fibroblasts showed only a modest responsiveness to IL-17, probably because baseline NF-κB is constitutively high in the absence of A20 (19). Nonetheless, Lcn2-promoter activity was markedly reduced upon transfection of ABIN-1 or A20, confirming that A20 is dispensable for the inhibitory function of ABIN-1 **(figure 4.2-B)**. As a third method to assess the role of A20, we transfected ST2 cells with the Lcn2 luciferase reporter with either a full-length ABIN-1 or a truncated mutant lacking the A20-binding domain (but retaining the NF-κB inhibitory C-terminal domain) **(figure 4.2-C)** (214). In response to IL-17, both the full-length and the truncated forms of ABIN-1 inhibited IL-17-dependent Lcn2-promoter activation **(figure 4.2-C)**. Collectively, these findings demonstrate that ABIN-1 suppresses IL-17 activity and tonic inflammatory signaling independently of A20.
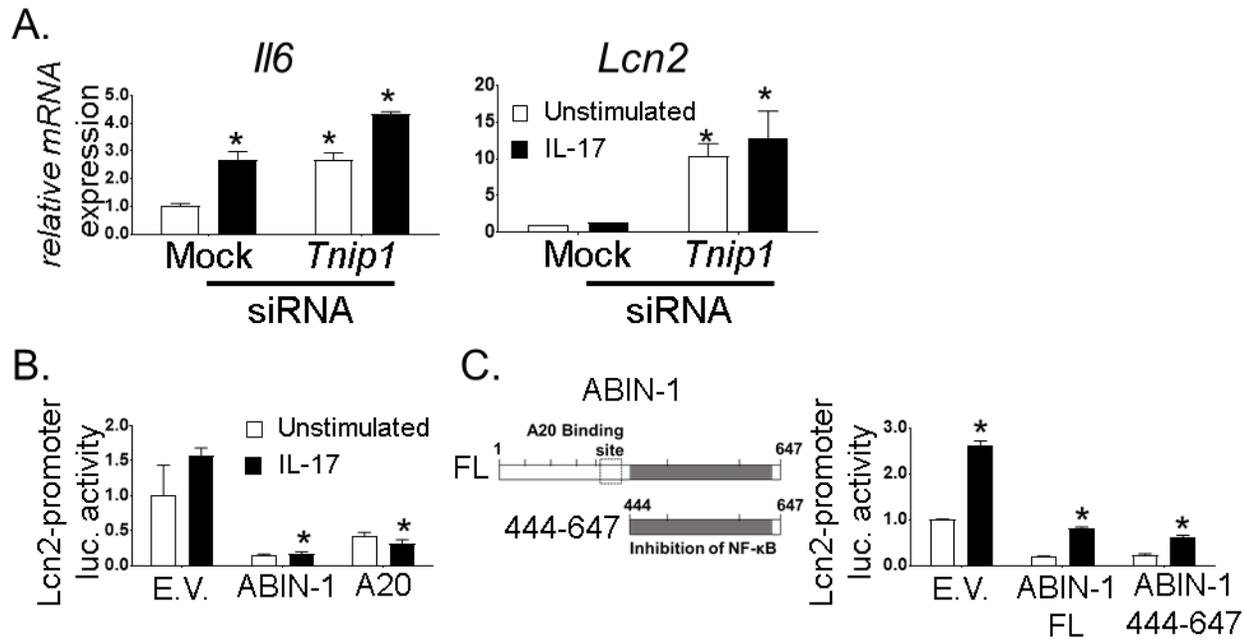
Figure 4.2: ABIN-1 inhibits IL-17 signaling independently of A20.

(A) ABIN-1 knockdown in *Tnfaip3^{-/-}* fibroblasts maintains enhanced *Il6* and *Lcn2* expression. *Tnfaip3^{-/-}* MEFs were transfected with ABIN-1 or mock siRNA and *Il6* and *Lcn2* expression were assessed by qPCR. (B) IL-17-induced *Lcn2*-promoter activation is inhibited in *Tnfaip3^{-/-}* cells by ABIN-1. *Tnfaip3^{-/-}* MEFs were transfected with the *Lcn2*-promoter together with plasmids expressing ABIN-1 or A20. After 24 h, cells were treated with IL-17 for 6 h and luciferase activity was measured. (C) An ABIN-1 mutant lacking the A20 binding domain inhibits IL-17-dependent *Lcn2*-promoter activation. Left: Schematic representation of ABIN-1 protein (FL, full length) and the 444-647 mutant lacking the A20 binding site. Right: ST2 cells were transfected with the *Lcn2*-promoter reporter together with FL or 444-647 forms of ABIN-1. 24 h later, cells were stimulated with IL-17 for 6 h and luciferase activity measured. *$P<0.05$, 2 way ANOVA with Tukey's multiple comparisons test compared to cells transfected with E.V. and treated with IL-17 in (C) and (B), or with cells treated with Mock siRNA and stimulated with IL-17 in (A). Data are representative of 3 independent experiments.

4.2.3   ABIN-1 regulates IL-17-induced NF-κB signaling

ABIN-1 is known to inhibit NF-κB in the context of TNFα stimulation (91,92,215), and the above results suggested ABIN-1 suppressed NF-κB-dependent genes in the IL-17 pathway as well. An early step in the canonical NF-κB signaling pathway is phosphorylation of the inhibitor I κBα, which leads to its degradation by the proteasome, releasing NF-κB for nuclear translocation. Moreover, the gene encoding IκBα is induced in an NF-κB-dependent manner, establishing a negative feedback loop (216). To determine whether ABIN-1 targets NF-κB in the IL-17 signaling cascade, we assessed IL-17-dependent IκBα degradation in $Tnip1^{-/-}$ fibroblasts. In $Tnip1^{-/-}$ cells transfected with a control (empty) vector (E.V.), IκBα was degraded within 15 minutes following IL-17 stimulation and remained at low levels for as long as 60 minutes post-stimulation (figure 4.3-A, lanes 5-8). In contrast, in $Tnip1^{-/-}$ fibroblasts reconstituted with ABIN-1, IκBα was restored to basal levels by 60 minutes (figure 4.3-A, lane 4). Therefore, there is prolonged activation of NF-κB signaling when ABIN-1 is deficient. These data indicate that ABIN-1 inhibits IL-17-dependent NF-κB signaling upstream of IκBα degradation.

To confirm that ABIN-1 inhibits NF-κB, we knocked down ABIN-1 in ST2 cells and evaluated NF-κB activation by IL-17. In ST2 cells transfected with a control (mock) siRNA, IκBα returned to normal levels 60 minutes after IL-17 stimulation (figure 4.3-B, lane 4). However, when ABIN-1 was reduced, cells deficient in this molecule could not re-establish pre-treatment levels of IκBα (figure 4.3-B, lane 8). These data suggest that ABIN-1 is required to inhibit IL-17-dependent NF-κB signaling upstream of IκBα.

To further define the mechanism by which ABIN-1 inhibits IL-17 signaling, we measured IL-17-dependent Lcn2 promoter activation in ST2 cells transfected with full-length ABIN-1 or a mutant with a non-functional ubiquitin binding domain (UBAN) (214). Whereas the

full-length ABIN-1 inhibited IL-17-dependent Lcn2 promoter activation, the UBAN mutant did not impair basal signaling and only partially inhibited IL-17 signaling (figure 4.3-C). Thus, ABIN-1 negatively regulates NF-κB activation in response to IL-17 signaling through IκBα. Additionally, the ubiquitin binding domain of ABIN-1 is required to inhibit both tonic and IL-17-induced signaling.
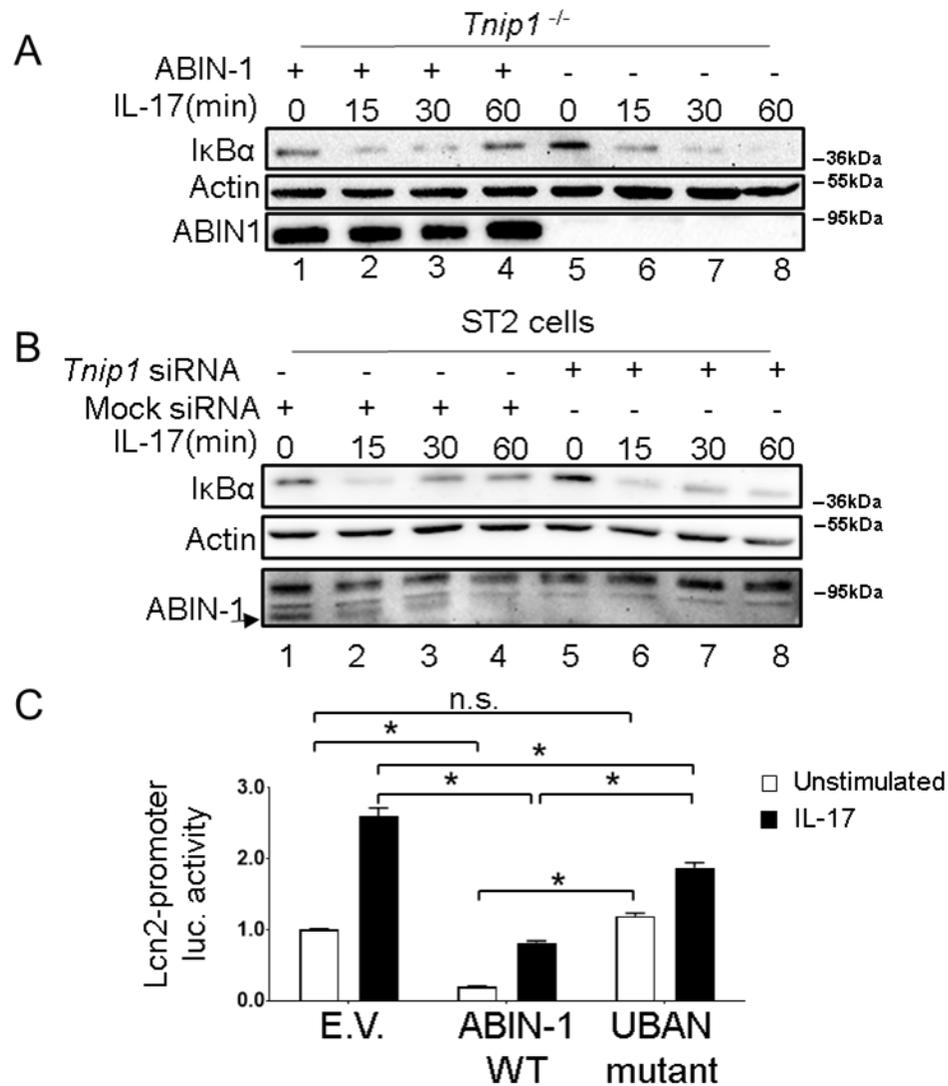
Figure 4.3: ABIN-1 inhibits IL-17-dependent NF-κB activation.

(A) *Tnip1*$^{-/-}$ fibroblasts exhibit prolonged activation of IL-17-dependent NF-κB signaling. *Tnip1*$^{-/-}$ MEFs were transfected with ABIN-1 or empty vector (E.V.), rested for 24 h, and stimulated with IL-17 (200 ng/ml) for the indicated times. IκBα degradation was assessed by immunoblotting of total cell lysates. (B) ABIN-1 knockdown prolongs activation of IL-17-induced NF-κB. ST2 cells were transfected with *Tnip1* or mock siRNAs for 48 h and treated with IL-17 for the indicated times. Whole cell lysates were immunoblotted for IκBα. Results are representative of 3 independent experiments. (C) The ubiquitin binding domain (UBAN) contributes to the inhibitory function of ABIN-1. ST2 cells were transfected with the *Lcn2*-promoter reporter together with and ABIN-1 or mutant lacking the UBAN domain (DF485/486NA). 24 h later, cells were treated with IL-17 for 6 h and

luciferase activity was measured. Data are representative of 3 experiments. *P<0.05, 2 way ANOVA with Tukey's multiple comparisons test.

4.2.4 IL-17 signaling differentially regulates *Tnip1* mRNA and ABIN-1 protein

IL-17 induces several known signaling inhibitors to establish feedback signaling loops, including IκBα, A20 and regnase-1/MCPIP1, all of which are regulated by NF-κB. *Tnip1* is also a known NF-κB target gene, raising the possibility that IL-17 might also induce its expression (217). Accordingly, we measured *Tnip1 m*RNA by qPCR following IL-17 stimulation. A linear regression model showed that IL-17 significantly, albeit modestly, induced *Tnip1* mRNA over time, resulting in ~2-fold increased levels over two hours **(figure 4.4-A)**. This induction was NF-κB-dependent, as expression was blocked in the presence of an IKK inhibitor **(figure 4.4-B)**. Moreover, IL-17 induction of *TNIP1* occurred at the level of the promoter, as verified in ST2 cells transfected with the full-length (6 kB) *TNIP1* proximal promoter (218,219) **(figure 4.6-A-B)**. Transfection of C/EBPβ or NF-κB p65, transcription factors regulated by IL-17, was also sufficient to drive TNIP1 promoter activation **(figure 4.6-C)**. These data support the hypothesis that IL-17 induces *Tnip1* mRNA through its proximal promoter, via C/EBPβ and NF-κB.

In probing regulation of the ABIN-1 protein, we were surprised that expression of ABIN-1 was markedly and reproducibly decreased after ~60 minutes of IL-17 stimulation **(figure 4.4-C)**. A similar trend was seen in MEFs **(figure 4.7-A-B)**. To determine whether the reduced ABIN-1 levels were due to proteasomal degradation, cells were treated with IL-17 in the presence of the proteasome inhibitor MG132. Blocking proteasome function prevented ABIN-1 protein degradation after IL-17 treatment, which was most notable at the early (15 minutes) time point **(figure 4.4-D)**. These data indicate that, even though the ABIN-1 promoter is activated by

IL-17, ABIN-1 is inducibly degraded in an IL-17-dependent manner. This unexpected finding suggests that IL-17 signaling releases a brake on inflammation that is normally controlled by ABIN-1.
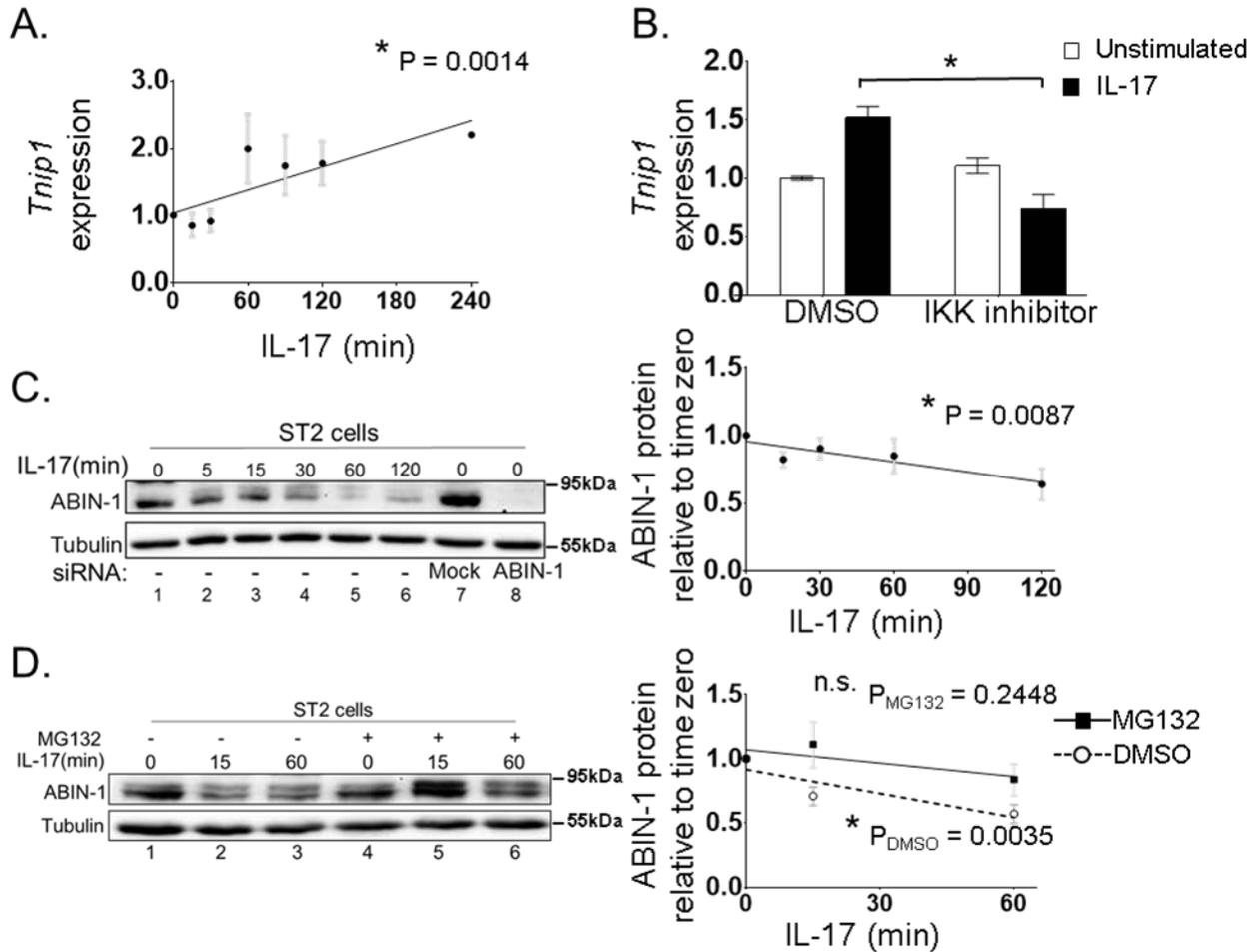
Figure 4.4: IL-17 signaling upregulates *Tnip1* mRNA by downregulates ABIN-1.

