FEEDBACK INHIBITION OF IL-17 RECEPTOR SIGNAL TRANSDUCTION

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IL-17 is a proinflammatory cytokine that mediates host defense against extracellular pathogens but also contributes to the pathogenesis of various autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis and psoriasis. Despite some homology with the toll-like receptors, IL-17 receptor subunits (IL-17RA and IL-17RC) do not recruit adaptors such as MyD88, but instead associate with and signal through the E3 ubiquitin ligase Act1. Upon recruitment, Act1 ubiquitinates tumor necrosis factor receptor associated factor 6 (TRAF6), resulting in the activation of downstream transcription factors, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), CCAAT-enhancer binding proteins (C/EBPs) and mitogen-activated protein kinases (MAPKs). Despite advances in our understanding IL-17 signaling, the details of molecular events involved in negative regulation of IL-17 signaling still remain unclear. Therefore, we focused our attention on feedback inhibitory mechanisms mediated by proteins that are regulated by IL-17. Here we describe three novel inhibitors of IL-17 signal transduction, A20, anaphase promoting complex subunit 5 (AnapC5) and monocyte chemotactic protein 1 induced protein 1 (MCPIP1). We uncovered non-overlapping function of these proteins in IL-17 pathway, signifying their importance in inhibiting inflammatory signaling. A20 is a deubiquitinating enzyme that restricts IL-17-induced TRAF6 ubiquitination, whereas AnapC5 associates with A20 and appears to function as an adaptor protein in the A20/IL-17 pathway. MCPIP1, on the other hand, is an RNase that degrades *Il17ra* and *Il17rc* mRNA, thereby restraining IL-17 signaling. Notably, gene expression of A20 and MCPIP1 is regulated by IL-17,

indicating feedback inhibition of IL-17 signaling pathway. In summary, our findings have advanced the understanding of IL-17 signaling inhibition, may provide a novel mechanistic approach for controlling IL-17-induced inflammation and ultimately treating IL-17 mediated autoimmune diseases.

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LIST OF ABBREVIATIONS

Act1	NF-κB Activator 1
AnapC5	Anaphase Promoting Complex subunit 5
BALF	Bronchoalveolar Lavage Fluid
CBAD	C/EBPβ Activation Domain
C/EBP	CAAT-enhancer-binding protein (C/EBP)
CFU	Colony Forming Unit
DUB	Deubiquitinase
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme Linked Immunosorbent Assay
FLS	Fibroblast like synoviocyte
IKK	Inhibitor of κB Kinase
IL	Interleukin
IL-17	Interleukin 17A
IL-17R	IL-17 Receptor
IL-17RA	IL-17 receptor subunit A
IL-17RC	IL-17 receptor subunit C
JNK	c-Jun N-Terminal Kinase
LPS	Lipopolysaccharide

MAPK	Mitogen Activated Protein Kinase
MCPIP1	Monocyte Chemotactic Protein-1 Induced Protein 1
miR	Micro RNA
MyD88	Myeloid Differentiation Primary Response 88
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
RA	Rheumatoid Arthritis
qPCR	Real-time Reverse Transcription Quantitative Polymerase Chain Reaction
SEFIR	Similar Expression to the Fibroblast Growth Factor Receptor (SEF)/IL-17R
siRNA	Small Interfering RNA
STAT	Signal Transducer and Activator of Transcription
T_H	T helper cell
TLR	Toll-like Receptor
TNFα	Tumor Necrosis Factor-alpha
TNFAIP3	TNFα Induced Protein 3
TNFR	TNFα Receptor
TRAF	TNF Receptor Associated Factor
USP	Ubiquitin Specific Peptidases
WT	Wild type
WCL	Whole Cell Lysate
Zc3h12a	Zinc Finger CCCH-type containing 12A

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1.0 CHAPTER ONE: INTRODUCTION

1.1 INFLAMMATION AND CYTOKINES

Over five decades ago, the discovery of soluble factors (Interferons, IFN) that confer protection against viral infections laid the foundation of cytokine research [1, 2]. Since then, numerous cytokines have been identified that are central players in a variety of immune functions, ranging from development of immune cells to counteracting infections (reviewed in [3, 4]).

One domain where the role of cytokines has been appreciated for a long time is the regulation of inflammation. Cytokines such as tumor necrosis factor-α (TNFα), granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1) and IL-6 have been recognized to affect the local tissue environment and recruitment of inflammatory cells [5-8]. Through these functions, cytokines contribute to host defense by helping the immune system control and eliminate pathogens. Conversely, chronic inflammation by these cytokines leads to several autoimmune conditions and can be detrimental to the host. Indeed, the neutralization of inflammatory cytokines by antibodies is one of the most effective treatments for autoimmunity (reviewed in [9]). This is clearly evident by the success of TNFα neutralizing antibodies in the treatment of rheumatoid arthritis [10]. However, lack of these cytokines can lead to an enhanced susceptibility to pathogens. For example, pateints undergoing anti-TNFα therapy are more susceptible to mycobacterial infections such as reactivated tuberculosis [11]. Moreover,

cytokine neutralization therapies are not successful in all patients. Therefore, it is essential to identify and study additional cytokine targets that can complement existing therapies. Additonally, it may be possible to minimize the side effects of cytokine neutralization by identifying specific mechanisms of targeting cytokine activity without losing the beneficial functions.

In this regard, the Gaffen laboratory focuses on the novel IL-17 cytokine family. IL-17A (commonly referred to as IL-17) is the signature cytokine of this family and has been associated with the development of several autoimmune diseases. Compared to previously identified cytokine families, the IL-17 family is unique in several aspects that will be explained in the following sections of this introduction, as well as in subsequent chapters in this thesis.

1.2 IL-17 FAMILY AND TH17 CELLS

Interleukin 17 was cloned from a subtractive rodent cDNA library and initially named CTLA8 [12]. Initial homology studies predicted that IL-17 lacked significant resemblance to existing cytokines, but shared approximately 57% homology to the ORF13 of *Herpesvirus saimiri* (HVS). Other IL-17 family members were identified on the basis of homology to IL-17A and the IL-17 family is currently composed of six cytokines (IL-17A through IL-17F) [13, 14]. Among the IL-17 family members, IL-17F was the first cytokine that was crystalized [15]. Although distinct in primary sequence, IL-17F was found to be structurally similar to cysteine knot cytokines such as platelet-derived growth factor (PDGF) and nerve growth factor (NGF). Furthermore, crystal structure and computer models confirm that IL-17 adopts a similar three-dimensional conformation [16].

Another group cloned the IL-17 receptor (IL-17R, now known as IL-17RA) by screening an EL4 cDNA expression library with an HVS13.Fc fusion protein [17]. Similar to its ligand IL-17, IL-17RA did not share detectable homology with other cytokine receptors. Likewise, four other members of the receptor family (IL-17RB to IL-17RE) were discovered on the basis of homology to IL-17RA [18]. While the lack of apparent homology of IL-17 and IL-17 receptor to other existing cytokine families made it challenging to predict the function of IL-17, this lack of homology simultaneously raised the prospect of a novel class of proteins that could help explain poorly understood immune activities.

Initial reports suggested that $CD4^+$ T cells are the primary source of IL-17 [13, 19]. However, it took nearly a decade after the cloning of IL-17 to determine the nature of IL-17 producing cells. In 2005, reports from several groups defined a distinct subset of T helper (T_H) cells that produce IL-17 as their signature cytokine [20-22]. Based on IL-17 production by these cells, the term 'T_H17' was coined.

Several seminal reports in quick succession established the phenotype and function of T_H17 cells. In addition to IL-17, T_H17 cells produce the related family member IL-17F, as well as other cytokines such as IL-21 and IL-22. T_H17 cells require a combination of cytokines to develop and mature, including IL-6, transformation growth factor (TGF)-beta (TGF-β), IL-1β and IL-23. IL-23 shares a common subunit with IL-12, which in IL-23 pairs with a distinct subunit, p19 (reviewed in detail elsewhere [23]). The transcription factors important for the development of T_H17 cells also differ from T_H1 and T_H2 cells. Specifically, T_H17 cells require the activation of RAR-related orphan receptor gamma **(**RORγt) and signal transducer and activator of transcription 3 **(**STAT3) for maturation, as opposed to T-box transcription factor TBX21 (Tbet) for T_H1 and GATA-binding factor 3 (GATA3) for T_H2 cells [24, 25].

Initial studies suggested that T_H17 cells drive autoimmune inflammation. Multiple autoimmune mouse models were used to prove that T_H17 cells (not T_H1 cells) are the primary drivers of autoimmunity through their production of IL-17. These models include experimental autoimmune encephalomyelitis [(EAE), mouse model of multiple sclerosis] [20] and collagen induced arthritis [(CIA), mouse model of arthritis] [26]. Subsequently, the role of IL-17 has been described in several autoimmune conditions, indicating that T_H17 cells are critical drivers of autoimmunity [27]. T_H17 cells and IL-17-producing cells are not only associated with autoimmune conditions but also help control infections by extracellular bacteria and fungi. For example, deficiency in IL-17 production or its function has been shown to increase susceptibility to *Klebsiella pneumoniae* and *Candida albicans* infections [28, 29].

While much of the early focus was on IL-17 production from T_H17 cells, there is a significant body of evidence to suggest that there are other lymphocyte populations that serve as "innate" sources of IL-17. These cells include γδT cells, LTi-like cells, natural T_H17 and innate lymphoid cells (ILC, especially the ILC3 subset) [30]. These cells appear to function primarily in a defensive capacity against pathogens at mucosal surfaces, providing an early source of IL-17 to recruit neutrophils to sites of infection. In addition, these innate IL-17 producers have been shown to participate in the initiation of certain autoimmune diseases. Further characterization of these cell types will shed more light on their respective contribution in the production of IL-17 and disease pathology.