(A) *Tnip1* mRNA is upregulated by IL-17. ST2 cells were stimulated with IL-17 for the indicated times, and *Tnip1* mRNA was analyzed by qPCR. Relative mRNA expression was assessed by linear regression modeling ($P = 0.0085$). Data are pooled from 5 independent experiments. (B) NF-κB regulates *Tnip1* mRNA. ST2 cells were pretreated with IKK inhibitor VII for 30 min or DMSO and treated with IL-17 for 3h. mRNA was analyzed by qPCR. *$P < 0.05$ 2 way ANOVA with Tukey's multiple comparisons test comparing to untreated sample. Data are representative of 3 experiments. (C) ABIN-1 protein is downregulated upon IL-17 signaling. Left: ST2 cells were treated with IL-17 (200 ng/ml) and whole cell lysates were analyzed by immunoblotting. Lanes 7-8: cells were transfected with mock or ABIN-1 siRNA to confirm the identity of ABIN-1 band. Right: Relative protein band intensities were compared to time of stimulation and analyzed by linear regression. Data are pooled from 6 independent experiments. (D) ABIN-1 is degraded by the proteasome. Left: ST2 cells were pretreated with

84

proteasome inhibitor MG-132 for 30 min or DMSO and treated with IL-17 for the indicated times. Whole cell lysates were analyzed by immunoblotting. Right: Relative protein band intensities were compared to time of stimulation and analyzed by linear regression. Data pooled from 4 independent experiments and presented as mean +/- standard error.

4.2.5   ABIN-1 is phosphorylated in an IL-17-dependent manner

As early as five minutes after IL-17 stimulation, a second band was observed above the ABIN-1 band **(figure 4.4-C)**, suggesting that ABIN-1 may be phosphorylated. To determine if ABIN-1 was phosphorylated, I immunoprecipitated endogenous ABIN-1 after 15 minutes of IL-17 stimulation and treated the fraction with alkaline phosphatase, which should remove all phosphate groups from the immunoprecipitated form of ABIN-1. Then, observing SDS-PAGE mobility of ABIN-1 I indirectly determine its phosphorylation status **(figure 4.5-A, lane 2)**. The comparison of the buffer-only treated sample in lane 1 with lane 2 demonstrates that ABIN-1 is phosphorylated after 15 minutes of IL-17 stimulation **(figure 4.5-A)**. In the case of IκBα, IL-17-mediated phosphorylation leads to proteasomal degradation. Since we observed a degradation of ABIN-1 after IL-17 stimulation, we tested if kinase activity had a role in this degradation. Interestingly, the use of the pan-kinase inhibitor staurosporine enhanced ABIN-1 degradation after IL-17 stimulation **(figure 4.5-B, lanes 7-12)** compared to control **(figure 4.5-B, lanes 1-6)**. This result confirms that phosphorylation of ABIN-1 is induced by IL-17 signaling and regulated by intracellular kinase activity. Since the IKK complex is downstream of IL-17 signaling, and plays a fundamental role in regulating signaling, we tested if the IKK complex can phosphorylate ABIN-1 in an overexpression system. Indeed, overexpression of IKKβ together with ABIN-1 was sufficient to phosphorylate ABIN-1 **(figure 4.5-C, lanes 2 and 3)**. Lastly, to determine if

IKK complex kinase activity is required to regulate phosphorylation and degradation of ABIN-1, we treated ST2 cells with the pan-IKK inhibitor Bay11-7082. Consistent with our previous staurosporine treatment, treatment of ST2 cells with Bay11-7082 was sufficient to enhance protein degradation **(figure 4.5-D, lanes 3 & 4)**. Overall, these data suggest that ABIN-1 is phosphorylated after IL-17 stimulation, which may be mediated by the IKK complex. Interestingly, in contrast to the case of IκBα in which phosphorylation leads to degradation, phosphorylation of ABIN-1 protects it from degradation.
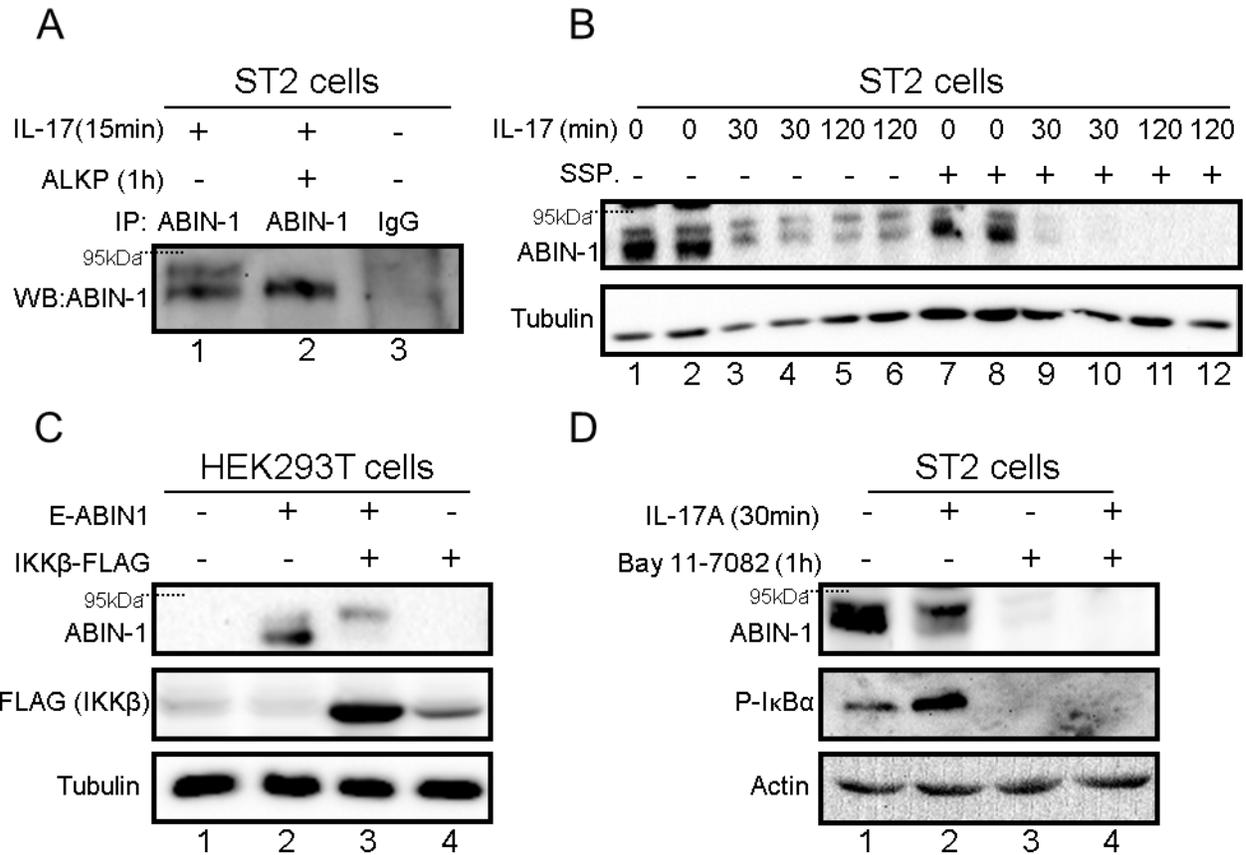
Figure 4.5: IL-17 induces a phosphorylation-independent mobility shift in ABIN-1.

(A) ABIN-1 is phosphorylated fifteen minutes after IL-17 stimulation. ST2 cells were stimulated with IL-17 and endogenous ABIN-1 protein was immunoprecipitated and treated with alkaline phosphatase (ALKP) to remove all phosphate groups. IP fractions were analyzed by WB. This image is representative of 3 independent experiments. (B) Kinase activity is necessary to repress ABIN-1 phosphorylation. ST2 cells were pre-treated with staurosporine (SPP) for 20 minutes before IL-17 was added to the medium at the indicated times. Whole cell lysates (WCL) were analyzed by WB. This image is representative of 2 independent experiments. (C) IKKβ kinase can phosphorylate ABIN-1. HEK293T cells were transfected with E-ABIN-1 and IKKβ-FLAG plasmids. WCLs were analyzed by WB. This image is representative of 2 independent experiments. (D) The IKK complex activity prevents ABIN-1 degradation. ST2 cells were pre-treated with Bay11-7082 for 60 minutes before IL-17 was added for 30 minutes. WCLs were analyzed by WB. This image is representative of 2 independent experiments.

4.2.6 IL-17 induces TNIP1 promoter

Since Brian Aneskievich group has robustly shown that NF-KB signaling induces TNIP1 promoter, we tested if IL-17 stimulation induced TNIP1 promoter. Using different fragments of the human TNIP1 promoter fused to luciferase **(figure 4.6-A)** we determined that the human TNIP1 promoter is induced 2.5-fold after IL-17 stimulation **(figure 4.6-B)**. To determine if NF-KB and CEBPB act on the TNIP1 Promoter, I transfected C/EBPB or p65 together with the luciferase reporter. Interestingly, both transcription factors activated the TNIP1 Promoter **(figure 4.6-C)**, suggesting that they could mediate the IL-17-dependent activation of TNIP1 promoter.
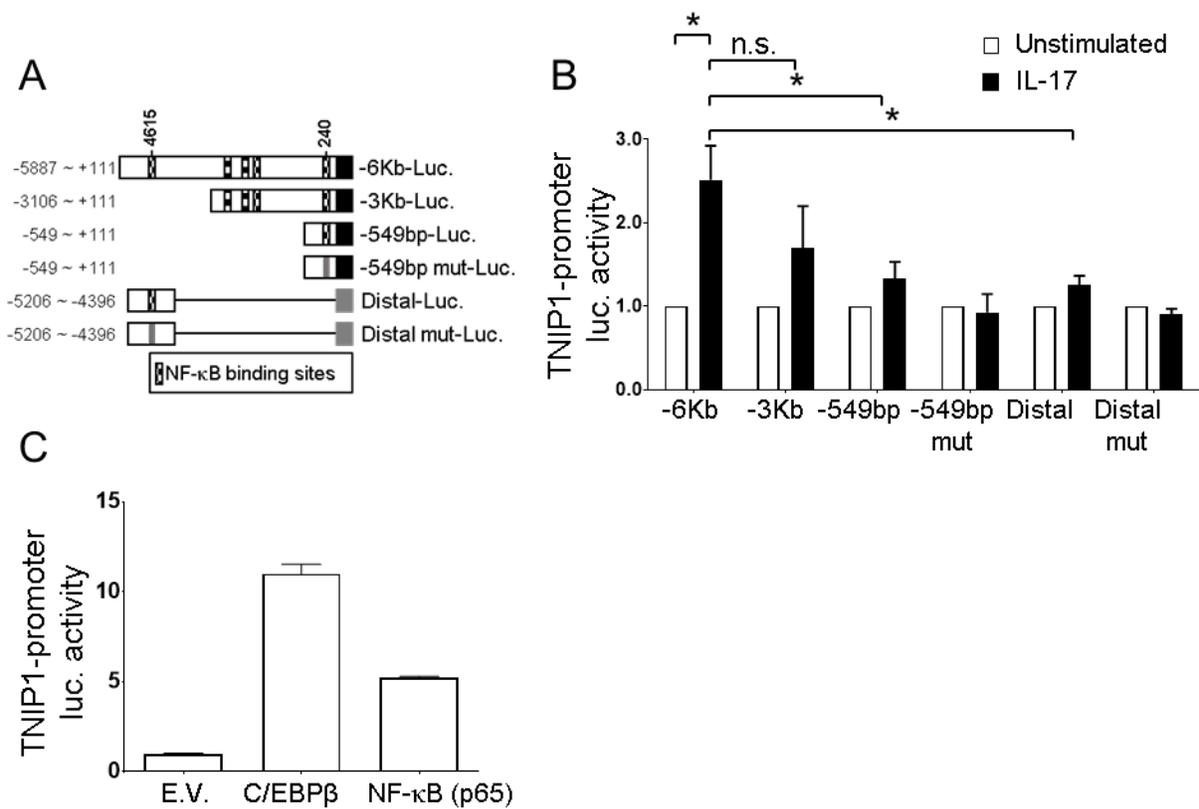
Figure 4.6: IL-17 induces *TNIP1*/ABIN-1 promoter activation.

(A) NF-κB binding sites are necessary to activate the *TNIP1*/ABIN-1 promoter. Schematic representation of different *TNIP1/*ABIN-1 promoters used in figure (B). (B) *TNIP1*/ABIN-1-promoter is activated by IL-17 and both distal and proximal sites are required for this activation. ST2 cells were transfected with the human *TNIP1*/ABIN-1-promoters from figure (A) and stimulated with IL-17 for 6h before luciferase activity was measured. (C) C/EBPβ and NF-κB (p65) activate *TNIP1*/ABIN-1 promoter. The -6kb *TNIP1*/ABIN-1 promoter was transfected in ST2 cells together with the C/EBPβ and p65 expressing plasmids. Luciferase activity was measured 24h later with no stimulation. These experiments are representative of two experiments.