The genes induced by IL-17 help explain its pro-inflammatory activities (reviewed in [31]). Chemokines regulated by IL-17 such as chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2 and CXCL5 promote neutrophil chemotaxis. IL-6 and granulocyte-colony stimulating factor (G-CSF) regulate the survival, proliferation and recruitment of myeloid lineages,

especially neutrophils. IL-17 also induces expression of CCL20, which promotes trafficking of mucosal-associated cells bearing the chemokine receptor CCR6; notably, CCR6 is a characteristic receptor on IL-17-expressing cells such as T_H17 cells and ILC3s [32]. Additionally, IL-17 is a strong inducer of antimicrobial peptides (AMPs), particularly β-defensins, which play vital roles at mucosal surfaces and skin in preventing infection, and which may also exert chemotactic activity. Lipocalin-2 is another antimicrobial protein induced by IL-17 that controls bacterial growth by restricting access to dietary iron. The majority of the inflammatory genes mentioned here are not exclusively IL-17 targets, but are also induced by other proinflammatory stimuli such as TNFα, lipopolysaccharide (LPS) and IL-1β. In fact, IL-17 synergizes with these proinflammatory proteins to further enhance its target gene expression [18, 31, 33]. Furthermore, the similarities of IL-17 induced gene expression to LPS/IL-1β have led to valuable insights into the *in vivo* role of IL-17 as well as signaling components of the IL-17 pathway.

Other IL-17 family members studied to date include IL-17E (IL-25) and IL-17C. Compared to IL-17, these cytokines have distinct activities and use other IL-17 receptor family members for signaling. IL-17E and IL-17C signal through IL-17RB and IL-17RE, respectively [34]. Intriguingly, both IL-17E and IL-17C use IL-17RA in addition to IL-17RB and IL-17RE, indicating that IL-17RA may be a shared receptor subunit for the IL-17 family cytokines. Therefore, at least some mechanisms of regulation dependent on IL-17RA are presumably applicable to IL-17E and IL-17C. However, a detailed discussion of IL-17C and IL-17E biology is outside the scope of this thesis [35-37].

1.3 IL-17: PARTNER IN HEALTH AND DISEASE

IL-17 is the hallmark cytokine of T_H17 cells and contributes to the inflammatory impact of these cells. As mentioned earlier, IL-17 exerts protective effects for the host in the context of host defense against pathogens whereas IL-17-induced autoimmune inflammation is detrimental for the host. The role of IL-17 in regulating different pathogens and autoimmune conditions will be described in this section.

1.3.1 IL-17 and Autoimmunity

Inflammation induced by IL-17 has been associated with several autoimmune conditions, including rheumatoid arthritis (RA), psoriasis, multiple sclerosis (MS), systemic lupus erythematosus (SLE), glomerulonephritis, ankylosing spondylitis, Crohn's disease and multiple lung disorders (reviewed in [38]). The role of IL-17 and IL-17R in the development of these autoimmune diseases has been confirmed in mouse models of some of these diseases [20, 22, 39-41]. Moreover, elevated levels of IL-17 have been detected in the serum of RA and psoriasis patients [42, 43]. In these autoimmune diseases, IL-17 targets tissue specific cells and activates inflammatory gene expression that ultimately drives the autoimmune phenotype. For example, in RA, IL-17 acts on synoviocytes by binding to its receptor, resulting in chronic inflammation of synovial tissues in multiple joints that has been associated with bone and cartilage damage [39, 44, 45]. Similarly, in EAE, IL-17 activates astrocytes and microglial cells to produce inflammatory cytokines and chemokines [46], aiding the recruitment of additional inflammatory innate and adaptive immune cells.

Finally, genome-wide association studies (GWAS) reported the IL-23 gene as a susceptibility locus in inflammatory bowel disease (IBD) [47]. As stated earlier, IL-23 drives the development and maintenance of T_H17 cells, further emphasizing the role of T_H17 cells in driving chronic autoimmune conditions. Unexpectedly, IL-17 and IL-17RA deficiency in T cells further exacerbated this disease, indicating a protective role for IL-17 in IBD [48, 49]. Interestingly, IL-17F was found to be pathogenic in this model and was shown to be the primary inducer of proinflammatory gene expression [50]. Cumulatively, these data indicate that IL-17 is a critical driver of autoimmunity.

1.3.2 Pathogen Control by IL-17

Although IL-17 is notorious for its role in inducing autoimmunity, IL-17 did not evolve to cause autoimmunity, but rather to promote effective host defense against pathogenic insult. Thus, IL-17- and IL-17R-knockout mouse models demonstrate increased susceptibility to a wide variety of infectious organisms, including gram-negative and gram-positive bacteria, parasites, fungi, and even some intracellular bacteria and viruses.

The importance of the IL-17/T_H17 axis in control of various pathogens is clearly evident in humans with mutations in genes along this axis. For example, patients with Job's Syndrome/Hyper-IgE Syndrome (HIES) have autosomal dominant-negative mutations in STAT3 that are associated with dramatically reduced T_H17 cell frequencies. These patients are susceptible to chronic mucocutaneous candidiasis (CMC, caused by the commensal fungus *C. albicans*), *Staphylococcus aureus* and *Streptococcus pneumonia* infections [51]. Similarly, mutations in STAT1 and caspase recruitment domain-containing protein 9 (CARD9) are also

associated with CMC and reduced T_H17 cell numbers [52, 53]. Therefore, T_H17/IL-17 deficiencies are strongly linked to increased susceptibility to fungal and bacterial infections.

1.3.3 IL-17 Neutralization for Autoimmune Treatment

The association of IL-17 and T_H17 cells with a number of chronic inflammatory diseases makes this pathway a promising target of monoclonal antibody based therapy. Indeed, antibodies targeting IL-17 or IL-17R are currently being tested in several clinical trials. For example, A Phase-II clinical trial with an antibody targeting IL-17 (Secukinumab/AIN457, Novartis) exhibited a significant reduction in psoriatic lesions as compared to the placebo-treated group. This antibody also displayed moderate success in treating RA and uveitis [54]. The potential success of IL-17 neutralizing antibodies presents an exciting opportunity in the treatment of autoimmune conditions where TNFα blockers and other therapies have had limited efficacy. Also, as IL-17 synergizes with TNFα and other inflammatory cytokines, IL-17 targeting antibodies could potentially be used in combination with other cytokine therapies. Detailed discussion of these trials is beyond the focus of this introduction and has been reviewed in detail elsewhere [55].

A side effect of successful anti-cytokine therapies in the past has been an increase in susceptibility to infections. Whether targeting IL-17 also results in a similar outcome is yet to be determined, but early data indicates increased incidence of oral candidiasis in patients being treated with Secukinumab (unpublished data, presented by Dhavalkumar D. Patel**,** Novartis Institutes for BioMedical Research, Switzerland, at the Keystone Conference, Emerging Cytokine Networks, January 2014). Therefore, it is important to identify drug targets that reduce IL-17-induced inflammation sufficiently to treat autoimmunity but that do not compromise its

host defense activity. Clues about such proteins are abundant in nature where inflammation is kept in check by inhibitors of signal transduction such as phosphatases, ubiquitin ligases and deubiquitinases. Despite our increasing understanding of the physiological role of IL-17 and regulation of IL-17 production, the mechanisms of regulation of IL-17 triggered signaling cascades remain elusive. Therefore, the objective of this thesis is to identify and understand the mechanisms by which IL-17 signaling is kept in check.

1.4 OVERVIEW OF IL-17 SIGNALING

Initial attempts to understand IL-17 biology largely focused on the regulation of IL-17 production and identification of the cells making IL-17 (T_H17 and innate sources). However, compared to other inflammatory cytokines, very little is known about IL-17 signal transduction and regulation of this pathway. This section outlines our current understanding of the IL-17 signaling pathway.

1.4.1 IL-17 Signaling Activation

IL-17 functions either as a homodimer or as a heterodimer with IL-17F [55]. Based on its induction of CXCL1, the IL-17A homodimer was found to be the most potent inducer of its target genes, followed by the IL-17A/F heterodimer, and finally the IL-17F homodimer [56].

The effects of IL-17 (IL-17A, IL-17F and IL-17A/F) are mediated through a receptor complex composed of two IL-17 receptor family members, IL-17RA and IL-17RC [55]. Among IL-17R family members, IL-17RA has the longest cytoplasmic region (521 amino acids, murine

IL-17RA), suggesting that IL-17RA is likely to be important in recruiting receptor proximal adaptor proteins. On the other hand, IL-17RC has a shorter cytoplasmic domain (214 amino acids, murine IL-17RC). Generation of IL-17RA and IL-17RC knockout mice and subsequent analysis of IL-17 signaling in these mice confirmed the importance of both subunits in the activation of IL-17 signaling. Furthermore, structure-function analysis of IL-17RA and IL-17RC demonstrated that the cytoplasmic domains of both receptor subunits are necessary for IL-17 signaling [33, 57-59].

The expression patterns of IL-17RA and IL-17RC are very different and determine the cell types that respond to IL-17. IL-17RA is expressed constitutively by most cell types including hematopoietic and non-hematopoietic cells. Conversely, IL-17RC is mainly expressed on non-hematopoietic cells. As IL-17RA and IL-17RC are both essential for IL-17 signal transduction, IL-17 is thought to mainly act on non-hematopoietic cells such as fibroblasts, synoviocytes, keratinocytes, epithelial and endothelial cells (reviewed in [34, 60]). These cells are also targets for other inflammatory cytokines like TNFα and contribute to tissue specific inflammation and autoimmunity. Thus, non-immune cells are pertinent tools for studying IL-17 signaling pathways.

IL-17R family members have considerable homology between their cytoplasmic domains but not to those of other receptor families, suggesting that the receptor proximal signaling events are unique to this family. The first insight into this pathway came from a landmark bioinformatics study that identified a conserved signaling motif among the IL-17 receptor family. The domain was related to the toll-IL-1 receptor (TIR) domain of the toll-like receptor (TLR) and IL-1 receptor (IL-1R) family and was named SEFIR (after similar expression to the fibroblast growth factor receptor (SEF)/IL-17R) [61]. The same report

identified Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) activator 1 (Act1), the only known protein outside the IL-17R family that has a SEFIR domain. At the time, Act1 (also known as CIKS (Connection to inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase (IKK) and stress-activated protein kinase (SAPK)/ c-Jun N-Terminal Kinase (JNK)), had only been described as an activator of NF-κB and a negative regulator of cluster of differentiation 40 (CD40) and B cell activating factor belonging to the TNF family (BAFF) signaling in B cells [62-64]. The presence of a SEFIR domain in Act1 raised the interesting possibility that Act1 is an adaptor protein that regulates IL-17 signaling, similar to the function of myeloid differentiation primary response 88 (MyD88) in TLR/IL-1R signaling. Indeed, subsequent studies using RNA interference and $Act1^{-/-}$ mice confirmed that Act1 is essential for signal transduction by IL-17 as well as for other IL-17 family members [65-67]. Engagement of Act1 by the IL-17R (through a homotypic SEFIR-SEFIR interaction) leads to the activation of downstream signaling cascades.

The primary function of Act1 is as an adaptor protein, linking IL-17 receptor to other activators of the pathway such as the TNF receptor associated factor (TRAF) family protein, TRAF6 [66, 68, 69]. Additionally, Act1 has enzymatic activity as an E3 ubiquitin ligase, and in that capacity mediates Lysine 63 (Lys-63 or K63) ubiquitination of TRAF6 [70] [Lys-63/K63 ubiquitination described in **Section 1.4.2**]. Recruitment and ubiquitination of TRAF6 leads to the activation of TGF-β-activated kinase-1 (TAK1) and TAK1 binding proteins 2/3 (TAB2/3). TAK1 further activates the IKK complex, leading to activation of the canonical NF-κB pathway [18]. The Act1/TRAF6 axis is also important for the phosphorylation and activation of mitogen activated protein kinase (MAPK) pathways. Finally, Act1 appears to be essential for the overwhelming majority of IL-17-dependent signaling events. Therefore, it is not surprising that

the phenotypes of Act1$^{-/-}$ mice are similar to those of IL-17RA$^{-/-}$ mice. For example, like IL-17RA$^{-/-}$ mice, Act1$^{-/-}$ mice are susceptible to *C. albicans* infection and have reduced severity of EAE as compared to wild-type (WT) mice [18, 46].

Heat shock protein 90 (Hsp90) also contributes to IL-17 signaling by regulating the function and stability of Act1. Lack of Hsp90 function results in proteasomal degradation of Act1, resulting in lack of IL-17 signaling. The D10N variant of Act1 (Act1-D10N) is defective in its interaction with Hsp90, which results in a global loss of Act1 function. Moreover, the Act1-D10N variant is linked to susceptibility to psoriasis, pointing to the physiological importance of Hsp90-Act1 association [71].

Another family of transcription factors, the CCAAT-enhancer-binding proteins (C/EBPs), is also involved in the regulation of IL-17 signal transduction. Two members of this family, C/EBPδ and C/EBPβ, are regulated by IL-17, albeit in different ways. C/EBPδ mRNA expression is induced by IL-17, whereas C/EBPβ is post-translationally modified (particularly by phosphorylation) as well as alternatively translated. C/EBP transcription factors cooperate with NF-κB to regulate IL-17-induced gene expression. Two examples of such genes are *Il6* and *lcn2* (lipocalin 2, also known as 24p3), which have both C/EBP and NF-κB sites in their promoters. Deletion of either site renders these promoters unresponsive to IL-17-mediated signals [72, 73].

As compared to TNFα and IL-1β, IL-17 and IL-17F are only modest activators of signaling on their own. However, IL-17 synergizes or cooperates with a variety of inflammatory effectors, particularly TNFα but also IFNγ, IL-22, lymphotoxin-beta (LT-β), IL-1β and LPS. This proves to be an important tool to study IL-17 signaling whenever IL-17 induced gene expression is too low or undetectable. More importantly, this is particularly relevant in

infections and autoimmune disorders, where IL-17 is induced in conjunction with other inflammatory proteins. The molecular basis for this synergy probably involves multiple mechanisms but to date remains poorly understood. In synovial tissue, IL-17 upregulates TNF receptor (TNFR) II expression, thereby enhancing responsiveness to TNFα signaling [74]. For some genes, cooperativity occurs at the level of the promoters (e.g., *Il6* and *lcn2*) as both IL-17 and TNFα activate transcription factors that bind to the promoters of these genes. IL-17 upregulates expression of inhibitor of kappa light polypeptide gene enhancer in B-cells, zeta (IκBζ, a positively activating member of the IκB/NF-κB family), which in turn promotes expression of some IL-17 target genes [75].

In addition, IL-17 synergizes with TNFα by regulating mRNA stabilization of genes induced by TNFα. These genes encode chemokines such as *cxcl1*, chemokine (C-C motif) ligand 2 (*ccl2*) and *csf3* (encodes G-CSF) [76-78]. Although Act1 is essential for IL-17-regulated enhanced mRNA stability, TRAF6 is surprisingly dispensable. Regulation of the stability of these mRNAs occurs via inducible Act1 phosphorylation, mediated by IKKi (Ikkε) and TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) [79, 80]. Phosphorylated Act1 binds to adaptors TRAF2 and TRAF5, which further recruit the splicing factor 2 (SF2) (also known as alternative splicing factor (ASF)). Sequestration of SF2 via Act1/TRAF2/TRAF5 results in reduced SF2-mediated mRNA splicing activity and as a result, enhanced mRNA stability of *cxcl1* mRNA [53, 79]. Furthermore, the Act1/TRAF2/TRAF5 axis promotes the recruitment of the mRNA stabilizing protein, HuR, to the *cxcl1* and *cxcl5* mRNA, resulting in enhanced mRNA stability [81]. IL-17 target mRNA regulation and synergy with TNFα is still not completely understood, but this is likely to be an important means by which inflammation is controlled by IL-17.

In conclusion, IL-17R plays an important role in the recruitment of early signaling activating proteins that regulate the downstream adaptor molecules as well as IL-17 target gene expression. Receptor structure-function analyses have demonstrated that, in addition to the IL-17RA and IL-17RC SEFIR domains, extended domains beyond SEFIR are crucial in NF-κB, C/EBPβ and C/EBPδ induction. Moreover, the distal C-terminal domain of IL-17RA (CBAD) is important in C/EBPβ regulation, and thus, IL-17 target gene expression. Although our understanding of the IL-17 targets has increased tremendously, the IL-17R proximal events and their regulation are far from fully characterized.

1.4.2 Ubiquitination and IL-17 Signaling

Among the various regulatory mechanisms for modulating signaling pathways, post-translational modifications of adaptor proteins are among the most important. One such modification is ubiquitination, in which ubiquitin (Ub) molecules are covalently linked to the target protein through specific lysine residues. A cascade of three proteins, E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase) are sequentially activated to carry out the ubiquitination **(Figure 1.2)**. In most cases, the E3 ligase provides substrate specificity by directly binding and linking ubiquitin molecules to the substrate. The outcome for the target protein depends on the lysine residues of ubiquitin used to form these polyubiquitin chains. Of the seven Lysine (K) residues on ubiquitin, K48 and K63 are most commonly used to form polyubiquitin chains on proteins. K48-linked Ub targets substrate proteins to proteasomal degradation, whereas K63-linked Ub is important in mediating non-proteolytic functions such as protein-protein interactions and cell signaling events (reviewed elsewhere in [82-84]). Proteins such as TRAF6, TAB2, TAB3 and TAK1 have ubiquitin binding domains in their

structure that specifically bind to K63-linked ubiquitin chains. K63-linked ubiquitination of adaptor proteins is crucial in activating several inflammatory pathways. For example, ubiquitination of receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP1) and TRAF6 in TNFα and TLR4/IL-1β signaling pathways, respectively, is central to the activation of downstream signaling cascades. Mutations of important residues targeted for ubiquitination on these adaptors render TNFα and TLR4/IL-1β signaling inactive.

IL-17 signal transduction also involves ubiquitination as an important post-translational modification. Most of the known IL-17R proximal proteins are E3 ubiquitin ligases, which further underscores the importance of ubiquitination in IL-17R signaling. In fact, the E3 ligase activity of Act1 was recently reported to mediate K63 ubiquitination of TRAF6 [70], which is a critical event in IL-17R signaling. Upon ubiquitination, TRAF6 recruits TAK1 and activates the canonical NF-κB pathway.