4.2.7 IL-17 regulates degradation of ABIN-1

To confirm that IL-17 induces ABIN-1 degradation in a different cell line, I stimulated wild-type murine fibroblasts with IL-17 and measured ABIN1 protein in whole cell lysate from these cells. Consistent with my findings from figure 4.4, ABIN1 protein was degraded after IL-17 stimulation **(figure 4.7-A-B)**. To determine whether degradation was controlled by IL-17 stimulation, I stimulated ST2 cells with IL-17 for one hour and washed cytokine out using a citrate pH 4.0 buffer, and I replace the medium with cytokine free media. Four hours after removal of IL-17, ABIN-1 levels were restored to basal conditions **(figure 4.7 C-D)**, demonstrating that IL17 signaling controls degradation of ABIN-1.
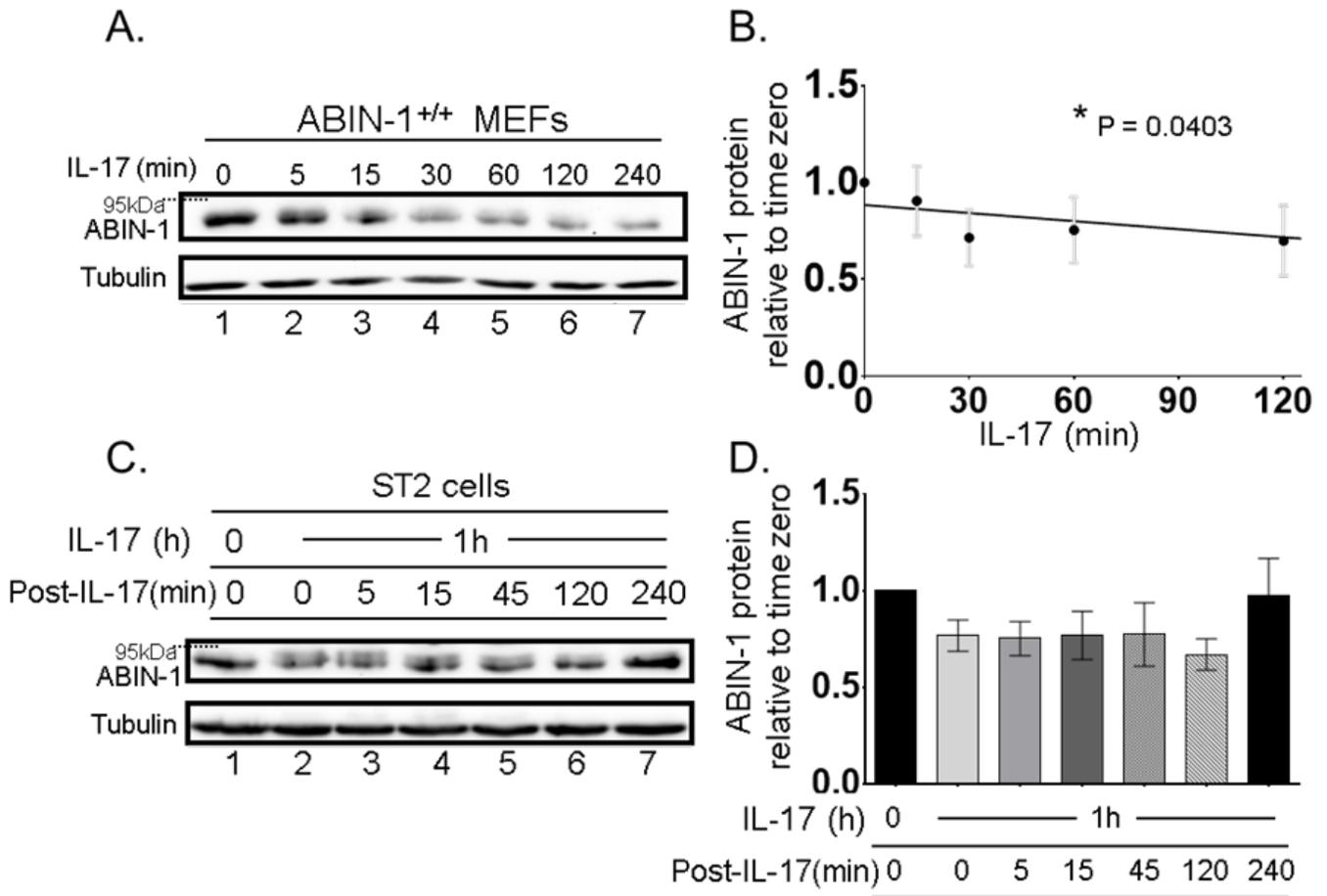
Figure 4.7: IL-17 downregulates ABIN-1 protein in mouse fibroblasts.

(A) ABIN-1 is downregulated in wild-type fibroblasts. Murine fibroblasts (MEFs) derived from ABIN-1+/+ mice were stimulated with IL-17 and ABIN-1 protein was assessed by WB. (B) Linear regression was used to model the effect of time of IL-17 stimulation on ABIN-1 protein expression in MEFs. ABIN-1 protein was significantly decreased and directly associated with time of stimulation (P = 0.0403). Data are from seven independent experiments and are presented as mean +/- standard error. (C) ABIN-1 protein levels returned to baseline four hours after removal of IL-17 from the environment. ST2 cells were treated with IL-17 for 1hour and the medium was removed, and the cytokine washed out from the cells using a citrate buffer pH 4.0. Cells were immediately incubated in complete medium for the indicated times and whole cell lysate was analyzed by western blot. This image is representative of three independent experiments. (D) Band density analysis of (C). Densitometry analysis of ABIN-1

expression after IL-17 stimulation and at various times after removal of IL-17. Data from three independent experiments are presented as mean +/- standard error.

4.2.8   Degradation of ABIN1 is not regulated by βTrCP1

To determine the mechanism of degradation of ABIN1, I investigated the effect of SCF$^{\beta TrCP}$ complex on degradation of ABIN1. SCF$^{\beta TrCP}$ functions downstream of IL-17 signaling to induce degradation of ACT1 and IKBα, thus I hypothesized that SCF$^{\beta TrCP}$ could also mediates degradation of ABIN1. However, knockdown of βTrCP1 and βTrCP2 together to disrupt the SCF$^{\beta TrCP}$ complex did not affect the IL-17-dependent degradation of ABIN1. These data suggest that degradation of ABIN1 is independent of SCF$^{\beta TrCP}$ complex activity.
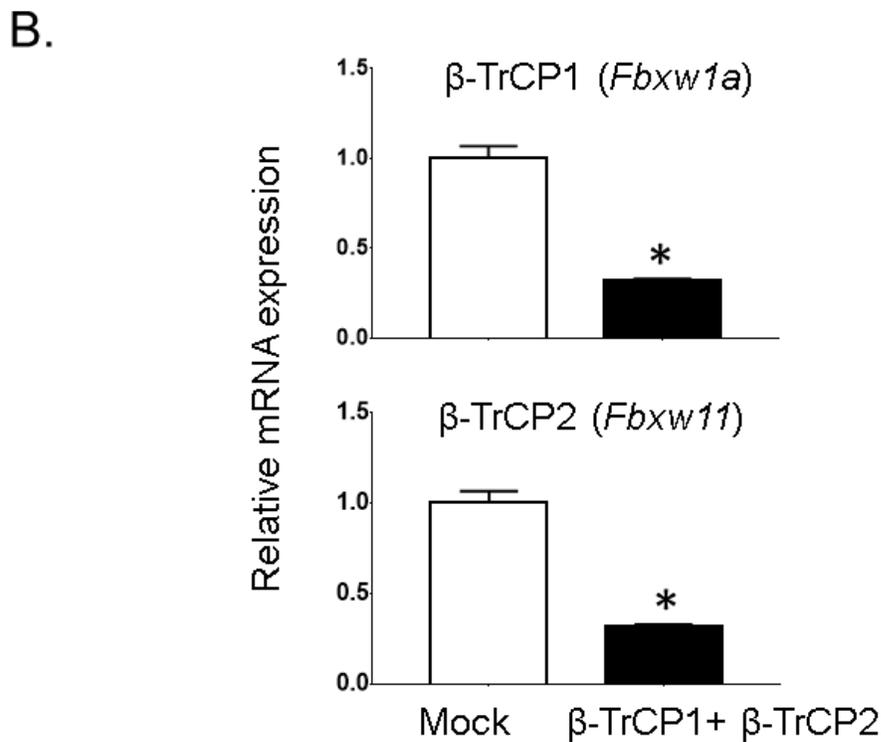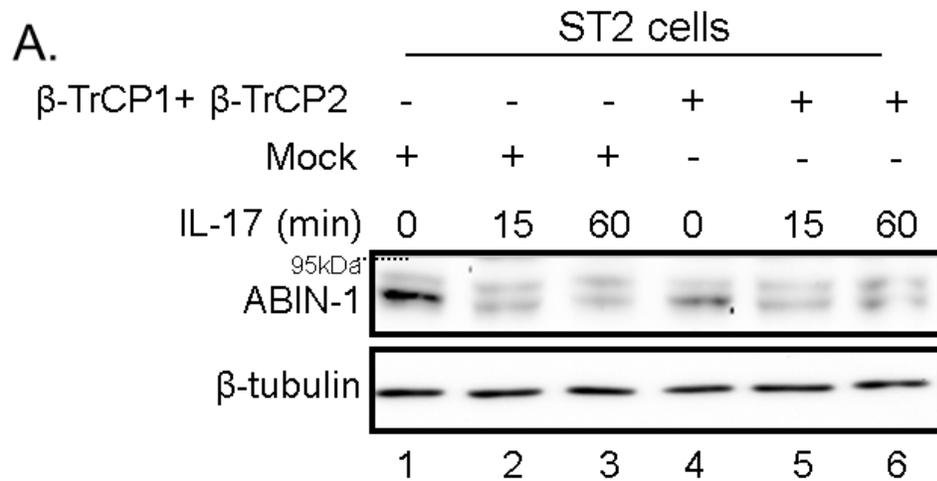
Figure 4.8: SCF$^{\beta TrCP}$ complex does not regulate ABIN-1 degradation.

(A) ABIN-1 degradation does not require β-TrCP1 and β-TrCP2 expression. ST2 cells were transfected with double siRNA for β-TrCP1 (Fbxw1a) and β-TrCP2 (Fbxw11) for 48h until cells were stimulated with IL-17A for indicated times. This experiment was repeated three times. (B) Knockdown of β-TrCP1 and β-TrCP2 was successful. ST2 cells from (A) were also analyzed by qPCR to assess gene expression and knockdown efficiency. Expression of both

genes was significantly decreased in cells treated with specific siRNA than cells treated with mock siRNA. P = 0.05 – t-test.

4.3   DISCUSSION

Sustained IL-17 receptor signaling is associated with psoriasis and other autoimmune conditions (34,220–222). Accordingly, it is not surprising that there are negative regulators that act on the IL-17 signaling cascade to prevent the over-exuberant inflammatory responses that underlie tissue pathology. Notably, many of these inhibitory pathways converge on NF-κB activation (11). The development of autoimmunity is associated with polymorphisms in genes that influence NF-κB activity, such as TNFAIP3 (encoding A20), NFKBIA (encoding IκBα) and TNIP1 (encoding ABIN-1) (223,224). In prior work, we found that A20 is part of a feedback loop that restricts IL-17-mediated TRAF6 ubiquitination, which is required for NF-κB and MAPK activation; that finding prompted the present analyses of the A20-binding protein ABIN-1 (19). Because of its A20-binding capacity, it is frequently assumed that ABIN-1 functions involve A20 (213,225,226). Indeed, ABIN-1 co-expression with A20 was shown to induce destabilization of the IKK complex and inhibition of NF-κB signaling in a lower chordate model organism (227). Similarly, ABIN-1 cooperates with A20 to inhibit antiviral signaling or TNF-regulated NF-κB signaling (92,228). In contrast, our data show that ABIN-1 acts independently of A20 in the context of IL-17 receptor signal transduction.

Most of what is known about ABIN-1 comes from studies of TNFα or LPS. ABIN-1 controls signaling by several reported mechanisms, including (i) inhibiting IKK complex activity by binding to NEMO (IKKγ) (92), (ii) inhibiting ubiquitination and degradation of p105 to block p50 production in the non-canonical NF-κB pathway (215), and (iii) by associating with and blocking TNF receptor adaptors such as RIP and TRAF2 (225). ABIN-1 requires its ubiquitin

binding domain (UBAN) for its function, as a D485N mutation within the UBAN suppressed binding to K63- or linear-poly-ubiquitin chains. *In vivo*, this mutation caused systemic inflammation and autoimmunity (229). This phenotype was considered to be LPS-mediated, but involvement of IL-17 was not explored. Here, we demonstrate that the same mutation in the UBAN eliminates ABIN-1-mediated inhibition of tonic and IL-17-dependent signaling, raising the possibility that some effects of the D485N mutation could be ascribed to IL-17.

An unexpected finding in this study was the dichotomy by which IL-17 regulates ABIN-1 mRNA versus protein. We consistently found that IL-17 triggered modest upregulation of ABIN-1 mRNA. Consistent with its mRNA induction, IL-17 activates the ABIN-1 proximal promoter. NF-κB regulates the gene promoter activity of ABIN-1, inducing its mRNA as a late expression gene, compared to the A20 expression after TNFα stimulation (218,230). These data thus suggested that ABIN-1 is part of a similar negative feedback loop, analogous to other IL-17 signaling inhibitors such as A20 and MCPIP1/Regnase-1 (19,81). Indeed, in HeLa cells NF-κB was found to induce expression of ABIN-1, while ABIN-1 in turn repressed NF-κB activity (218). However, the impact of IL-17 on ABIN-1 protein levels contrasted strikingly and unexpectedly with its transcriptional activation, since the net effect of IL-17 was downregulation of ABIN-1 protein levels. Interestingly, the phenomenon of ABIN-1 degradation after an inflammatory stimulus is evident in the published literature, although it has never, to our knowledge, been remarked upon. For example, ABIN-1 levels decreased after TLR and TNFα stimulation of bone marrow derived macrophages with approximately the same kinetics as we observed with IL-17 signaling (229). Additionally, studies in human psoriatic skin lesion biopsies showed increased ABIN-1 mRNA, but decreased protein, indicating that this phenomenon occurs in a setting where both IL-17 and ABIN-1 are known to be clinically

relevant (134). Mice in which ABIN-1 is conditionally deleted in keratinocytes develop more severe skin inflammation than control mice after imiquimod-induced dermatitis, which is associated with increased expression of IL-17 target genes such as Cxcl1 and Ccl20 (175). These findings therefore confirm the physiologic connection between ABIN-1 and IL-17.

How do we reconcile the observation that IL-17 and other stimuli induce ABIN-1 transcription yet triggers protein loss? We speculate that the answer lies in the persistence of the stimulus and the kinetics of activation. In the early phase of inflammatory signaling, ABIN-1 protein is degraded within 1-2 hours, which serves to enhance inflammation. Simultaneously ABIN-1 mRNA expression is upregulated by NF-κB and C/EBPβ, replenishing at least somewhat the pool of translated ABIN-1. In washout experiments, we found that ABIN-1 protein levels return to baseline approximately four hours after IL-17 withdrawal (data not shown), thus returning the cell to a constitutively inhibited state. However, when IL-17 was able to signal continuously, ABIN-1 degradation was maintained. Thus, the inhibitory activity of *Tnip1* is compromised during ongoing stimulation. This might explain the observation that in patients with psoriasis there is elevated mRNA, but decreased protein, for ABIN-1 (134). Psoriasis is linked to several inflammatory cytokines, with a predominant role for IL-17A signaling, based in part on the success of anti-IL-17A and anti-IL-17RA biologic drugs (220)(127,231,232). Therefore, the idea that ABIN-1 is both a tonic inhibitor of inflammation and a negative regulator of IL-17 signaling fits well with our data and with findings in pre-clinical mouse models and human studies (134,174,175).

Interestingly, during early IL-17 stimulation, ABIN-1 was phosphorylated. In contrast to IκBα, which has less degradation when phosphorylation is inhibited, blocking phosphorylation of ABIN-1 increased ABIN-1 degradation. These data suggest that phosphorylation-mediated by

the IKK complex induces ABIN-1 degradation by a mechanism that has yet to be discovered. We suggest that there are two possible mechanisms by which the IKK complex can mediate ABIN-1 degradation: (i) the IKK complex directly phosphorylates ABIN-1, which promotes ABIN-1 interaction with stabilizing proteins, and/or restricting the access of an E3-ligase that would K48-ubiquitinate ABIN-1 to induce its proteasomal degradation, or (ii) the IKK complex phosphorylates a different enzyme, like an E3-ligase that would target ABIN-1, inactivating its function, therefore, indirectly protecting ABIN-1. Future experiments to describe in more detail the link between phosphorylation and degradation of ABIN-1 will lead to a better understanding of the IL-17-mediated regulation of ABIN-1.

There are certainly other examples of inhibitors that are downregulated by the signals that they regulate. In the case of IL-17, the proximal adaptor Act1 is degraded following phosphorylation to temper all downstream signaling events (75), and IκBα is degraded following phosphorylation to unmask the NF-κB nuclear import signal (233). IL-17 is just one of multiple immune effectors that can initiate ABIN-1 degradation and presumably thereby enhance inflammation. Moreover, IL-17 functions synergistically with many of these cytokines, particularly TNFα (11,234). Thus, ABIN-1 appears to regulate signaling at the intersection of these inflammatory stimuli, namely the IKK complex.

We show that ABIN-1 degradation is at least partially mediated by the proteasome, with no apparent contribution of caspases or the lysosome (data not shown). These data have possible implications for therapeutic interventions. Therapeutics targeting the proteasome are used for cancer (235), and might be useful in diseases where ABIN-1 is implicated, i.e., those where TNIP1 SNPs are found. Another therapeutic opportunities involves settings where over-production of ABIN-1 activators such as IL-17 or TNFα (e.g., autoimmunity, microbiome

dysbiosis) might lead to under-expression of ABIN-1 and chronic inflammation. Clearly more work needs to be done to further dissect the role of this important, but enigmatic molecule in the context of inflammation.

5.0 CHAPTER FIVE: IL-17R SIGNALING IS ACTIVATED BY CAVEOLIN-1

5.1 BACKGROUND

Caveolin-1 is an integral membrane scaffolding protein, which organizes signaling proteins within a specific region of the plasma membrane, the caveolae (236). Caveolin-1 is the main structural protein of the caveolae that shapes the inner surface of the plasma membrane in a flask-like structure (237). Caveolin-1 is widely express in endothelial cells, smooth muscle cells, skeletal muscle cells, fibroblasts, type-I alveolar cells, fat cells, brain and spinal cord neurons, and glial cells (238,239). Caveolin-1 primary functions include: lipid and cholesterol transport, tumor suppression, endocytosis, exocytosis, and signal transduction in the cell (240–242). Caveolin-1 functions are determined by caveolin-1 location **(figure 5.1)** and by the nature of the protein that interacts with caveolin-1. For instance, Caveolin-1 interacts with TLR4 and TNFR1 through their specific binding motifs to regulate inflammatory signaling (176,177,243–246)(196,247). Furthermore, caveolin-1 inhibits TLR4 signaling in macrophages, and activates TLR4 signaling in endothelial cells (177). Additionally, caveolin-1 regulates TNFR1 subcellular localization and TNFR1 signaling by both regulating the trafficking of the receptor and activating signaling by binding to TRAF2 (243). Altogether, these findings suggest that caveolin-1 acquires specific functions by interacting with different proteins.

In addition to regulating formation of signaling complexes, caveolin-1 also plays a role inducing receptor desensitization. The dopamine 1 receptor (D1R) interacts with caveolin-1, and the caveolin-1 binding site in D1R mediates receptor desensitization through a mechanism that is not fully understood (248). However, in the same study, cells transfected with a mutant containing mutations in the caveolin-1 binding site, D1R failed to internalize or to transduce signal, which suggests that caveolin-1 mediates receptor surface localization and desensitization (248). Likewise, desensitization of the IL-17R is induced via phosphorylation and degradation of ACT1 after persistent IL-17 stimulation (75). This suggests that desensitization of IL-17R can happen when proteins that transduce its signaling are removed. Thus, I also explore in this chapter whether caveolin-1 is regulated by IL-17R signaling.

The IL-17R signaling cascade is initiated upon binding of IL-17 to the extracellular portion of the heterodimeric receptor formed by subunits IL-17RA and IL-17RC (249). Since IL-17RA is the most important signaling subunit, it is the focus of this work (250). IL-17RA has two extracellular fibronectin-III-like (FN) domains, FN1 and FN2, which bind IL-17 (13). After IL-17 engages IL-17R, ACT1 is recruited to the intracellular domain of IL-17R to initiate signaling. ACT1 promotes IL-17R signaling by forming different functional complexes downstream of IL-17R (14,59). Because caveolin-1 initiates similar signaling complexes in other cytokine receptor signaling, it is possible that it could be playing a role in IL-17R (177,196,243). However, whether caveolin-1 regulates IL-17R signaling by forming a complex with the IL-17R has not been studied. For this reason, I tested the hypothesis that the caveolin-1/IL-17R complex regulates IL-17R signaling; data supporting this hypothesis are presented in this chapter.

In this chapter, I report that the IL-17RA subunit of the IL-17R complex has a caveolin-1 binding site in the FN1 domain of the extracellular portion of the receptor. Furthermore, I present

data demonstrating that caveolin-1 also regulates IL-17R signaling and activation of NF-κB. These findings suggest that caveolin-1 activates IL-17R signaling by a mechanism that involves both regulation of intracellular signaling and IL-17R localization to the plasma membrane.
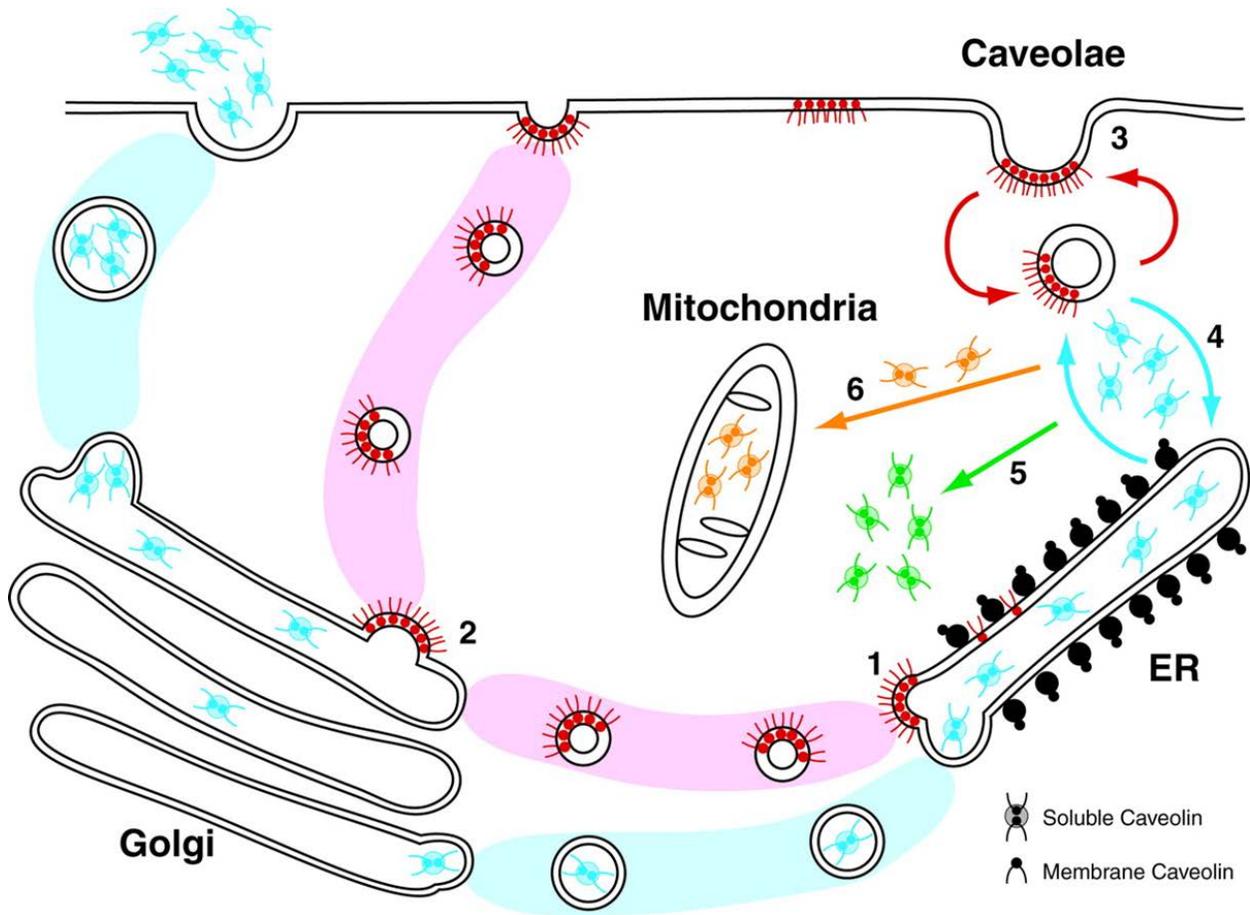
Figure 5.1: Intracellular trafficking of caveolin-1.

Diagram representing the different routes of caveolin-1 inside a cell. (1) Caveolin-1 (in red) is inserted co-translationally into the ER membrane, and has both N- and C-terminal domains in the cytoplasm (red path). Caveolin-1 traffics to the Golgi via intracellular vesicles (251). (2) In the Golgi, caveolin-1 oligomerizes (252), which is required to transit to the plasma membrane (red path). Once in the plasma membrane, caveolin-1 is incorporated into caveolae, and (3) caveolin-1 is internalized and recycled. (4) Caveolin-1 from internalized vesicles enters the cytoplasm by interacting with lipids and forming lipid particles. Soluble caveolin-1 can return to the vesicles (return blue arrow) or go to the endoplasmic reticulum (ER). In the lumen of the ER, soluble caveolin-1 binds High-density lipoprotein (HDL)-like particles that are secreted by the cell (253). (5) Alternatively, caveolin-1 can bypass the ER and stay in the cytosol (green path) as part of lipid droplets (254). (6) Lastly, soluble caveolin-1 can reach mitochondria (orange). Figure taken from Liu *et al*. J. Biol. Chem. 2002.

5.2   RESULTS

5.2.1   IL-17RA has a putative caveolin-1 binding site in the FN1 extracellular domain.

To determine if IL-17R can potentially bind to caveolin-1, I scanned the protein sequences of IL-17RA and IL-17RC for the presence of putative caveolin-1 binding sites (255). Interestingly, I identified that the extracellular portion of the IL-17R subunit A (IL-17RA) has a putative Cav-1 binding site between aa142 and aa149 (WRFSFSHF) **(figure 5.2-A).** In addition to IL-17RA, IL-17RC also has a consensus caveolin-1-binding sequence (not shown), but since IL-17RA is the major signaling subunit of IL-17R signaling, I focused on the study of IL-17RA and caveolin-1 interaction. A sequence alignment between human and murine IL-17RA revealed that the caveolin-1 binding site was conserved across both species. This evolutionary piece of evidence supports my hypothesis that this binding domain is functional, and therefore maintained through evolution **(figure 5.2-A)**. The caveolin-1 putative binding site is within the FN1 domain of IL-17RA, which has been previously described as one of the two domains involved in engaging the IL-17 ligand (13). Since the FN1 domain is important for the binding of IL-17, I analyzed the previously published crystal structure of IL-17RA to determine whether the putative caveolin-1 binding site would interfere with the binding of IL-17 to IL-17RA (9,256). I found that the putative caveolin-1 binding site is on the opposite side of the ligand binding pocket, suggesting that binding to caveolin-1 would not interfere with binding to IL-17 **(figure 5.2-B)**. These analyses suggest that caveolin-1 may bind to the FN1 domain in IL-17RA, and set the stage to pursue the study of the interaction of caveolin-1 with IL-17RA.

A.

Putative Cav1 binding site

IL-17RA

| | | 140 | | | 160 |
| | | | | | |

Murine FLSMLQHHRK RWRFSFSHFV VDPGQEYEVT
Human   FLSKLRHHHR RWRFTFSHFV VDPDQEYEVT
Consensus FLSᴋLᴏHHᴋᴋ RWRFₛₜFSHFV VDPᴅGQEYEVT
                        * *   *

B.

IL-17RA

IL-17A

FN1

IL-17A

FN2
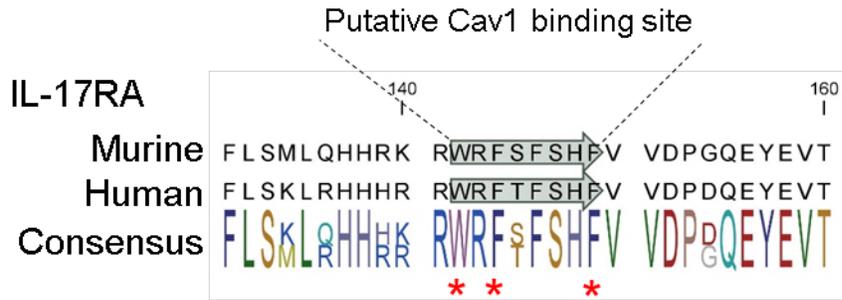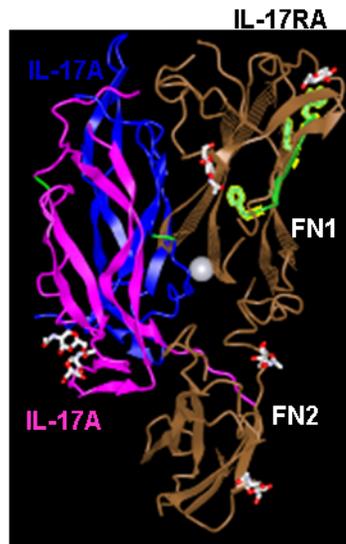
Figure 5.2: IL-17RA has a putative caveolin-1-binding site in the FN1 extracellular domain.

(A) Alignment and identification of caveolin-1 binding site. Consensus sequence used to scan protein sequence of IL-17RA was AXAXXXXA where A is an aromatic residue (Y, W, F) and X any amino acid. **(B)** Crystal structure of the extracellular portion of the IL-17RA (brown) bound to IL-17A homodimer (pink and blue). Green is highlighting the putative caveolin-1 binding site. PDB file: 3JVF_C. from (256).