Conversely, removal of ubiquitin chains from adaptor proteins leads to the disruption of protein complexes and subsequent inactivation of inflammatory signaling **(Figure 1.2)**. This process is carried out by enzymes known as 'deubiquitinases' (DUBs). The human genome is predicted to encode over 100 DUBs, but the identity and function of the vast majority of these is still unknown. A20 (TNFα induced protein 3, tnfaip3), CYLD (Cylindromatosis (Turban Tumor Syndrome)), A20 binding inhibitor of NF-κB (ABIN1) and several USP (Ubiquitin Specific Peptidases) proteins are some examples of deubiquitinase proteins that regulate inflammatory signaling pathways. The inhibition of inflammatory signaling by DUBs is essential to keep inflammation in check and to avoid any associated collateral damage. Furthermore, the importance of DUBs is well established by the association of gene loci encoding these deubiquitinases with susceptibility to several autoimmune conditions. For example, the human

A20 gene locus has been linked to susceptibility to Crohn's disease, RA, SLE and psoriasis (reviewed in [85]) and human CYLD is a susceptibility locus in IBD [86-88]. The role of deubiquitination in regulating IL-17 induced inflammation is one of the main mechanisms discussed in this thesis and will be elaborated in detail in subsequent chapters **(Chapters 3-4).**

1.4.3 IL-17 Signaling Inactivation

Given the association of aberrant IL-17 production and signaling with the development of several autoimmune diseases, it is not surprising that there are multiple layers of restriction that control IL-17-mediated inflammatory signaling. A variety of inhibitory mechanisms have been identified (**Figure 1.1**).

As mentioned, C/EBP proteins regulate IL-17-induced gene expression. Interestingly, C/EBPβ is both a positive and negative regulator of the IL-17 pathway. Although C/EBPβ can positively transactivate IL-17 target genes, C/EBP phosphorylation correlates with a decrease in IL-17-induced gene expression. Specifically, C/EBPβ is sequentially phosphorylated on two threonine (Thr) residues located within in the regulatory domain 2 (RD2) by the kinases extracellular signal-regulated kinases (Erk) and glycogen synthase kinase 3 beta (GSK-3β). Whereas ERK is activated via TRAF6 and the SEFIR domain of the IL-17RA, phosphorylation through GSK-3β is dependent on the IL-17RA distal C-terminal domain (hereafter mentioned as CBAD, C/EBPβ activation domain) [89].

TRAF proteins also play both positive and negative roles in regulating the IL-17 signaling pathway. TRAF6 is important for the activation of this signaling pathway. However, TRAF3 is a negative regulator of IL-17 signal transduction. TRAF3 inhibits IL-17 signaling by competing with Act1 for binding to IL-17RA, thereby dampening IL-17-dependent signals [90].

TRAF3 also associates with IL-17RA through a consensus TRAF binding site in the CBAD domain of the receptor. CBAD-mediated recruitment of inhibitory proteins, GSK-3β and TRAF3, suggests a model where the IL-17RA CBAD is involved in the inhibition of IL-17 signaling. Indeed, the deletion of the CBAD was linked to enhanced IL-17 signaling, confirming an inhibitory role of this domain. Receptors often function as docking sites for adaptors that activate inflammatory signaling pathways. Therefore, receptor-mediated recruitment of inhibitory proteins is an intriguing mechanism of limiting inflammation at the source. IL-17RA is the shared receptor for other IL-17 family cytokines, such as IL-17E and IL-17C. Thus, these means of regulation could potentially be applicable to the other IL-17 family cytokine signaling pathways. Similar to TRAF3, TRAF4 directly binds Act1 upon IL-17 stimulation and competes with TRAF6 for association with Act1 [91]. Consistently, TRAF3 and TRAF4 limit IL-17-mediated development of EAE. Taken together, these findings show that TRAF proteins are essential in the regulation of IL-17 signal transduction.

Ubiquitination plays a vital role in a variety of proinflammatory cytokine signaling pathways, including IL-17, and a prolonged activation of these pathways leads to autoimmune disease. Thus, targeting ubiquitination as a mechanism for downregulating these pathways is a plausible strategy. Indeed, deubiquitination as a mechanism of inhibition was shown by a recent report describing a similar function of the DUB, USP25, which tempers IL-17-mediated signal transduction by targeting TRAF proteins. Here, USP25 removes K63-linked ubiquitin chains on both TRAF6 and TRAF5, resulting in inhibition of IL-17 target gene transcription as well IL-17-induced mRNA stability. USP25 deficiency is associated with enhanced IL-17-dependent pulmonary inflammation and increased susceptibility to EAE [92]. The role of another DUB,

A20, and its accessory proteins in inhibiting IL-17 signaling is the focus of subsequent chapters and will be introduced later **(Chapter 3-4).**

In contrast to K63-linked ubiquitination, K48-linked ubiquitination regulates inflammatory signaling by promoting the proteasomal degradation of proteins. For example, K48-linked ubiquitination of Inhibitor of κB alpha (IκBα) drives its degradation and permits nuclear import and activation of NF-κB. IκBα ubiquitination is carried out by the ubiquitin ligase complex, beta-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) [93-95]. β-TrCP also targets phosphorylated forms of Act1, leading to its degradation and loss of signaling [96].

These mechanisms suggest that post-translational modifications are vital for inhibition of IL-17 signaling. However, inhibition at the transcriptional level is also important in the negative regulation of this pathway. MicroRNAs (miR) have been implicated in regulating both T_H17 development and IL-17 signaling. MiR-155, expressed in $CD4^+$ T cells, positively regulates T_H17 differentiation, which may be at least in part due to targeting of SOCS1 by miR155 [97]. Consequently, $miR155^{-/-}$ mice are susceptible to EAE. In terms of signaling, miR-23b keeps IL-17-induced NF-κB activation in check by targeting the mRNA degradation of TAB2, TAB3 and IKKα. Consistently, patients with RA and SLE show reduced expression of miR-23b, which is regulated by IκBζ. Downregulation of miR-23b by IL-17 supports the role of IL-17 in the development of these autoimmune conditions [98].

In addition to regulation by microRNA, adaptor mRNA transcripts as well as target gene mRNA can be targeted for degradation by RNases. These enzymes target the secondary structure of certain transcripts and lead to the degradation of mRNA. The activity and function

of an RNase, MCPIP1, in regulating IL-17 signaling will be introduced and expanded in this thesis **(Chapter 5)**.

1.5 SUMMARY

Our fundamental understanding of IL-17-mediated functions in the immune system is increasing at a fast pace. The importance of IL-17 is evident from the variety of innate and adaptive immune cells that produce this cytokine. It is equally essential to study the mechanisms by which IL-17 target gene expression is activated and inhibited. In this thesis, I report three proteins; (i) A20, (ii) Anaphase Promoting Complex subunit 5 (AnapC5), and (iii) Monocyte chemotactic protein (MCP-1) induced protein 1 (MCPIP1), that all function as negative regulators of IL-17 signaling. Although they have overlapping roles in inhibiting IL-17 signaling, the mechanisms by which they act are completely different. A20 acts by deubiquitinating TRAF6, whereas the AnapC5/7 complex binds A20 and IL-17RA, thus potentially acting as a link between A20 and the receptor. Finally, MCPIP1 degrades *Il17ra* and *Il17rc* mRNA, thereby inhibiting IL-17 signaling. Taken together, findings in this thesis have greatly improved our understanding of IL-17 signaling inhibition as well as identified novel mechanisms of inflammatory signaling inhibition that can be applicable to other inflammatory pathways.