5.2.2 IL-17RA interacts with caveolin-1 via FN1 domain.

To determine whether caveolin-1 interacts with IL-17RA via the FN1 domain, I used an overexpression system in HEK293T cells. I sub-cloned a mutant form of IL-17RA that expresses a truncated IL-17RA-FN2 protein, which lacks the FN1 domain **(Figure 5.3-A)**. Using a co-immunoprecipitation assay, I observed that the full-length of IL-17RA interacted with caveolin-1. Interestingly, the truncated IL-17RA- FN2 protein did not interact with caveolin-1 **(figure 5.3-B)**, suggesting that the FN1 domain is necessary for the IL-17RA/caveolin-1 interaction. These results confirm the *in silico* findings and demonstrate that caveolin-1 interacts with IL-17RA through the FN1 domain of IL-17RA. To determine that the putative caveolin-1-binding domain contained in the FN1 domain regulates IL-17RA/caveolin-1 interaction, I made a mutant of IL-17RA to use in a similar immunoprecipitation assay as above. The IL-17RA mutant plasmid had two of the three phenylalanine (F) of the caveolin-1-binding site substituted with alanine, and I called this construct delta F144A/F149A **(figure 5.4-A)**. Immunoprecipitated caveolin-1 associated with full-length IL-17RA similar as shown in figure 5.3-A, however, the association with the IL-17RA delta F144A/F149A was diminished **(figure 5.4-B)**. Altogether, these results suggest that the predicted caveolin-1-binding site can mediate, at least partially, the association of IL-17RA with caveolin-1. Further characterization with a mutant of all three phenylalanines could additionally confirm the function of the putative caveolin-1-binding domain in IL-17RA.
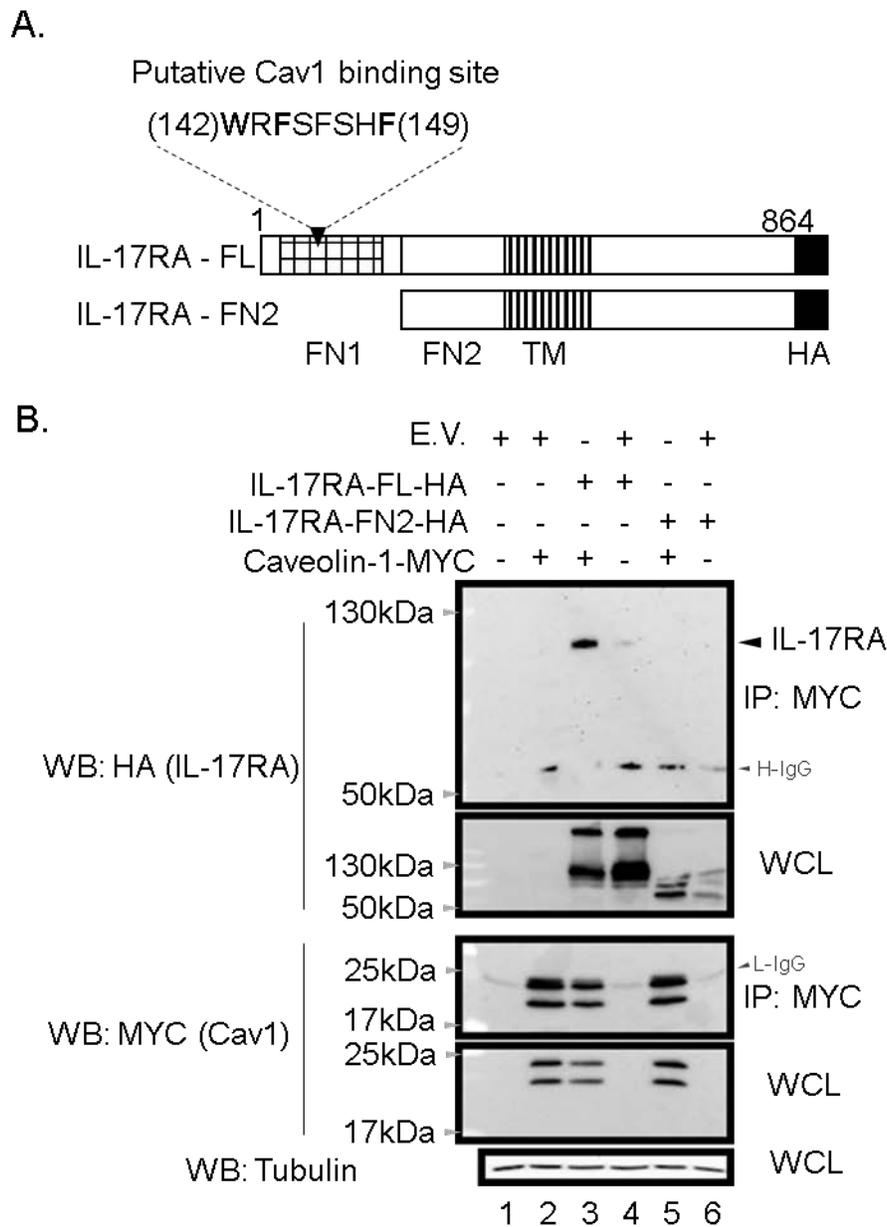
Figure 5.3: IL-17RA FN1 domain interacts with caveolin-1.

(A) Schematic diagram of murine IL-17RA showing the full-length (FL) and fibronectin-III-like (FN) domains. These constructs were sub-cloned from constructs made by Kramer et al. 2007 and inserted in a vector containing an HA-tag. (B) IL-17RA interacts with caveolin-1 via FN1 domain. HEK293T cells were transfected with the indicated plasmids and lysed 24 hours later. Cell lysates were incubated with an anti-MYC antibody to immunoprecipitate caveolin-1-myc protein and interacting partners. Immunoprecipitates and whole cell lysates were analyzed by western blotting. The immunoprecipitation of caveolin-1 with full-length IL-17RA is representative of four

independent experiments. The immunoprecipitation of caveolin-1 with truncated IL-17RA-FN2 is representative of two independent experiments.
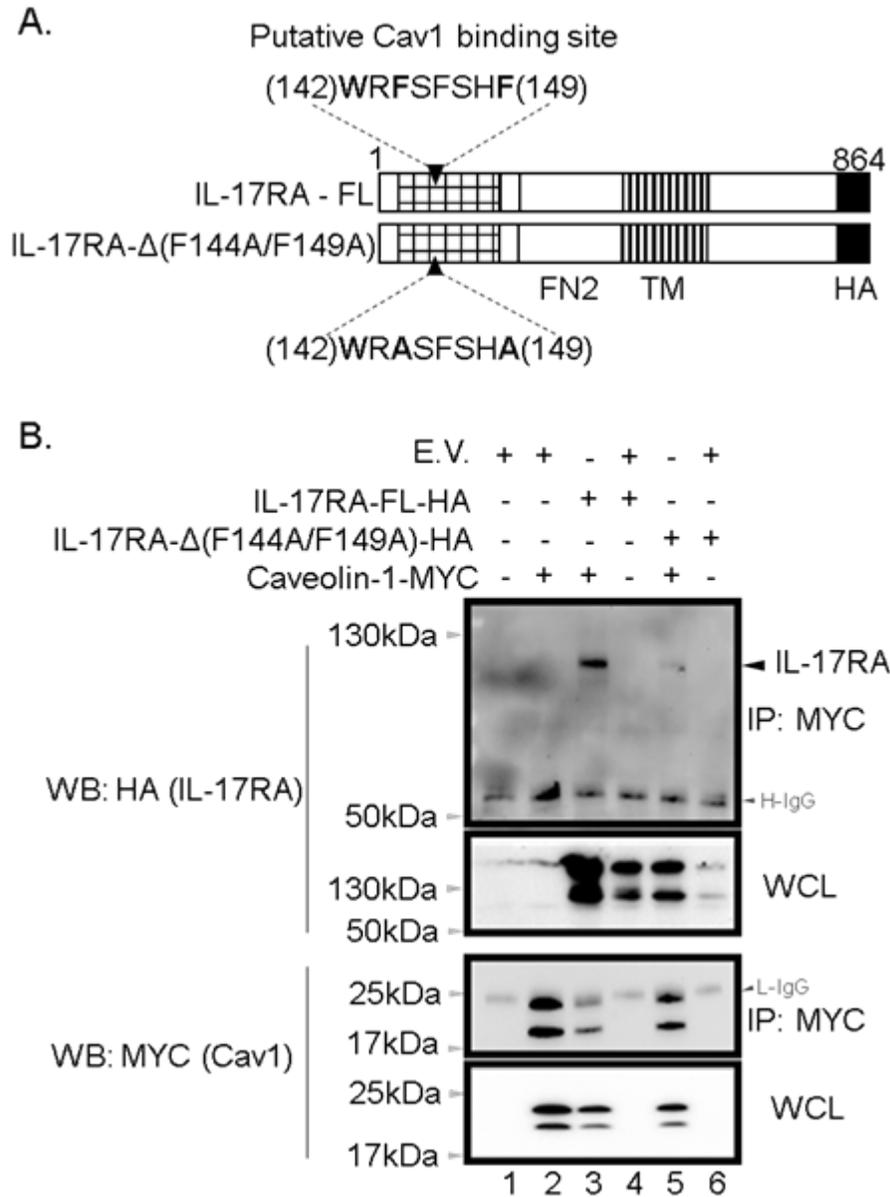
Figure 5.4: IL-17RA/caveolin-1 interaction is partially mediated by two phenylalanine residues.

(A) Schematic diagram of murine IL-17RA showing the full-length (FL) and mutant in two of the three relevant amino acids of the caveolin-1-binding site (ΔF144A/F149A). These constructs were cloned by inserting the double mutation using PCR. (B) IL-17RA interacts with caveolin-1 via caveolin-1-binding domain. Similar as in (B), HEK293T cells were transfected and lysed 24 hours later. Caveolin-1-myc protein was immunoprecipitated to determine interaction with FL or mutant IL-17RA-HA. Immunoprecipitates and whole cell lysates were analyzed by western blotting. This experiment was repeated twice.

5.2.3 Caveolin-1 partially facilitates the surface localization of IL-17RA.

Caveolin-1 regulates surface localization of TNFR1, which influences receptor signaling (176,247). Additionally, caveolin-1 regulates intracellular trafficking of signaling proteins to different intracellular compartments (257–260). To determine whether caveolin-1 regulates surface localization of IL-17RA, I analyzed IL-17RA receptor levels on the surface of $Cav1^{-/-}$ fibroblasts by flow cytometry. Compared to wild-type $Cav1^{+/+}$ mice fibroblasts, cells deficient in $Cav1$ had lower surface expression of IL-17RA **(figure 5.5-A-B)**. Interestingly, surface IL-17RA did not change in ST2 cells after knockdown of $Cav1$ (not shown), although IL-17R signaling decreased **(figure 3.2)**. These results suggest that acute knockdown of caveolin-1 is enough to interrupt intracellular signaling, but is not enough to disrupt the pool of receptor that is already on the surface. Altogether, these data demonstrate that caveolin-1 regulates intracellular signaling and surface localization of IL-17RA.
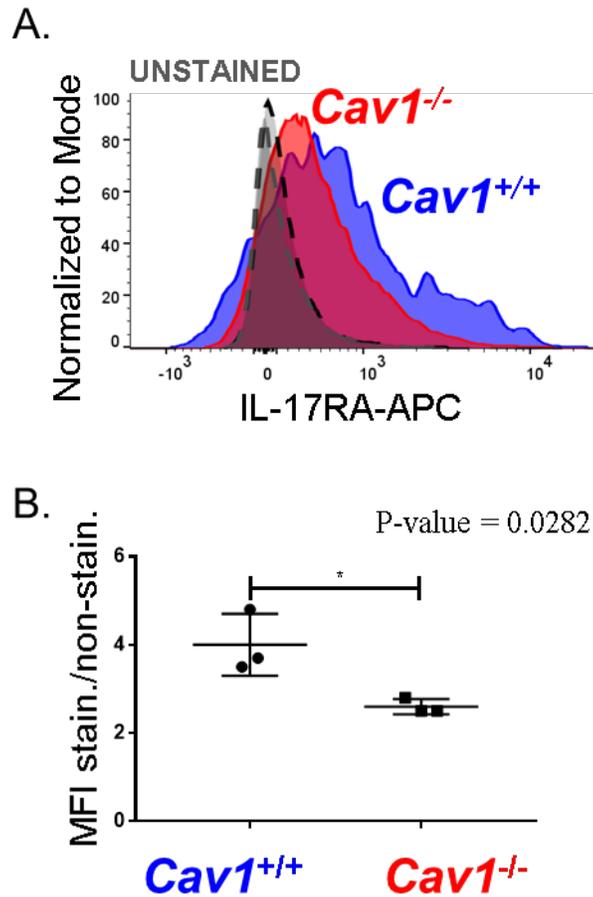
Figure 5.5: Caveolin-1 may partially facilitate the surface localization of IL-17RA.

(A) *Cav1*^{-/-} fibroblasts exhibit less IL-17RA on the surface. Flow cytometry analysis of expression of IL-17RA on the surface of *Cav1*^{+/+} (blue) or *Cav1*^{-/-} (red) tail fibroblasts. (B) MFI analysis from 3 independent experiments show a statistically significant difference between the surface expression of IL-17RA on *Cav1*^{-/-} and *Cav1*1^{+/+} fibroblasts. Ratio of MFI was analyzed for each sample, compared to unstained control. Statistical analysis: Students *t* test p = 0.0282.

5.2.4 Caveolin-1 overexpression increases IL-17R signalling.

I reported in chapter 3 that caveolin-1 knockdown impaired IL-17R signaling. To confirm that IL-17R signaling is regulated by caveolin-1, I transiently transfected ST2 cells to overexpress Myc-tagged caveolin-1. Consistent with findings from chapter 3, caveolin-1 overexpression increased production of IL-17-dependent IL-6, Lcn2 and CXCL1 in the supernatant of cultured ST2 cells **(figure 5.6-A)**. Expression of caveolin-1 in ST2 cells was verified by immunoblotting whole cell lysates with anti-MYC antibody **(figure 5.6-B)**. These findings indicate that overexpression of caveolin-1 activates IL-17R signaling.
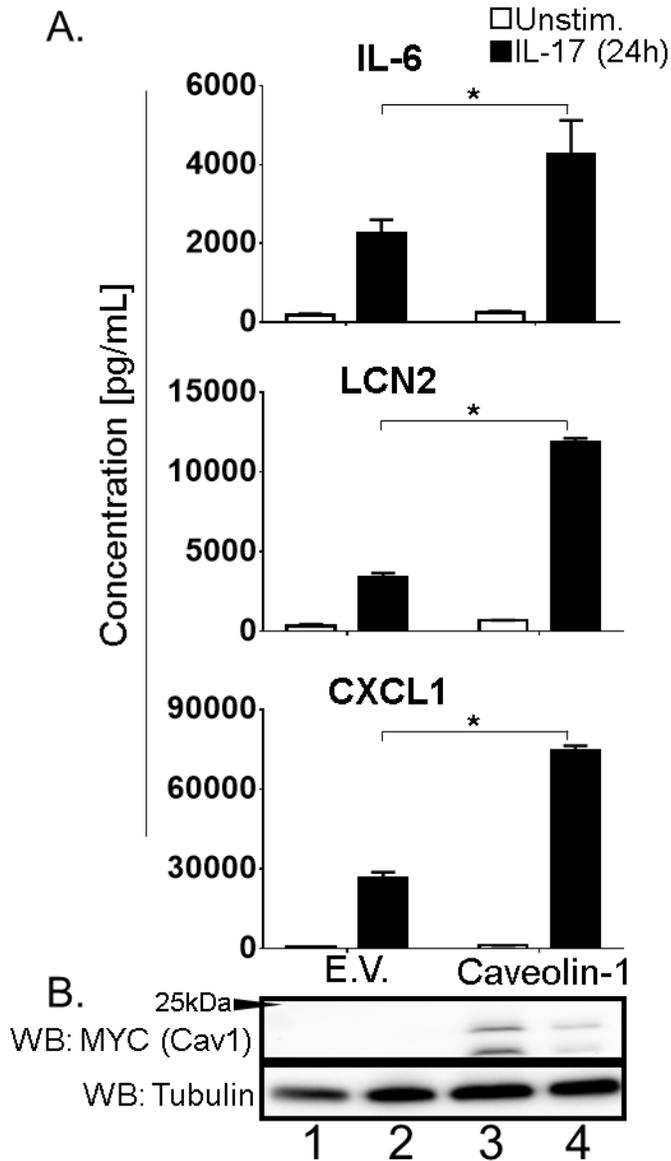
Figure 5.6: Caveolin-1 increases IL-17R signaling.

(A) IL-17-dependent expression of IL-6, Lcn2 and CXCL1 are increased after overexpression of caveolin-1. ST2 cells were transiently transfected with caveolin-1, 24 hours later these cells were stimulated with IL-17 for 24 hours. The supernatant of the cells was analysed for the indicated protein by ELISA. Results representative of four independent experiments. Statistics: one-way ANOVA – $p < 0.05$. (B) Ectopic expression of caveolin-1 was successful in ST2 cells. The same cells from (A) were lysed and their whole cell content was analysed by western blotting.

5.2.5 Lack of caveolin-1 decreases activation of IL-17-dependent NF-κB signaling.

Activation of NF-κB is among the main effector cascades triggered by IL-17R signaling (261–263). To determine if caveolin-1 regulates IL-17-dependent NF-κB signaling, I assessed IκBα degradation in Cav1$^{-/-}$ and Cav1$^{+/+}$ (WT) fibroblasts after IL-17 stimulation. As expected, IκBα was degraded 15-30 minutes after IL-17 treatment, and returned to basal levels by 60 minutes **(figure 5.7-A)**. Interestingly, IκBα degradation following IL-17 treatment was compromised in Cav1$^{-/-}$ fibroblasts compared to WT fibroblasts **(figure 5.7-A-B)**. These results suggest that in Cav1$^{-/-}$ fibroblasts IL-17-mediated NF-κB signaling is impaired in compared to their WT counterparts.

A.
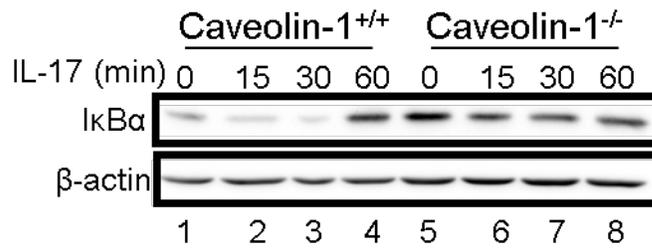
B.
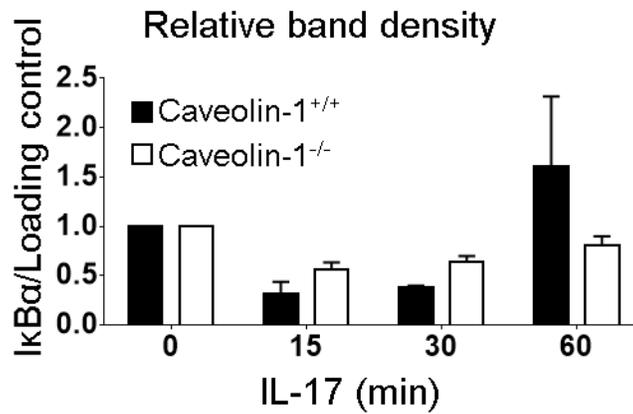
Figure 5.7: Lack of caveolin-1 increases activation of IL-17-dependent NF-κB signaling.

(A) Cav1^{-/-} fibroblasts exhibit reduced NF-κB signaling. Tail fibroblasts from Cav1^{+/+} or Cav1^{-/-} mice were stimulated with IL-17A for the indicated time. Whole cell lysates were analyzed by western blotting. This experiment is representative of two independent experiments. (B) Caveolin-1^{-/-} fibroblasts exhibit less degradation IκBα after IL-17 stimulation. The ratios of IκBα to loading control (actin) were normalized to the densitometry at time zero for each cell type. This plot has summary data from both biological replicates (A).

5.2.6 IL-17 stimulation dynamically regulates caveolin-1 protein expression.

 I previously noted in chapter 3 that levels of caveolin-1 mRNA were decreased upon IL-17 stimulation in ST2 cells. These results are consistent with another report indicating that inflammatory signaling, including IL-17 stimulation, decreased caveolin-1 mRNA in endothelial cells (139). To confirm the regulatory effect of IL-17 on caveolin-1 protein levels, I assessed caveolin-1 protein in whole cell lysates of ST2 cells stimulated with IL-17. Consistent with our findings about mRNA, caveolin-1 protein levels were also decreased after IL-17 stimulation **(figure 5.8-A)**. These results suggest that caveolin-1 protein is regulated by IL-17R signaling.

A.

ST2 cells

IL-17 (min)   0   5   15   30   60   120 180 240 1440

Caveolin-1

Tubulin

1   2   3   4   5   6   7   8   9
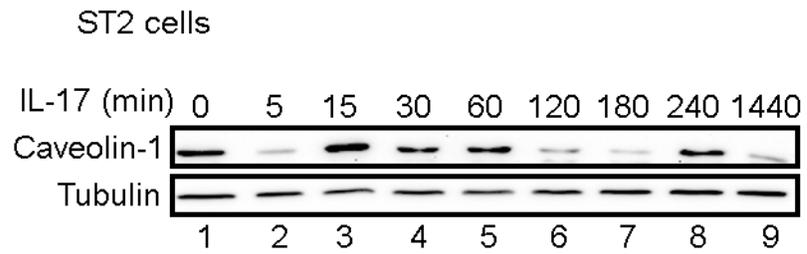
Figure 5.8: IL-17 stimulation downregulates caveolin-1 expression.

(A) Caveolin-1 protein decreases after IL-17 stimulation. ST2 cells were stimulated at the indicated times and cells were harvested and analyzed by western blotting. This is a representative experiment from 4 biological replicates.

5.2.7   Role of caveolin-1 in the synergic effect of IL-17 and TNFα *in vivo*.

To investigate that role of caveolin-1 in IL-17R signaling *in vivo*, I used $Cav1^{+/+}$ and $Cav1^{-/-}$ mice that I injected with IL-17 and TNFα combined. Because Li and colleagues demonstrated that a single intraperitoneal (i.p.) injection with IL-17 combined with TNFα increases IL-6 and CXCL1 in serum of mice after four hours (59), I used a similar assay to determine the role of caveolin-1 in IL-17R signaling *in vivo*. Age and gender matched mice were i.p. injected and blood was collected four hours after the injection. The content of IL-6 and CXCL1 was quantified in serum by ELISA. Since there was no statistically significant difference between male and female mice, their measurements were combined. To confirm that the technique was well performed, I injected ACT1-deficient mice to analyze same cytokines in the blood serum **(figure 5.10-C-D)**. Consistent with findings from Li and colleagues, ACT1-deficient mice had no response to IL-17 in synergy with TNFα, but the wild-type control mice had a statistically significant increase of IL-6 and CXCL1 in serum after the injection of IL-17+TNFα. When Caveolin-1-deficient mice were injected, although there was an increased in IL-6 after the injection, the baseline levels of IL-6 were also elevated respect to wild-type mice. Multiple comparison analysis of the 4 different groups (wild-type vs knock-out, and PBS vs IL-17+ TNFα injected) resulted in no statistically difference among these for groups. However, between mice injected with IL-17+ TNFα, there was difference that almost reached to significance (p = 0.06), suggesting that caveolin-1-deficient mice had a higher production of IL-6 compared to wild-type **(figure 5.9-A)**. The levels of CXCL1 in serum followed a similar pattern, but with differences that reached to statistically significance. Caveolin-1-deficient mice injected with IL-17+ TNFα exhibited more CXCL1 in their serum than wild-type controls, however, I should also indicate that there was an

increased production of CXCL1 in mice injected with PBS compared to wild-type controls **(figure 5.9-B)**. These data indicate that production of IL-6 and CXCL1 was increased in caveolin-1-deficient mice before any stimulation, and that these mice do not phenocopy ACT1-deficient mice.
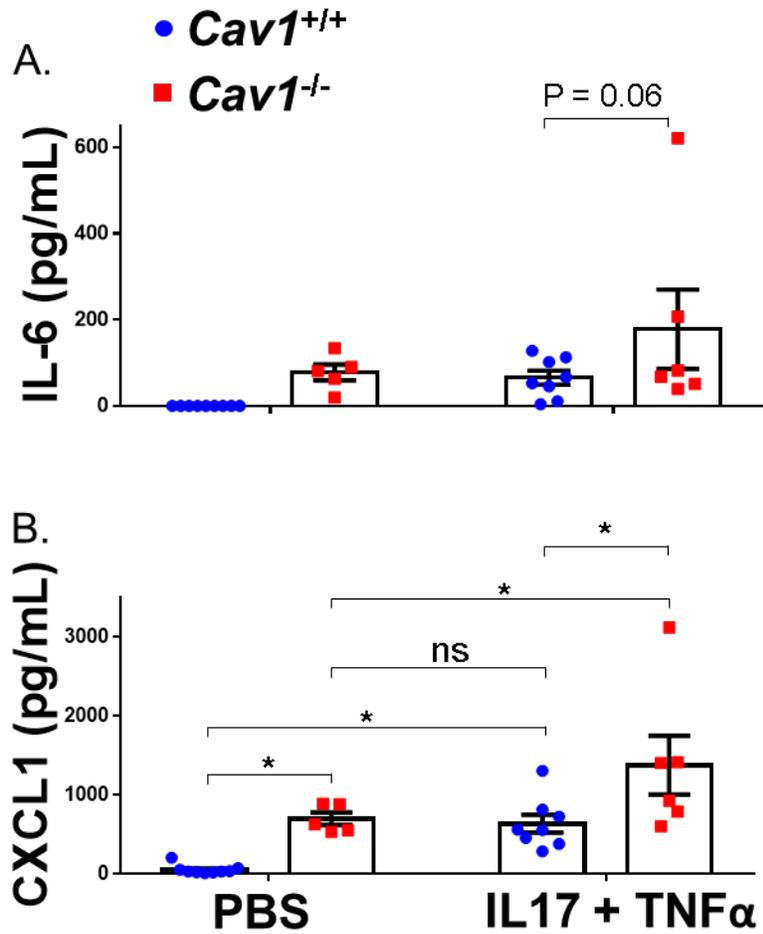
Figure 5.9: Caveolin-1-deficient mice exhibit increased baseline inflammation.

Cav1⁻ᐟ⁻ mice were injected with IL-17A (1mg/mouse) combined with TNFα (200ng/mouse), and four hours later the mice were sacrificed, and blood was collected by cardiac puncture. Blood serum was used to measure (A) IL-6 and (B) CXCL1. There was not significant difference between genders, so these plots show results for both male and female mice combined. Statistics: two-way ANOVA – Uncorrected Fisher's LSD test p = 0.05.
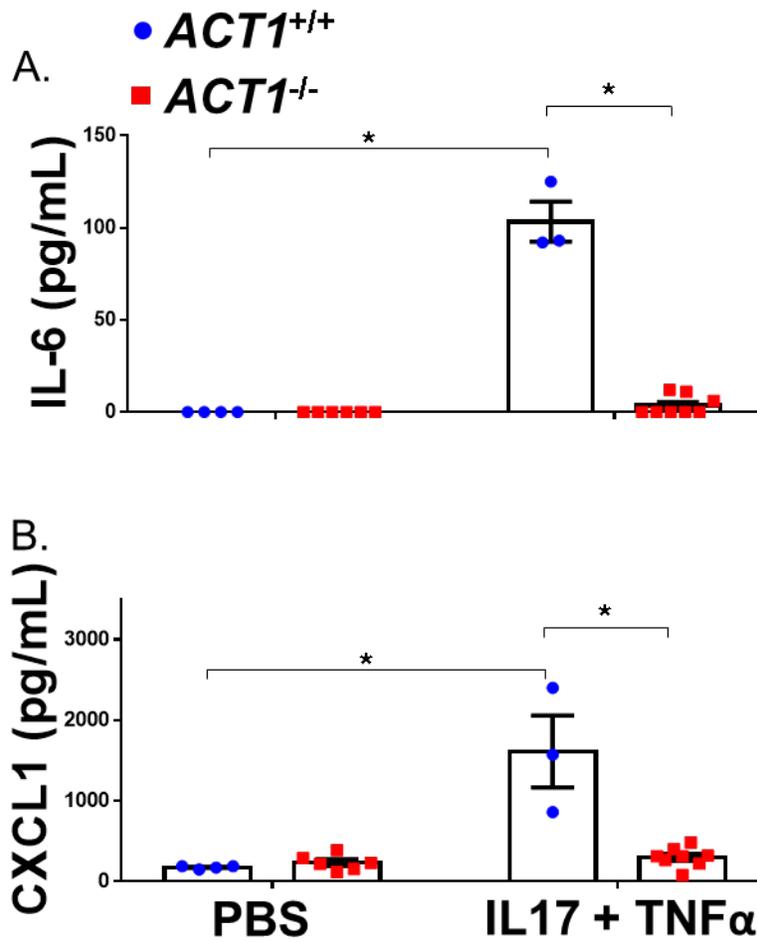
Figure 5.10: ACT1-deficient mice are non-responsive to IL-17R signaling.

(A) IL-6 and (B) CXCL1 measured in the serum of ACT1-deficient mice. There was not significant difference between genders, so these plots show both male and female mice combined. Statistics: two-way ANOVA – Uncorrected Fisher's LSD test $p = 0.05$.

5.3   DISCUSSION

Caveolin-1 regulates inflammatory signaling by binding to receptors like TLR4 or TNFR1 to promote their interaction with downstream intermediate molecules (177,196,243). In this chapter, I present findings that suggest that caveolin-1 can also regulate IL-17R signaling. Upon IL-17 binding to IL-17R, signaling is activated by the recruitment of ACT1 to the IL-17R intracellular SEFIR domain (59). ACT1 is an E3-ligase that ubiquitinates both itself and TRAF6 to initiate downstream IL-17R signaling, and is also a scaffolding protein that mediates the binding and functional interaction of adaptor proteins with IL-17R (60). ACT1 is an example of the crucial role of scaffolding proteins in the activation of IL-17R signaling. In this chapter, I present results showing that caveolin-1 interacts with IL-17R through a putative caveolin-1 binding site located within the FN1 extracellular domain of IL-17R, and that downregulation or overexpression of caveolin-1 in ST2 cells, respectively, decreased or increased IL-17R signaling. Additionally, caveolin-1$^{-/-}$ fibroblasts had less IL-17R on the cell surface, but ST2 cells with caveolin-1 knockdown have no changes in surface levels of IL-17R (not shown). Based on these data, I can provisionally conclude that IL-17R signaling is positively regulated by caveolin-1.

TLR4 has a putative caveolin-1 binding site located in the C-terminal portion of its cytoplasmic domain (196,264), whereas TNFR1 has a putative caveolin-1 binding site located in the transmembrane/cytoplasmic region of the receptor (247). Combined with my results regarding the putative caveolin-1 binding site in IL-17R, I conclude that caveolin-1 binds these three inflammatory receptors through a consensus sequences like AXXAXXXA, where A is an aromatic residue and X is any other residue (255). Interestingly, these binding sites are in

different domains of the three receptors, suggesting that binding to caveolin-1 could have distinct functions depending on the specific receptor domain with which these interactions occur. Furthermore, my data indicate that mutation of two out of the three aromatic residues decreased the association of IL-17RA with caveolin-1 **(figure 5.4-B)**, suggesting that this putative caveolin-1-binding site may indeed mediate this association. Further analysis of the subcellular distribution of this mutant of IL-17RA are yet to be performed.

My studies showing that binding to caveolin-1 may regulate signaling and sub-cellular localization of IL-17R, are reminiscent of similar findings with TNFR1. Knockdown of caveolin-1 in human endothelial cells decreases TNFR-signaling and total TNFR1 protein levels, due to lysosomal degradation; however, the cell-surface levels of TNFR1 do not change (247). Likewise, knockdown of caveolin-1 in murine ST2 cells impaired IL-17R-signaling without changing the surface levels of IL-17R. Although the total IL-17R protein levels after caveolin-1 knockdown in ST2 cells are yet to be determined, the decreased surface levels of IL-17R observed in caveolin-1$^{-/-}$ fibroblasts, compared to WT fibroblasts, suggest that caveolin-1 facilitates intracellular trafficking of IL-17R. I speculate that absence of caveolin-1 re-distributes IL-17R to lysosomes for degradation. Lastly, I hypothesize that caveolin-1 is acting as a scaffolding protein inside the cell, interacting with IL-17R and a complex of other proteins. Consequently, caveolin-1 may prevent IL-17R lysosomal degradation before the receptor gets to the surface of the plasma membrane.

Caveolin-1 can not only regulate signaling by associating with IL-17R, but it can also mediate signaling by activating downstream receptor signaling molecules, such as TRAF proteins (243,265). My data indicated that knockdown of caveolin-1 decreased, while overexpression of caveolin-1 increased, IL-17 signaling. This is partially mediated by activation

of NF-κB upstream of IκBα. In a previous report, caveolin-1 activated TLR4 signaling in endothelial cells via caveolin-1-Y14 phosphorylation (177). Moreover, caveolin-1 interacted with TLR4 and stabilized the TLR4/MYD88 complex to activate NF-κB signaling (177). For future work, questions remain regarding whether phosphorylation of caveolin-1-Y14 plays a role in regulating of IL-17R signaling, and whether this phosphorylation site regulates the interaction of caveolin-1 with IL-17R.

Finally, a preliminary analysis of the effect of caveolin-1 in IL-17R signaling *in vivo* indicates that caveolin-1 and ACT1 have different functions. My data with fibroblasts and ST2 cells suggested that caveolin-1 could positively regulate IL-17R signaling, an effect that is similar to the function of ACT1 in cells. However, in contrast *in vivo*, caveolin-1-deficient mice had an increased production of IL-6 and CXCL1 in baseline and in stimulated conditions while ACT1-deficient mice had no increase in these cytokines. In sum, these data suggest that although caveolin-1 regulates IL-17R signaling in cells in culture, caveolin-1 functions are broader in organisms. Additionally, caveolin-1 and ACT1 have no redundant functions, suggesting that a possible function of caveolin-1 in IL-17R signaling involves regulation of the receptor localization rather than control of signaling.