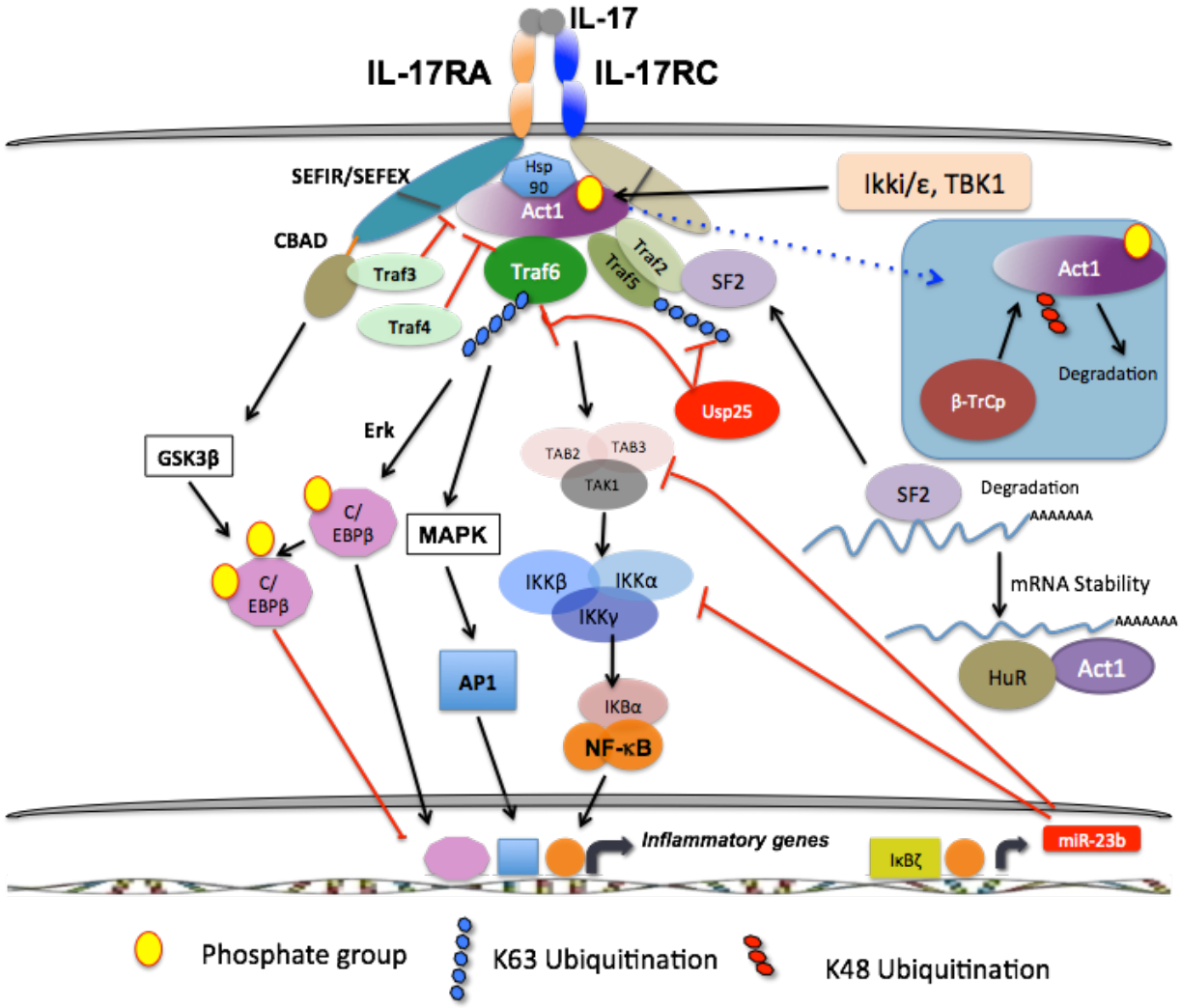

Figure 1.1: Schematic diagram of IL-17 signaling.

Overview of IL-17 signaling pathway. Multiple proteins are involved in the activation and inhibition of IL-17 signaling and have been explained in detail in Chapter 1.

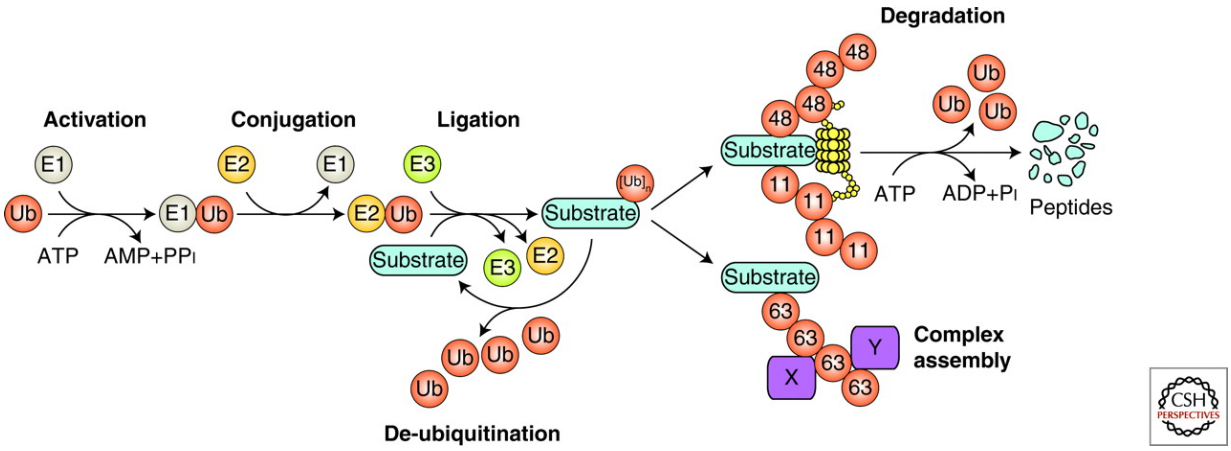

Figure 1.2: Ubiquitination and Deubiquitination.

A cascade of enzymes sequentially act and link ubiquitin chains on the substrate in a process called ‘Ubiquitination’. Removal of these ubiquitin chains is referred to as ‘Deubiquitination’ and is carried out by a different class of enzymes,

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2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Culture and Reagents

All cell lines were cultured in α-MEM (minimum essential medium, Sigma) containing 10% fetal bovine serum (FBS) supplemented with L-glutamine and antibiotics (Invitrogen), unless otherwise stated. Human FLS cells were cultured in α-MEM with 15% FBS, L-glutamine, and antibiotics.

Plasmid transfection in Human Embryonic Kidney **(**HEK) 293T cells were carried out using the calcium phosphate method, whereas primary cells such as, $A20^{-/-}$ mouse embryonic fibroblasts (MEFs) and $MCPIP1^{-/-}$ fibroblasts were transfected with FuGENE 6 (Roche) or FuGENE HD (Promega). siRNA transfections in all cell types were performed using Dharmacon Transfection Reagent 1, purchased from Thermo Scientific (Catalog Number: T-2001). Luciferase assays were performed as described previously [73] with the Dual-Luciferase Reporter Assay System, Promega (Catalog number: E1980). Human subject research was performed in accordance with protocols approved by the Institutional Review Board (IRB) of the University of Pittsburgh.

2.1.2 Plasmids

Plasmids encoding murine IL-17RA, its point mutants, and truncation mutants were constructed as described previously [33, 90, 99]. Plasmids encoding murine A20 and its mutants were obtained from the plasmid repository at BCCM/LMBP (Belgian Coordinated Collections of Micro-organisms/ Laboratory of Molecular Biology–Plasmid collection) (Belgium) [100].

Plasmids encoding human MCPIP1 as well as MCPIP1 mutants were obtained from Dr. Pappachan Kolattukudy at University of Central Florida and have been previously described [101]. Other plasmids mentioned in this study were cloned using primers listed in Table 2.2.

2.1.3 Cytokines

Murine IL-17 used to stimulate cells *in vitro* was obtained from PeproTech (Catalog number: 210-17). Murine TNF-α (315-01A) and Human IL-17 (300-01A) were also purchased from PeproTech. All the above-mentioned cytokines were reconstituted to appropriate concentrations using PBS containing carrier protein (final concentration: 0.1% BSA).

Murine IL-17 used for intranasal challenge in mice was purchased from R&D Systems (421-ML-025/CF) and was reconstituted in PBS without the carrier protein (BSA).

2.1.4 Inhibitors

IKK inhibitor VII was purchased from Calbiochem, EMD Millipore (Catalog number: 401486) and used at a final concentration of 0.1uM.

2.1.5 Mice

MCPIP1$^{-/-}$ mice were acquired from Dr. Pappachan Kolattukudy, University of Central Florida under an approved materials transfer agreement (MTA). These mice were crossed to IL-17RA$^{-/-}$ mice (MTA with Amgen) to generate MCPIP1$^{-/-}$IL-17RA$^{-/-}$. WT mice were ordered from The Jackson Laboratories (Bar Harbor, ME) in the C57BL/6 background. In all experiments, mice

were matched according to gender and age (ranging from 7 to 11 weeks). Animal protocols were approved by the University of Pittsburgh IACUC, and adhered to guidelines in the Guide for the Care and Use of Laboratory Animals of the NIH.

2.1.6 Antibodies

The primary antibodies used for western blot analysis have been listed in Table 2.1 along with the dilutions used for the detecting protein of interest.

Table 2.1: List of Antibodies

Antibody	Company	Catalog #	Dilution
A20	Cell Signaling	5630	1:1000
Act1 (H-300)	Santa Cruz	sc-11444	1:1000
β-TUBULIN	Invitrogen	32-2500	1:1000
ERK- Total	Cell Signaling	9102	1:1000
ERK-Phospho	Cell Signaling	9106	1:1000
FLAG-tag	Sigma	F 3165	1:1000
HA-tag	Sigma	H 3663	1:1000
IkBα (C-21)	Santa Cruz	sc-371	1:1000
IkBα (Phospho)	Cell Signaling	9246	1:1000
IL-17RA	R&D	AF448	1:200
JNK- Total	Cell Signaling	9252	1:1000
JNK- Phospho	Cell Signaling	9251	1:1000
MCPIP1 (P-12)	Santa Cruz	sc-136750	1:400
Myc-tag (9B11)	Cell Signaling	2276	1:1000
MyD88	Santa Cruz	sc-11356	1:1000
p38- Total	Cell Signaling	9211	1:1000
p38- Phospho	Cell Signaling	9212	1:1000
TRAF3 (C-20)	Santa Cruz	sc-949	1:1000
TRAF6	Santa Cruz	sc-7221	1:1000

2.1.7 Cloning Primers

Table 2.2: Cloning Primers

Gene	Description	Primer Sequence (5’ to 3’)
Anapc7	Fwd	GAC AAG CTT CG GTT CCC GGG AGT TCT GTC C CGT
Anapc7	Rev w/d stop codon	GC TCT AGA TCA CTG CAT GCC GAA CCA CTG CT
Anapc7	Rev w/o Stop codon	GC TCT AGA CTG CTG CAT GCC GAA CCA CTG CT
Act1	ΔSEFIR Fwd	TG GGA TCC ATC AAC CGA AGC ATT CCC GTG
Act1	ΔSEFIR Rev	CT GAA TTC TCA TTC TGG CAA ATT GCT GGT
Act1	ΔUbox-set1 Fwd	TG GGA TCC ATC AAC CGA AGC ATT CCC GT
Act1	ΔUbox-set1 Rev	GTA GAG CTG GTC TTC AAA CTG AGG TGG CAA TTC
Act1	ΔUbox-set2 Fwd	CCT CAG TTT GAA GAC CAG CTC TAC CGC CCA
Act1	ΔUbox-set2 Rev	CT GAA TTC TCA CAA GGG TAC CAC CTG AAG
MCPIP1	Fwd with Flag tag	GC AAG CTT ATG GCC GAC TAC AAA GAC GAT GAC GAC AAG GCC AGT GAC CCT TGT GGA
MCPIP1	Rev	AGCA GC GGC CGC TTA CTC ACT GAG GTG C
Anapc5	Fwd	GA ATG ATG ACC AAC GGG GTA GTG
Anapc5	Rev w/o stop	GT TCT AGA TGC GAG GTG GTT AAT CAA GGG
Anapc5	Rev w/d stop	GT TCT AGA CTA GAG GTG GTT AAT CAA GGG

2.1.8 Quantitative Reverse Transcriptase PCR Primers

Primers used in qPCR assays were either designed in-house (Table 2.3) or purchased from Qiagen (Table 2.4).