In summary, this study expands the understanding of the mechanism of activation of IL-17R signal transduction, and reveals a role for caveolin-1 in IL-17R localization and signaling. Future investigations of the role of caveolin-1 in the regulation of IL-17R signaling may provide insight into activation and intracellular trafficking of IL-17R.

6.0   CHAPTER SIX: SUMMARY AND DISCUSSION, FUTURE DIRECTIONS AND

CONCLUDING REMARKS

6.1   SUMMARY AND DISCUSSION

IL-17 receptor signaling is both an important line of defense against pathogens and a significant contributor to inflammation in autoimmune diseases. The downstream intracellular signaling of the IL-17 receptor (IL-17R) requires activation of intermediate proteins that transduce signals through a cascade of events, involving protein-protein interactions and post-translational modifications like phosphorylation and ubiquitination of target proteins (reviewed in (11,34)). Together, these molecular events organize the stabilization of mRNA transcripts, and/or the activation of terminal effectors like the NF-κB transcription factor that induces gene expression. In this thesis, I sought to define the molecular mechanisms by which the ubiquitin-binding protein ABIN-1 and the membrane-bound scaffolding protein caveolin-1 regulate IL-17R signal transduction. Additionally, I provide evidence for deubiquitinases CYLD and Otulin having potential regulatory roles in IL-17 signaling.

In chapter 3, I reported results of an siRNA screen for candidate genes that could regulate IL-17R signaling. From the literature, I selected ten candidate genes that fell into one or more of the following categories: (i) genes known to regulate NF-κB downstream of TLR4 or TNFR1 signaling, (ii) genes that regulate signaling pathways via ubiquitination, and (iii) genes linked to

IL-17-dependent autoimmune diseases. Among these, I found five genes with possible regulatory effects on IL-17R signaling. Two of these genes, caveolin-1 and anaphase-promoting complex (AnapC3 or *Cdc27)*, were possible activators, as their knockdown reduced IL-17-mediated signaling. I chose not to pursue the study of AnapC3 *(Cdc27)*-mediated activation of IL-17R signaling because this effect is likely to be a consequence of disrupting the cell cycle, a major known function of AnapC3 (206). The other activator was caveolin-1, a multifunctional scaffolding protein. Caveolin-1 regulates various intracellular signaling pathways, including signaling by receptor and non-receptor tyrosine kinases, G-protein coupled receptors (GPCR), and TNFR1 and TLR4 (266). Since caveolin-1 is known to regulate trafficking and signaling of several surface receptors (267,268), I further explored the specific role of caveolin-1 downstream of IL-17R. Additionally, IL-17 stimulation of ST2 cells down-regulated caveolin-1 mRNA and protein, suggesting the presence of a feedback loop. Three other putative intermediates discovered from this screen showed inhibitory activity on IL-17R signaling: ABIN-1, CYLD, and Otulin. CYLD and Otulin are deubiquitinases, while ABIN-1 binds poly-ubiquitinated K63-linkages with its ubiquitin-binding domain homologous to that of NEMO (IKKγ), a critical component of the IKK complex of NF-κB signaling. I decided to focus further investigation on determining the role of ABIN-1 in IL-17R signaling for the following reasons: (i) Among ABIN-1, Otulin and CYLD, ABIN-1 (*TNIP1*) is the only gene that has been genetically associated with psoriasis, a complex autoimmune skin disease that is successfully treated by blocking IL-17R signaling, (ii) ABIN-1 binds to NEMO and A20, two key intermediates of NF-κB signaling, (iii) ABIN-1 inhibits TNF-dependent NF-κB signaling (91,92).

In chapter 4, I presented data that suggest a molecular mechanism of action for ABIN-1 in IL-17R signaling. ABIN-1 is a tonic inhibitor of inflammatory signaling functioning at the

level of NEMO and the IKK complex (92) **(figure 6.1)**. I discovered that ABIN-1 can inhibit IL-17R signaling independently of A20 and dependent on a functional ubiquitin-binding domain **(figures 4.2 & 4.3)**. Although ABIN-1 mRNA is induced by NF-κB (217), the levels of ABIN-1 protein did not return to baseline until four hours after removal of IL-17 stimulation (figure 4.7). To our knowledge, this is the first study that has described that ABIN-1 protein is degraded after IL-17 stimulation. Furthermore, several studies have reported that ABIN-1 transcript levels increase after TNFα stimulation, but no studies were done to query the protein levels (217,269). My data, presented in **figure 4.4**, show that while ABIN-1 mRNA levels increase after IL-17 stimulation, surprisingly, there is a decrease in ABIN-1 protein. This decrease in ABIN-1 protein levels is particularly important in the "RNAseq era". Technological advances have improved our capacity to analyze gene induction on a large-scale by measuring mRNA. These data emphasize the principle that, even when mRNA levels are increased, protein levels are not necessarily altered concomitantly. I was able to reconcile ABIN-1 mRNA induction, degradation of ABIN-1 protein, and ABIN-1 inhibitory function by suggesting that ABIN-1 serves as a constitutive brake on signaling. Thus, as a tonic inhibitor of inflammatory signaling, ABIN-1 would somehow need to be degraded to permit activation of IL-17-dependent NF-κB signaling. In turn, activation of NF-κB would induce ABIN-1 mRNA, as part of a feedback signaling circuit. Furthermore, I speculate that while IL-17R signaling remains activated, ABIN-1 protein continues to be degraded, which could explain a previous observation that ABIN-1 protein is decreased in skin biopsies of psoriatic patients, a disease that is characterized by increased IL-17 signaling (134). Further studies are of course necessary to elucidate the complete molecular mechanism by which ABIN-1 is degraded and how IL-17R signaling contributes to this

127

degradation. Nonetheless, I speculate that molecular events like phosphorylation and ubiquitination of ABIN-1 mediated by IL-17R signaling will be part of this mechanism.

Furthermore, in chapter 4, I explored the mechanism by which IL-17 induces phosphorylation and degradation of ABIN-1. Specifically, I investigated whether these two processes are related, as is the case for another tonic inhibitor of NF-κB, IκBα. To initiate signaling, IκBα is first phosphorylated at serine 32 (Ser 32) and serine 36 (Ser 36), and then K48-ubiquitinated, and finally degraded (270). For IκBα, these three processes are sequentially dependent on each other, as shown by substitution of Ser32/36 with alanine residues to originate an IκBα protein that does not undergo signal-induced degradation (271). To test the relationship between phosphorylation and degradation of ABIN-1, I used staurosporine, a pan-kinase inhibitor, to non-specifically block intracellular phosphorylation. Surprisingly, inhibiting phosphorylation of ABIN-1 increased ABIN-1 degradation **(figure 4.5-B)**, leading me to conclude that intracellular phosphorylation activity protects ABIN-1 from degradation.

To characterize further the phosphorylation of ABIN-1 and to delineate the kinase responsible for its phosphorylation, I inhibited the IKK kinase complex, as it is known to function downstream of IL-17R signaling (261). Consistent with the staurosporine experiment, inhibition of both IKKα and IKKβ together enhanced degradation of ABIN-1 even before stimulation **(figure 4.5-D)**. Based on my results, I speculate that IL-17 induces phosphorylation of ABIN-1 at one or more sites (tyrosine (Y), serine (S) or threonine (T) residues). S and T residues are commonly seen as part of the PEST (Proline-Glutamic-Serine-Threonine) signal sequence that promotes protein degradation (272). However, no PEST signal sequence was found in ABIN-1 (not shown), suggesting that phosphorylation of ABIN-1 has a role other than inducing protein degradation. Together, these data suggest that kinase activity of the IKK

complex prevents degradation of ABIN-1. However, important questions remain, including: (i) does the IKK complex directly phosphorylate ABIN-1? and (ii) is degradation of ABIN-1 dependent on, or independent of, its phosphorylation? These and other remaining questions are discussed later in this chapter. In summary, I found that IL-17R signaling induces ABIN-1 phosphorylation, but further studies are required to map the phosphorylation site in ABIN-1 and to identify the specific kinase that catalyzes the phosphorylation.

In addition, in chapter 4, I provide evidence that IL-17-dependent ABIN-1 degradation occurs at least partly via the proteasome, a process that requires K48-ubiquitination. The SCF-β-TrCP complex, containing Skp1, Cullin-1, β-TrCP1 and β-TrCP2 proteins, is known to mediate K48-ubiquitination of IκBα to target it for proteasomal degradation (273). Based on published reports that the SCF-β-TrCP complex is activated downstream of IL-17R signaling to target IκBα and ACT1 for degradation (75,274), I hypothesized that the SCF-β-TrCP complex (formed by β-TrCP1 and β-TrCP2 as aforementioned) regulates degradation of ABIN-1. However, knocking-down β-TrCP1 and β-TrCP2 to disrupt the SCF-β-TrCP complex had no effect on ABIN-1 degradation **(figure 4.8)**, suggesting that this complex does not mediate ABIN-1 ubiquitination and degradation. Nevertheless, possible caveats are that the incomplete knockdown of ABIN-1 siRNA was insufficient to fully restrict the β-TrCP complex activity (knockdown efficiency was 60%, and protein levels were not tested). Another explanation may relate to the half-life of β-TrCP1, which was reported to be between three to ten hours in two different cell lines (275). To-date, the half-life of β-TrCP2 is unknown. Consequently, if the β-TrCP complex half-life exceeds 48 hours, siRNA treatment may not have sufficiently reduced the existing pool of β-TrCP protein, and thus not interrupted ABIN-1 ubiquitination and degradation. Alternatively, a different E3-ligase may be activated by IL-17R signaling to induce

K48-ubiquitination and degradation of ABIN-1. If the latter is the case, it would be important to identify this ligase and to identify the other signaling intermediates that are targeted by it. In the future directions sections of this chapter, I discuss how to address some of these remaining questions in more detail.

In chapter 5, I presented results to support the hypothesis that caveolin-1 activates IL-17R signaling. Caveolin-1 regulates intracellular signaling by binding to various signaling intermediates and restricting or promoting their function in the context of TLR4 and TNFR1 signaling (176,177,196). One possibility that seemed promising is that caveolin-1 binds to IL-17R. I proposed this possibility because I discovered *in silico* that both subunits of the heterodimeric receptor IL-17R (formed by IL-17RA/IL-17RC) contain a putative caveolin-1 binding site in the FN1 (Fibronectin III-like 1) domain of their extracellular region **(figure 5.1)**. I experimentally confirmed the functionality of this caveolin-1 binding site in IL-17RA, the most important signaling subunit of the IL-17 receptor (14,16). Indeed caveolin-1 associates with IL-17RA upon overexpression of both proteins in HEK293T cells **(figure 5.2)**. Interestingly, a truncated form of IL-17RA, lacking the FN1 domain that encompasses the putative caveolin-1-binding site, did not associate with caveolin-1. To my knowledge, these preliminary results are the first report that caveolin-1 associates with IL-17RA, and set the groundwork for more in-depth analysis of this pathway.

In chapter 5, I also observed that fibroblasts deficient in caveolin-1 express lower levels of IL-17RA on the cell surface as shown by flow cytometry **(figure 5.3)**. Caveolin-1 is well-known to regulate receptor trafficking to the cell surface and signaling of receptors such as the bone morphogenic protein receptor II (BMPRII), transforming growth factor beta receptor I (TGF-βRI), D2 dopamine receptor (Drd2) and TNFR1 (176,248,258,259,276). Accordingly, it is

tempting to speculate that there is reduced IL-17 signal transduction due to the decreased levels of IL-17RA on the cell surface. As a preliminary conclusion, I propose that caveolin-1 facilitates signaling and surface localization of IL-17R by direct binding to IL-17RA. However, it must be pointed out that this conclusion only partially explains my results, because IL-17RA on the cell surface was unchanged in ST2 cells following caveolin-1 knockdown (**not shown**). ST2 cells are a bone marrow stromal cell line that robustly responds to IL-17 stimulation (212). Nonetheless, as I showed in chapter 3, ST2 cells in which caveolin-1 was knocked down exhibited a decreased response to IL-17 stimulation as measured by IL-6 production (**figure 3.1**). Thus, in addition to regulating IL-17RA surface localization, these data suggest that caveolin-1 may also have a direct signaling role downstream of IL-17R signaling.

In addition to regulating surface localization of receptors, caveolin-1 also regulates signaling of TLR4 and TNFR1 (176,277). In chapter 5, I also described the finding that caveolin-1 functions downstream of IL-17R signaling (**figure 5.4**). To corroborate this finding, I overexpressed caveolin-1 in ST2 cells and measured IL-17-dependent IL-6 production. Consistent with the knockdown experiments, overexpression of caveolin-1 increased IL-17R signaling (**figure 5.4**). Together, these data suggest that IL-17R is positively activated by caveolin-1 in our experimental system. Moreover, adaptor molecules in the IL-17R signaling cascade like ACT1 or TRAF6 may interact with caveolin-1. For instance, TRAF6 interacts with caveolin-1 downstream of IL-1β receptor signaling, another inflammatory pathway (265), and my *in silico* analyses revealed a putative caveolin-1 binding site in the sequence of ACT1 (**not shown**). It is therefore tempting to hypothesize that caveolin-1 may also interact with ACT1 or TRAF6 downstream of IL-17R signaling to facilitate the formation of signaling complexes. Additionally, I also discovered evidence of mutual regulation between caveolin-1 and IL-17R

signaling. A similar mechanism of mutual regulation between IL-17R signaling with ACT1 has been reported (75). Specifically, in chapters 3 and 5, I showed that IL-17 stimulation decreased caveolin-1 mRNA **(figure 3.3)** and protein **(figure 5.6)** in ST2 cells. These data suggest that caveolin-1 is downregulated because of the activation of IL-17R signaling. Furthermore, Yamaguchi et al. described that stimulation of keratinocytes with IL-17 or TNFα decreased caveolin-1 mRNA, which supports my findings. The authors of that study correlated their findings in keratinocytes with decreased caveolin-1 mRNA in skin biopsies of patients with psoriasis (127,139). However, Yamaguchi et al. did not investigate whether caveolin-1 regulates IL-17 signaling, and to my knowledge, this thesis is the first report describing a mutual regulation between caveolin-1 and IL-17R signaling. Moreover, to test further the role of caveolin-1 in IL-17R signaling, caveolin-1$^{-/-}$ cells could be reconstituted with a plasmid expressing a dominant negative mutant of caveolin-1 protein. Phosphorylation of caveolin-1 at tyrosine-14 has been shown to mediate its signal transduction function, and mutations of this residue originate a dominant negative form of caveolin-1 (177,278). Thus, I hypothesize that caveolin-1$^{-/-}$ cells reconstituted with this dominant negative tyrosine-14 mutant of caveolin-1 would have decreased IL-17 signaling. Finally, extending the hypothesis to *in vivo* models, absence of caveolin-1 could potentially reduce IL-17-mediated inflammation. To test this hypothesis, I suggest using caveolin-1$^{-/-}$ mice in models of IL-17-mediated inflammation like the mouse model of multiple sclerosis (MS) and/or the imiquimod-induced psoriasis-like disease model. Thus, work presented here identified that both ABIN-1 and caveolin-1 function downstream of IL-17R, by different mechanisms.

IL-17R signaling synergizes with other signaling receptors, like TNFR1 (212,279–281). Integration of all the responses from various receptors determines how cells respond to multiple

stimuli. ABIN-1 and caveolin-1 act downstream of both IL-17R (this thesis) and TNFR (174,211,229) so ABIN-1 and caveolin-1 could mediate receptor cross-talk. For example, ABIN-1 and caveolin-1 protein levels are down-regulated by IL-17. ABIN-1 is degraded for as long as IL-17 is stimulating its receptor **(figure 4.7)**, while caveolin-1 protein levels dynamically change during IL-17 stimulation **(figure 5.6)**. Degradation of ABIN-1 can prime cells to having an enhanced response to a secondary stimulation with TNFα, but dynamic changes in caveolin-1 can modulate the final signaling response. An approach to understanding the contribution of ABIN-1 and caveolin-1 in the cross-talk between signaling pathways is to build computational models of these signaling networks. Computational models have previously been used to determine the cross-talk of pathways via NF-κB signaling (reviewed in (282)). Experimental data from my studies on ABIN-1 and caveolin-1 could be used as input to build and calibrate a model of the IL-17 response. Kinetics of down-regulation, target gene expression, and the status of different proteins from the IL-17R pathway can be also used as inputs for this model. Additional data should be accumulated regarding the role of ABIN-1 and caveolin-1 in TNFR1 signaling, and added to this computational model. Furthermore, this model could be used to suggest new hypotheses regarding IL-17R signaling, and to understand the cross-talk between TNFα and IL-17 pathways.

The addition of my findings to the IL-17R signaling pathway are illustrated in figure 6.1**(figure 6.1)**. In this model, I depict that ABIN-1 and caveolin-1 are constitutively expressed prior to IL-17 stimulation. At baseline, ABIN-1 inhibits the IKK complex, while caveolin-1 facilitates localization of IL-17R to the cell surface. Upon IL-17 stimulation, ACT1 is recruited to the receptor and caveolin-1 may contribute to ACT1-dependent signaling by possible stabilization of the ACT1/IL-17R complex, leading to activation of NF-κB. As a distal signaling

133

event upon receptor activation, IL-17R signaling induces degradation of ABIN-1 to increase NF-κB activation. In this model, caveolin-1 is downregulated due to the activation of IL-17R signaling. Furthermore, downregulation of caveolin-1 restricts IL-17-mediated inflammation and helps to dampen inflammatory signaling, while IL-17 continues to stimulate the receptor.

In summary, my thesis described a screen of novel intermediates of IL-17R signaling, and is the first study to propose mechanistic roles for ABIN-1 and caveolin-1 acting downstream of IL-17R signaling. Here, I described a potential mechanism by which the ubiquitin-binding domain of ABIN-1 regulates baseline and IL-17-stimulated signaling in an A20-independent manner. In the case of my preliminary studies on caveolin-1, my data suggest that caveolin-1 promotes IL-17 signaling as well as regulates surface localization of the receptor (IL-17RA). Together, the results described in this thesis contribute to the characterization of downstream intermediate molecules of IL-17R signaling.
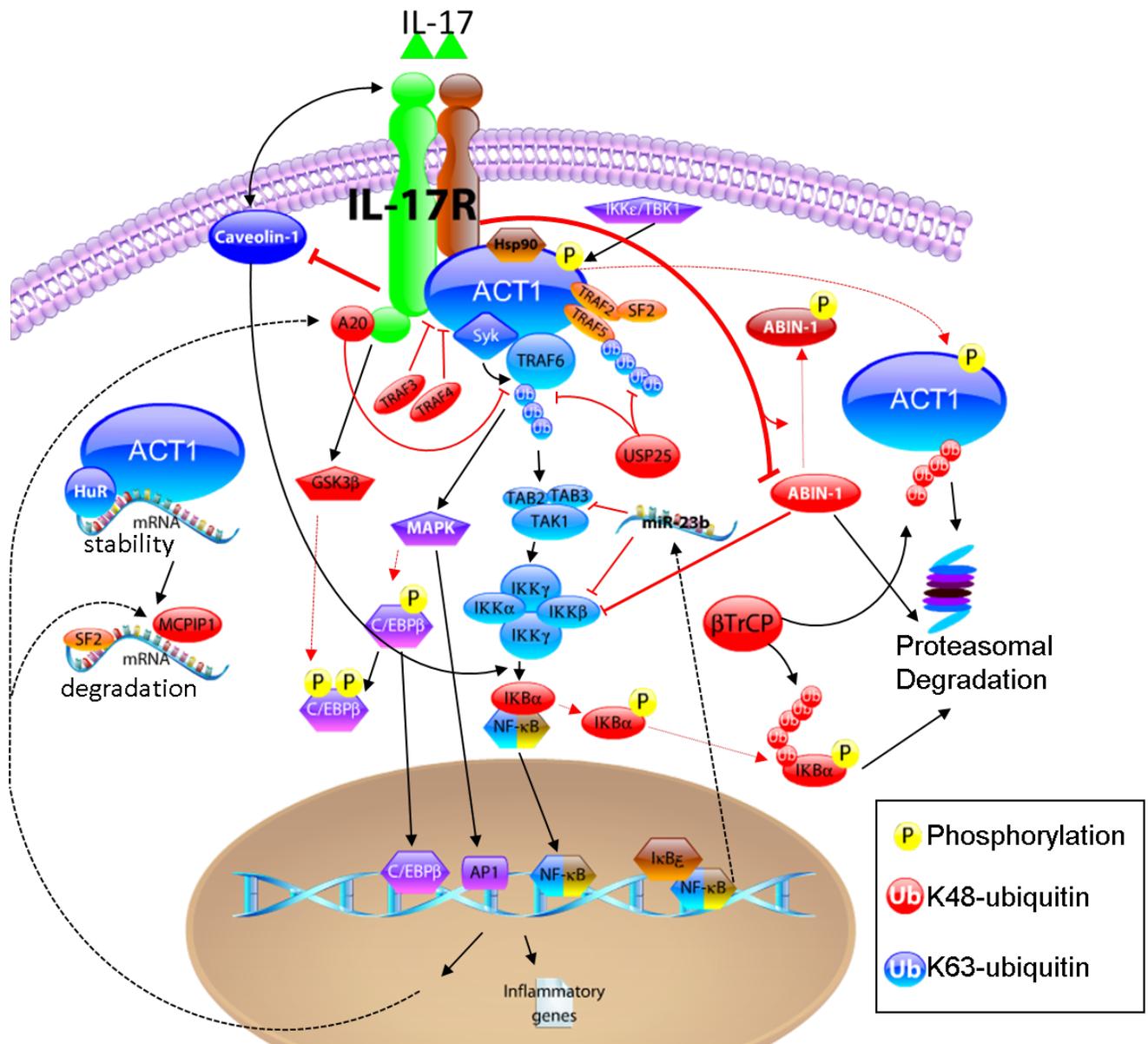
Figure 6.1: Schematic representation of IL-17R signaling

Proposed roles of ABIN-1 and caveolin-1 are included in this model as described in this thesis.

The red arrows with flat ends indicate inhibition and the black arrows with pointed ends indicate

induction or activation. Dotted black arrows indicate feedback regulation, dotted red arrows

represent phosphorylation events, and double arrows indicate the interaction of caveolin-1 with the extracellular domain of IL-17RA.

6.2 FUTURE DIRECTIONS

The work presented in this thesis illuminates some aspects of the regulation of IL-17R signaling, while generating many other possible avenues of investigation. Questions remain regarding the molecular mechanism of phosphorylation and degradation of ABIN-1, as well as the specific role of caveolin-1 in activation of IL-17R signaling. Potential *in vivo* relevance of these signaling molecules could be tested in murine models of IL-17-related diseases to investigate the physiological relevance of ABIN-1 and caveolin-1 in IL-17R signaling. Since a goal of studying signaling is to provide the basis for therapy, I propose exploration of small molecules and peptides for studying, and for possible therapeutic regulation of, IL-17R signaling.

6.2.1 <u>Molecular approaches to study ABIN-1 in IL-17R signaling.</u>

A possible avenue to extending my studies involves the identification of the kinase(s) and E3-ligase(s) responsible for phosphorylation and ubiquitination of ABIN-1 downstream of IL-17R signaling. Achieving this aim would be useful in determining what other proteins interact with ABIN-1 (its "interactome") after IL-17 stimulation in ST2 cells. The Gaffen lab has widely used this cell line to study IL-17R signaling (15,19,283). Using mass spectrometry can provide insight into the identity of proteins that bind to ABIN-1 upon IL-17 stimulation. Understanding the interactome of ABIN-1 as regulated by IL-17R signaling could provide information about the

136

mechanism by which ABIN-1 regulates IL-17R signaling and vice-versa. Specifically, I speculate that this approach could help to narrow the list of possible kinase(s) or E3-ligase(s) that regulate ABIN-1 after IL-17 stimulation. Specific findings from this mass spectrometry analysis could be validated in other relevant cells that respond to IL-17, like murine keratinocytes or human oral epithelial cells (35,284).

An additional future direction could be to identify the role of β-TrcP proteins in ubiquitination and degradation of ABIN-1 by using β-TrCP1-deficient fibroblasts. As I discussed above, siRNA knockdown of β-TrcP1 and β-TrcP2 could have been incomplete in disrupting the functional β-TrcP complex. Thus, using β-TrcP1-deficient fibroblasts, which have impaired degradation of IκBα (285), could help to determine if ABIN-1 is K48-ubiquitinated by the SCF-β-TrcP complex after IL-17 stimulation.

In addition, *in vitro* analyses could be used to investigate ABIN-1 phosphorylation and ubiquitination in cells. Endogenous ABIN-1 could be incubated in an assay *in vitro* with recombinant IKKβ protein to assess if ABIN-1 can be phosphorylated by the IKK complex. If so, these results would strengthen our findings in stromal cells (chapter 4). A similar assay could be used with other kinases. Possible limitations of this approach include the source of purified active protein and the requirement to immunoprecipitate sufficient quantities of endogenous ABIN-1 protein. These limitations could be addressed by purifying overexpressed protein from HEK293T cells. All in all, this assay would provide evidence that ABIN-1 could be phosphorylated (or not) by the tested kinases. Lastly, since the ABIN-1-phosphorylating kinase could have different roles within a cell, the relevance of these findings should be validated in IL-17-responding cells like ST2 cells, keratinocytes, or epithelial cells.

In vivo approaches to study caveolin-1 in IL-17R signaling

How might one apply the findings from this thesis to a physiological relevant model? I propose to analyze the relationship between caveolin-1 with IL-17R signaling using murine models of autoimmune diseases like psoriasis or multiple sclerosis (MS) (127,286). Caveolin-1$^{-/-}$ mice have been previously used with the autoimmune encephalitis (EAE) model, a murine model of MS that has been shown to depend on IL-17 signaling (142,287–289). Specifically, results from two studies using caveolin-1$^{-/-}$ mice in the EAE model are consistent with my hypothesis that caveolin-1 promotes IL-17R signaling in stromal cells. Wu et al showed that caveolin-1$^{-/-}$ mice developed less disease than wild-type mice (290). They explained that their results were a consequence of a decreased expression of adhesion proteins (ICAM and VCAM) in endothelial cells of caveolin-1$^{-/-}$ mice, which increased the selectivity of the blood-brain barrier and decreased trafficking of T cells into the central nervous system (CNS) (291). In a different study, Lutz et al. confirmed that caveolin-1$^{-/-}$ mice develop less MS-like disease severity by demonstrating that caveolin-1$^{-/-}$ endothelium had a significant reduction in transcellular and paracellular migration of T_H1 cells, but not of T_H17 cells. Accordingly, they found similar numbers of T_H17 cells in the CNS of wild-type and caveolin-1$^{-/-}$ mice used in the EAE model. Lastly, they attributed the decrease in EAE severity in caveolin-1$^{-/-}$ mice to a decrease in T_H1 cells in the CNS (292). These two studies confirm a role of caveolin-1 in the development of EAE. Together, these findings raise the question of why there is a decreased disease severity if IL-17-producing cells do get into the CNS? Putting their findings in the context of my results, it is tempting to speculate that the lack of caveolin-1 in glial cells – which are responsible for the development of IL-17-dependent EAE (142) — makes them less responsive to IL-17R signaling. To test my hypothesis that caveolin-1 activates IL-17R signaling *in vivo*, mice with a deletion of caveolin-1 in glial cells could be used in the EAE model. Another model to test for an IL-17-

dependent disease with the caveolin-1$^{-/-}$ mice is the murine imiquimod-induced psoriasis-like disease model (284,293). This model of psoriasis-like disease has not been tested in caveolin-1$^{-/-}$ mice, nor has it been used to study the specific role of caveolin-1 in the non-hematopoietic compartment. Thus, I recommend performing the imiquimod murine model with bone marrow chimeras.

To better understand the cell types involved in using caveolin-1 as a signaling intermediate, murine studies incorporating a bone marrow chimera approach in the imiquimod-induced psoriasis-like disease model could be used. If the hypothesis that caveolin-1 activates IL-17R signaling is correct, caveolin-1$^{-/-}$ mice would develop less imiquimod-induced psoriasis-like disease, perhaps comparable to a control group of ACT1$^{-/-}$ mice that are less susceptible to developing disease (121). Next, to corroborate that the role of caveolin-1 is in the non-hematopoietic compartment, a bone marrow chimera approach could be added. Based on my hypothesis, I predict that the recipient caveolin-1$^{-/-}$ mice would be less susceptible to developing a psoriasis-like disease. Results from such *in vivo* experiments would bolster our understanding of the regulation of IL-17R signaling mediated by caveolin-1, and further demonstrate its potential clinical utility as a target.

6.2.2 <u>Pharmacological approaches to study ABIN-1 and caveolin-1 in IL-17R signaling.</u>

Another compelling area for future investigation at the molecular level is the study of IL-17-dependent ABIN-1 degradation using specific proteasome inhibitors. In my studies, I used MG132, a non-specific inhibitor of proteasomal degradation, which partially inhibited ABIN-1 degradation. Perhaps using a more specific inhibitor could be more effective blocking ABIN-1 degradation. Several new inhibitors of the proteasome are in clinical trials as therapy for

neoplasia (235), and these could be tested for their effectiveness in inhibiting IL-17R signaling. This *in vitro* study could lead to the identification of effective drugs to block degradation of ABIN-1. The inhibitor IκBα is also degraded via the proteasome; thus, blocking IκBα and ABIN-1 proteasomal degradation could enhance inhibition of IL-17R signaling. These experiments, using a pharmacological approach *in vitro*, could set the groundwork to test these specific proteasome inhibitors for IL-17-mediated autoimmune diseases.

To study the role of oligomerization of proteins to caveolin-1 in IL-17R signaling, I propose to use the commercially available peptide that specifically inhibits protein binding to caveolin-1, known as the WL 47-dimer (294). WL 47 is a small, high-affinity, selective disrupter of caveolin-1 oligomerization. It was identified in a screen using a phage-displayed library for selection of novel binding peptides targeting residues from the caveolin-1 oligomerization domain. From this screen, WL 47 had the highest affinity for caveolin-1. Thus, in principle, this peptide works by binding to the site of the caveolin-1 oligomerization domain, displacing other proteins. This peptide could be used in IL-17-responding cells to determine if it interrupts IL-17R signaling. I speculate that disrupting the IL-17RA and caveolin-1 interaction may decrease IL-17-dependent production of IL-6. Furthermore, if this peptide safely inhibits IL-17R signaling, it could prove to be a promising new molecule to investigate IL-17R signaling.

6.3   CONCLUDING REMARKS

This thesis contributes to our understanding of the mechanisms of activation and inhibition of IL-17R signaling. I demonstrated that ABIN-1, an ubiquitin binding protein, inhibits IL-17R signaling in a ubiquitin-binding-dependent manner (chapter 4). Interestingly, IL-17R signaling triggered the degradation of ABIN-1, which I hypothesize is a necessary step to allow signaling to proceed. I also demonstrate that caveolin-1, a scaffolding protein best known for binding most G-protein-coupled receptors, binds to the IL-17R and positively activates signal transduction. I showed evidence supporting a hypothesis that caveolin-1 functions both as an IL-17R chaperone that facilitates the surface localization of the receptor and as a signaling adaptor protein that functions downstream of IL-17R, albeit by an unknown mechanism. Future work validating the function of ABIN-1 and caveolin-1 in the non-hematopoietic compartment *in vivo* is still needed. These findings add a baseline inhibitor, ABIN-1, and an activator, caveolin-1, to the set of molecules that could be targets for therapies to control IL-17-mediated inflammation.

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179

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