Table 2.3: Self-designed qPCR Primers

Gene Symbol		Primer Sequence (5' to 3')
Anapc7	Fwd	GCG TCC AGG CTC TGT TAC TC
	Rev	CGT AAC AGT CCA AGC GAC AA
Anapc5	Fwd	GTC CTT TGC CGT CGC TCT CT
	Rev	TCG GTC CTT CAA GTG CTT TA
Zc3h12a (MCPIP1)	Fwd	GTG TCC CTA GAG GCT GAA CC
	Rev	GCA CAG GTC TGC GGT GAG CC
Tnfaip3 (A20)	Fwd	ACC CCA GCT ATC ACT CAT GG
	Rev	TTT GGG AGC TTG CTC TGA TT
Traf3	Fwd	CCC TGG AGA AGA AGG TTT CC
	Rev	CCT TCT GCC TCT CAA TCT CG
Cyld	Fwd	CGC ATG CAG TTC TGT TTG TT
	Rev	ACG TCT ACC TGG AGG CCT TT
Traf3ip2 (Act1)	Fwd	CTT CCA AAC TGC GAT TGA CA
	Rev	GGG GCT GAT TGC TAC GAT TA
Traf6	Fwd	AAA CCA CGA AGA GGT CAT GG
	Rev	TCC CTG TCT TAA GGC TTC CA
Il17rc	Fwd	GGT ACT GTC CCC AGG GGT AT
	Rev	TTC ACA GTG GCG TTC TTC TG
Il17ra (C-terminal)	Fwd	TGC TTA GGT TCC AGG AGT GG
	Rev	CAG CAG TGG GTC TTC AAA CA

Table 2.4: Commercial qPCR Primers

Gene Symbol	Catalog Number
Anapc7 (m)	PPM28440A
Anapc5 (m)	PPM41082B
Ccl20 (m)	PPM03142B
Cd4 (m)	PPM04028F
Cd8 (m)	PPM04031A
Csf2 (m)	PPM02990F
Csf3 (m)	PPM02989B
Cxcl1 (m)	PPM03058C
Cxcl2 (m)	PPM02969E
Cxcl5 (m)	PPM02966F
Gapdh (m)	PPM02946E, QT01658692
Gapdh (h)	PPH00150F
Ifng (m)	PPM03121A
Nfkbz (m)	PPM34845A
Il17a (m)	PPM03023A
Il17ra (m)	QT00112063
Il17ra (h)	QT02451078
Il6 (m)	PPM03015A
Il6 (h)	PPH00560C
Itch (m)	QT01048684
Lcn2 (m)	PPM03770A
Tax1bp1 (m)	PPM60164A
Tnf (m)	PPM03113G
Tnfaip3 (m)	PPM03207A
Traf3 (m)	PPM03088E
Zc3h12a (m)	PPM24532A, QT01166480
Zc3h12a (h)	QT00229838

2.1.9 Small Interfering RNA (siRNA)

SMARTpool ON-TARGETplus siRNA targeting the gene of interest as well as Non-targeting scrambled siRNA (control) were acquired from Dharmacon, Thermo Scientific. The siRNA pool were reconstituted in 1X siRNA buffer (Catalog number: B-002000) at a concentration of 50uM and used at a final concentration of 50nM.

Table 2.5: On Target plus siRNA Sequences

Gene Symbol	Organism	siRNA Sequence (5' to 3')
Anapc7	Mouse	GGA UGG AUG UUU ACG GCU A CGU GUG GAU UAA AGC GUA U CGA UAA GGA GUA CCG CAA U GCA AAC GCU CAG ACG CUU A
Traf3ip2 (Act1)	Mouse	GCU CAG AUA UAG UAG UAG A CCG UGG AGG UUG AUG AAU C CAG AUG UGC CCA CGA UAG A UGC CAG AAG AAU UAC GGA A
Anapc5	Mouse	UCA CAA AAC GAG UGU AGU A CCG CUU UGU UGA AGA AUG A AUG CUG AUA UGG ACC GAG A AGG AAG AGC UCG ACG UGU C
Zc3h12a (Mcpip1)	Mouse	GGA AAC GCU UCA UCG AGG A UGG ACA ACU UCC UUC GUA A CGA GAA GGC UCA CCG CAG A AGU ACU GGU CUG AGC CGU A
Tnfaip3 (A20)	Mouse	AGA GAC AUG CCU CGA ACU A GCU GUG AAG AUA CGA GAG A UGU UAC UGC CUC UGC GAA A GCA CCU AAG CCA ACG AGU A
Traf3	Mouse	GAC AAG UAC AAG UGC GAG A GAA AUG UAA CCC UUC GAU A CCU CAC AAG UGC AGC GUU C CUA GAG AAC GGG ACG UAU A

Tnfaip3 (A20)	Human	CUG CAG UAC UUG CUU CAA A CAA CUC AUC UCA UCA AUG C UCU GGU AGA UGA UUA CUU U CAA CGA AUG CUU UCA GUU C
Itch	Mouse	CAU CUC AGC CAA ACU UAA A CAG CUU GAU UCC AUG GGU A GAA ACG UCA UGC UCC GAG A AUU GGG AAC UGC UGG AUU A
Tax1bp1	Mouse	UGA AAU UCG UGG AGC AAG U GAA CGA UGC UUC AAU AAA U CUA ACU ACG AUC AGA CCA A GAU GAA GGC AAU UCC GAU A
Usp25	Mouse	GCA CGA ACU CUG UGA GCG A UAA UGU ACA UAG ACG ACA A AAA CAA UAA ACC CGA GAA U GAG AAA UGU CCU CUA CUA A
Cdc27	Mouse	CUA UAU GGC AAG CGC UAA A AGG CAA UUG ACA AGC GCU A CGG CGG AAA GUG ACG AAU U CAA AUU ACG CUU ACG CCU A

2.2 METHODS

2.2.1 Mouse Tail Tip Fibroblast generation

Mice were euthanized and about one inch of the distal tail was harvested. The tails were cut into smaller pieces using a sterile razor blade in a 10 cm tissue culture plate containing approximately 500 ul of 2% PenStrep in 1X PBS. The tail pieces were transferred to 15 ml conical tubes and additional PBS with PenStrep added to make the volume 1 ml. Next, an equal amount of collagenase (final concentration= 1000U/ml) was added and the tubes incubated at 37°C for 20-30 minutes. The tubes were mixed every few minutes to ensure effective collagenase treatment. After the incubation, the tubes were spun at 1200 RPM for 5 minutes and washed once with Hank's Balanced Salt Solution (HBSS). Then, 2-3 ml of trypsin was added and tubes were incubated at 37°C for 20 minutes. After this incubation, equal amount of media with FBS was added to the tubes and the tubes were spun at 1200 RPM for 5 minutes. The pellet of tissue and cells was re-suspended in 3-4 ml of complete media (with 20% FBS) and transferred to T25 tissue culture flasks. The tissue pieces were kept in the flask until there was a layer of fibroblasts covering the whole flasks after which the cells were split into two flasks and cultured. To immortalize the cells, the fibroblasts were transfected with 1 ug SV40 T antigen (6-well plate) and cultured for a few passages until the growth advantage was clearly visible. Both primary as well as immortal cells were frozen and stored in liquid nitrogen for later use.

2.2.2 ELISA, Immunoprecipitation and Immunoblotting

Murine and human IL-6 enzyme linked immunosorbent assay (ELISA) kits were from eBioscience, and the CCL20 ELISA kit was from R&D Systems. For each experiment, each sample was analyzed in duplicate or triplicate, and minimum of three replicate samples were included per experiment. Manufacturer's protocols were followed to run these assays.

Cells were lysed using 1% NP-40 lysis buffer containing protease inhibitors, sodium orthovandate and PMSF, unless otherwise stated. Total protein in the lysis buffer was quantified using a BCA assay kit (Thermo Scientific, Catalog number: 23227). In most cases, approximately 30-35 ug of total protein was loaded for analysis. Antibodies used have been listed in Table 2.1. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Catalog number: 34080). For detecting endogenous protein, the substrate was spiked with 20-40 ul of SuperSignal West Femto Reagent (Thermo Scientific, Catalog number: 34094).

Immuno blotting analysis and immunoprecipitations were performed as described previously [57, 99], and bands on blots corresponding to proteins of interest were analyzed by ImageJ software or on a ProteinSimple FluorChem E instrument/software.

2.2.3 In vitro Cytokine Stimulation

IL-17 (murine and human) was used at a final concentration of 200 ng/ml, unless otherwise stated. Final concentration for TNF-α was 2ng/ml when used by itself or in synergy with IL-17. Stimulation times varied between experiements and are mentioned along with individual experiments.

2.2.4 siRNA Transfection

ON-TARGETplus SMARTpool siRNA targeting the gene of interest as well as scrambled controls were obtained from Dharmacon/Thermo Scientific. Lyophilized siRNA were reconstituted in 1X siRNA buffer at a stock concentration of 50 uM. The siRNA stocks were frozen at -20°C in 10 ul aliquots. The stocks were diluted to 5 uM at the time of transfection and used to make the final transfection mixture.

For transfecting siRNA, ST2 cells (approximately 10-15 thousand cells per well) were seeded overnight in antibiotic-free medium (0.5ml per well) in 24-well tissue culture plates. The next day, these cells were transfected with 50 nM siRNA with DharmaFECT Reagent 1 (Dharmacon/Thermo Scientific) according to the manufacturer's protocol. DharmaFECT Reagent 1 is a liposomal transfection reagent and works well with a majority of murine and human cell lines. Twenty-four hours after transfection, the culture medium was changed. The cells were incubated in medium alone for additional 24 hours after which these cells were stimulated with IL-17 for the times indicated with each individual experiment. For each experiment, three replicate samples were analyzed separately. Cells were visually checked periodically to make sure that the transfection of siRNA was not toxic to these cells. To assess the efficiency of knockdown by immunoblotting, the siRNA knockdown protocol was scaled up to a 6 cm or 10 cm (diameter) dish. The cells were lysed in minimal volume of lysis buffer (50 ul) and quantified for total protein. Approximately 30 ug of total protein was loaded for western blot analysis.

2.2.5 mRNA Extraction and qPCR

Total RNA from cells and tissues was isolated with an RNeasy Mini Kit (Qiagen). Cells in the lysis buffer were homogenized by passing them 10 times through a 20-gauge needle, whereas tissues (Kidneys, lungs, spinal cords, intestines) were homogenized by using M tubes and gentleMACS Dissociator (Miltenyi Biotech). The amount of lysis buffer used for tissue lysis was dependent on the amount of tissue, and approximately 25-30 ul of lysis buffer was used per mg of tissue. The homogenates were spun down 3 times to get rid of all the tissue debris before proceeding with addition of 70% ethanol and proceeding with the steps suggested in the supplier protocol. RNA concentration was measured using a nanodrop.

Complementary DNA synthesis was performed with SuperScript III First-Strand (Invitrogen). The extent of expression of genes was determined by quantitative PCR (qPCR) analysis with PerfeCTa SYBR Green FastMix ROX (Quanta BioSciences). The PCRs were performed on a 7300 Real-Time PCR System (Applied Biosystems). The abundances of the mRNAs of interest were normalized to that of Gapdh. Primers for this assay were either self designed or purchased from Qiagen and have been listed in Table 2.3.

2.2.6 *Candida albicans* Culture and Disseminated Candidiasis mouse model

For infection, *C. albicans* (strain CAF2-1) was grown overnight (approximately 16 hours) in YPD broth at 30°C with continuous agitation.

For the disseminated candidiasis infection model, the number of *C. albicans* cells per ml of YPD broth were counted using haemocytometer. On average, overnight growth resulted in 3-4 x 10^8 *C. albicans* cells per ml. *C. albicans* was serially diluted in sterile PBS to obtain a final

concentration of $2x10^6$ *C. albicans* cells per ml. Mice were injected with 100ul of this dilution in their tail veins using a 27 gauge needle.

Assessment of fungal burden in the kidney was done two days post infection. Mice were euthanized and both the kidneys were harvested and sliced longitudinally. One half of a kidney was snap frozen in micro-centrifuge tubes using liquid nitrogen whereas another half from the other kidney was fixed using 10% NBF for histological analysis. The two remaining halves were weighed and added to 1 ml of PBS. Homogenization of these tissues was carried out in C-tubes (Millipore). Several dilutions of these tissue homogenates were plated on plates with YPD agar containing ampicillin. The plating was either carried out by spreading the homogenates on the plates or by spotting 25 ul aliquots of these dilutions. All the samples were plated in triplicates and the calculations were modified based on the amount of homogenate plated. These plates were incubated at 30°C for 48 hours and the *C. albicans* colony forming units (cfu) were counted. The fungal load was reported as total cfu per gram of tissue.

2.2.7 Intranasal IL-17 challenge and Pulmonary Inflammation model

For IL-17-induced pulmonary inflammation, age- and sex-matched mice were treated with IL-17 (300ng per mouse) by intranasal injection or left unchallenged. 24 hours later, 0.5 ml PBS (containing 0.5 mM EDTA) was used to obtain bronchoalveolar lavage fluid (BALF) though the trachea. Furthermore, 4ml PBS was used to acquire BALF from the lungs. Supernatants from the first lavage were used for ELISA and precipitates from all 5ml of BALF were combined and analyzed for lung-infiltrating cells. Cells were counted using haemocytometer and then were stained with anti-CD11b, anti-Gr-1 and anti-F4/80 antibodies, followed by flow cytometry. Left lobe of lung was snap frozen in eppendorfs for qPCR analysis, whereas the rest of the lungs

were homogenized in 1 ml of ice cold PBS containing protease inhibitors (Roche). These homogenates were also analyzed for inflammatory cytokine and chemokine protein levels.

2.2.8 Experimental Autoimmune Encephalomyelitis (EAE) - Mouse Model of Multiple Sclerosis

C57BL/6 mice (both WT and MCPIP1$^{+/-}$) were immunized subcutaneously in four sites on the back with a total of 100 mg myelin oligodendrocyte glycoprotein (MOG) peptide (35–55) in 200 ul incomplete CFA mixed with 100 mg M. tuberculosis strain H37Ra (DIFCO, Michigan, USA). All mice also received 100 ng pertussis toxin (List Biological Laboratories) intraperitoneally on days 0 and 2. Mice were assessed daily for symptoms of paralysis and assigned clinical scores for EAE according to the following scale: 1, flaccid tail; 2, impaired righting reflex and hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, hindlimb paralysis with partial forelimb paralysis; 6, moribund. If an animal had a clinical score or 5 or more for two consecutive days, it was euthanized for humane reasons. At the end of the experiment, mice were euthanized and their spinal cords were harvested and snap frozen using liquid nitrogen for RNA analysis.

2.2.9 Flow Cytometry

For staining surface IL-17RA, one million HEK293T cells were washed and treated with 100 ul Fc block antibodies (1:100). These cells were then washed and stained with a non-commercial primary antibody (1:1800) from Amgen (Clone: m751: 78997-88, Concentration: 4.62 mg/ml)

for approximately 45 minutes on ice. Finally, the cells were counterstained with a secondary antibody (1:100) (anti-Mouse PE or anti-Mouse APC) from Becton Dickinson (BD).

To assess the neutrophil infiltrate in the BALF, the cells were first treated with Fc block. For staining neutrophils and macrophages, the cells were stained with anti-Gr1 (Alexa Fluor 700) and anti-F4/80 (PE) respectively at a dilution of 1:100. The samples were analyzed using LSRII in the Department of Immunology, University of Pittsburgh.

2.2.10 Statistics

To assess statistical significance, we used Student's t test (for pairwise comparisons) or ANOVA with post Tukey's analysis (for more than two comparisons in an experiment). In some cases, Chi Square test was used to compare experimental replicates. $P < 0.05$ was considered statistically significant. Error bars reflect the means ± SEM of biological replicates within individual experiments. All experiments were repeated a minimum of two times to ensure reproducibility.

3.0 CHAPTER THREE: THE DEUBIQUTINASE A20 MEDIATES FEEDBACK INHIBITION OF IL-17 RECEPTOR SIGNAL TRANSDUCTION

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'**The Deubiquitinase A20 Mediates Feedback Inhibition of Interleukin-17 Receptor Signaling'**

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3.1 BACKGROUND

Inflammatory cytokines, such as TNF-α, have long been recognized to promote the pathogenesis of devastating autoimmune diseases such as RA, among many others [102]. It is not an exaggeration to say that biologic therapies targeting TNF-α and other inflammatory cytokines revolutionized the clinical management of many of these diseases. Despite these advances, many patients fail to respond to available anti-cytokine therapies, and some diseases do not respond to anti-TNF drugs. In the last several years, IL-17 emerged as a key player in autoimmune inflammation, and new clinical trial data indicate exciting promise for anti-IL-17 drugs in treating psoriasis and other autoimmune conditions [54, 103-105]. IL-17 is produced by a recently defined subset of $CD4^+$ T cells termed T_H17 cells, and a fast-moving body of literature has described many mechanisms by which T_H17 cells are generated and regulated [23, 106]. In addition, it is increasingly apparent that IL-17 is produced by many innate cell types, which bear marked similarities to classic T_H17 cells and participate in mediating autoimmune inflammation [30].

In contrast to the efforts focused on investigating the immunology of T_H17 cells, there has been far less emphasis on understanding how IL-17 activates downstream signaling pathways. IL-17 is the founding member of a distinct subclass of cytokines and receptors that exhibit unique signaling properties compared to those of the better-defined cytokine receptors, such as the TNFR superfamily or the IL-1 receptor and TLR families [107]. However, IL-17 shares similar signaling endpoints to those of other inflammatory cytokines, particularly in terms of activation of NF-κB and MAPK signaling, as well as induced expression of genes encoding pro-inflammatory cytokines [such as IL-6 and G-CSF], antimicrobial peptides

[including lipocalin 2 (also known as 24p3)], S100A proteins, and β-defensins, and chemokines (including CXCL1, CXCL5, and CCL20) [31]. The net effect of IL-17 signaling is effective host defense against bacterial and especially fungal infections. Indeed, in humans, mutations in the gene encoding IL-17R or in genes whose products control T_H17 development, such as *STAT3* or *STAT1*, cause increased susceptibility to infections by the commensal fungus *Candida albicans* [108, 109]. Conversely, excess IL-17 is associated with numerous autoimmune diseases, and many genes identified as risk loci for autoimmunity in genome-wide association studies (GWAS) (for example, *IL23R* and *STAT3*) are associated with regulation of IL-17 or the T_H17 differentiation pathway [47, 110].

IL-17 mediates signaling through a heterodimeric receptor composed of IL-17RA and IL-17RC [18]. Both subunits contain a signaling motif unique to the IL-17R family known as a SEFIR domain [61]. The SEFIR provides a platform for binding of Act1 (also known as CIKS), a SEFIR-containing adaptor protein and E3 ubiquitin ligase [61, 66, 67, 70, 111]. IL-17 engagement recruits Act1 to the receptor complex. In turn, Act1 recruits and activates TRAF6, ultimately leading to activation of transcription factors, such as NF-κB and C/EBPβ and C/EBPδ, as well as MAPKs [65, 112]. The C-terminal domain of IL-17RA, in contrast, is required for activation of C/EBPβ and is linked to inhibitory signaling through GSK-3β and TRAF3 [33, 89, 90, 99].

Ubiquitination is a posttranslational modification that is essential for modulating pro-inflammatory pathways [113]. Ubiquitination involves covalent linking of ubiquitin (Ub) moieties to target proteins through specific lysine residues, and is regulated by a cascade of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligase) enzymes. Of the seven lysines in ubiquitin, K48 and K63 are most commonly used to form polyubiquitin chains. K48-linked Ub

generally targets proteins for proteasomal degradation, whereas K63-linked Ub triggers non-degradative functions, such as protein-protein interactions and cell signaling events [114]. Notably, many of the currently identified IL-17R–proximal proteins are E3 ubiquitin ligases. For example, the E3 ligase activity of Act1 is required for the K63 ubiquitination of TRAF6, which is also an E3 Ub ligase [70, 115, 116]. Ubiquitination of TRAF6 is a key event in the downstream activation of both NF-κB as well as MAPK pathways [18, 68, 70].

Reversal of ubiquitination is equally important in regulating inflammation, particularly to keep potentially damaging signals in check [117-119]. A20 is a DUB and tumor suppressor encoded by *TNFAIP3* (TNF-α–induced protein 3), and was first identified as an inhibitor of the TNFR signaling pathway [120, 121]. Subsequent studies identified roles for A20 in inhibiting the TLR, IL-1R, and Nod-like receptor (NLR) pathways [117, 122, 123]. In the TNFR pathway, the E3 ubiquitin ligase and adaptor protein TRAF2 and the kinase ribosome inactivating protein (RIP) 1 are targets of A20, whereas in IL-1R and TLR signaling, TRAF6 is a key A20 target **(Figure 3.1)**. Deubiquitination of these adaptors restricts activation of the NF-κB and MAPK pathways [114, 117, 124]. Confirming its essential role in restraining inflammation, A20-deficient ($A20^{-/-}$) mice develop spontaneous multi-organ inflammation and die shortly after birth [124], and mice with cell-type specific knockout of A20 are prone to multiple autoimmune diseases [123, 125-127]. Moreover, polymorphisms in the *TNFAIP3* locus are associated with increased susceptibility to RA, lupus, systemic sclerosis, Crohn's disease and psoriasis [126, 128]. Here, we show that A20 is a feedback inhibitor of the IL-17 signaling pathway. IL-17 increased the expression of *Tnfaip3* mRNA and subsequent production of A20 protein, which inhibited IL-17-mediated activation of TRAF6, NF-κB, and MAPK as well as downstream gene expression. Furthermore, A20 bound to a domain of IL-17RA that is associated with inhibiting

receptor signaling [99]. Thus, these findings expand the known regulatory role of A20 in regulating inflammatory signaling, and lend new insight into how IL-17-driven inflammation is controlled.

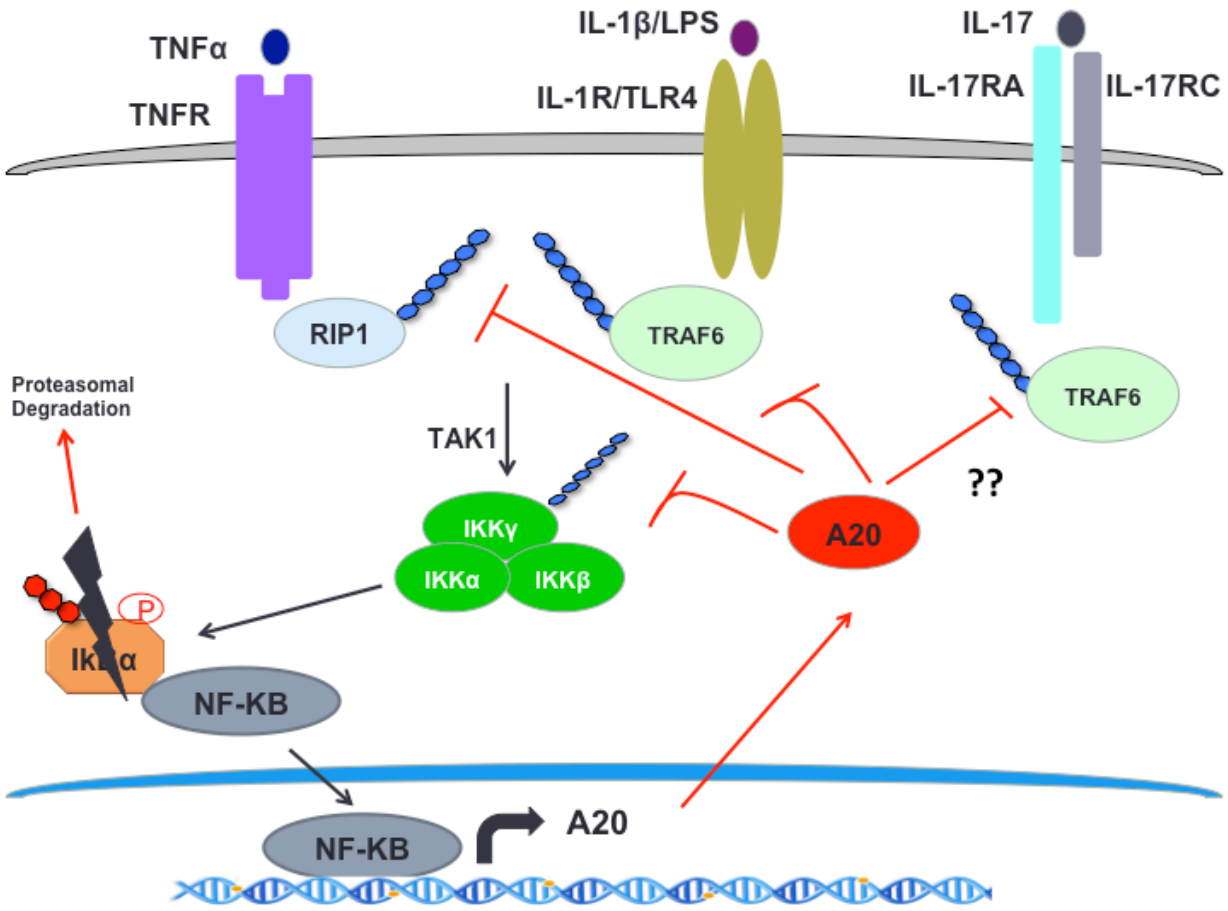


Figure 3.1: A20-mediated inhibition of proinflammatory signaling pathways.

A20 is induced by multiple proinflammatory stimuli and acts as a feedback inhibitor of its inducing signaling pathways.

3.2 RESULTS

3.2.1 IL-17 induces *TNFAIP3* expression and production of A20, which is dependent on NF-κB.

As its name implies, *TNFAIP3* was first identified as a TNF-α–induced gene [129], and its gene product A20 serves as a feedback inhibitor of NF-κB [124]. Based on similarities in the downstream pathways regulated by IL-17 and TNFα, we hypothesized that A20 might also participate in inhibition of the IL-17 signaling pathway. Although produced primarily by T cells, IL-17 exerts its biological effects primarily in the non-hematopoietic compartment, particularly on mesenchymal cell types such as fibroblasts and stromal cells [130]. Accordingly, we stimulated ST2 cells (a murine stromal cell line) with IL-17 over a time course of 24 hours and assessed endogenous *TNFAIP3* mRNA abundance by quantitative real-time reverse transcription polymerase chain reaction (qPCR). IL-17-induced expression of *TNFAIP3* occurred within 15 min of stimulation **(Figure 3.2-A)**. Expression peaked at 30 min after treatment and remained increased at 4- to 5-fold above baseline for at least 24 hours. These kinetics are similar to those reported for TNF-α–induced expression of *TNFAIP3* [124]. We verified that endogenous A20 protein was also induced by IL-17, at slightly delayed kinetics compared to mRNA expression **(Figure 3.2-A)**. A20 transcription was previously reported to be regulated at the promoter level by various transcription factors, including NF-κB [129]. Consistently, an IKK inhibitor blocked IL-17-mediated induction of A20 mRNA in ST2 cell, implicating NF-κB in this pathway **(Figure 3.2-B)**.

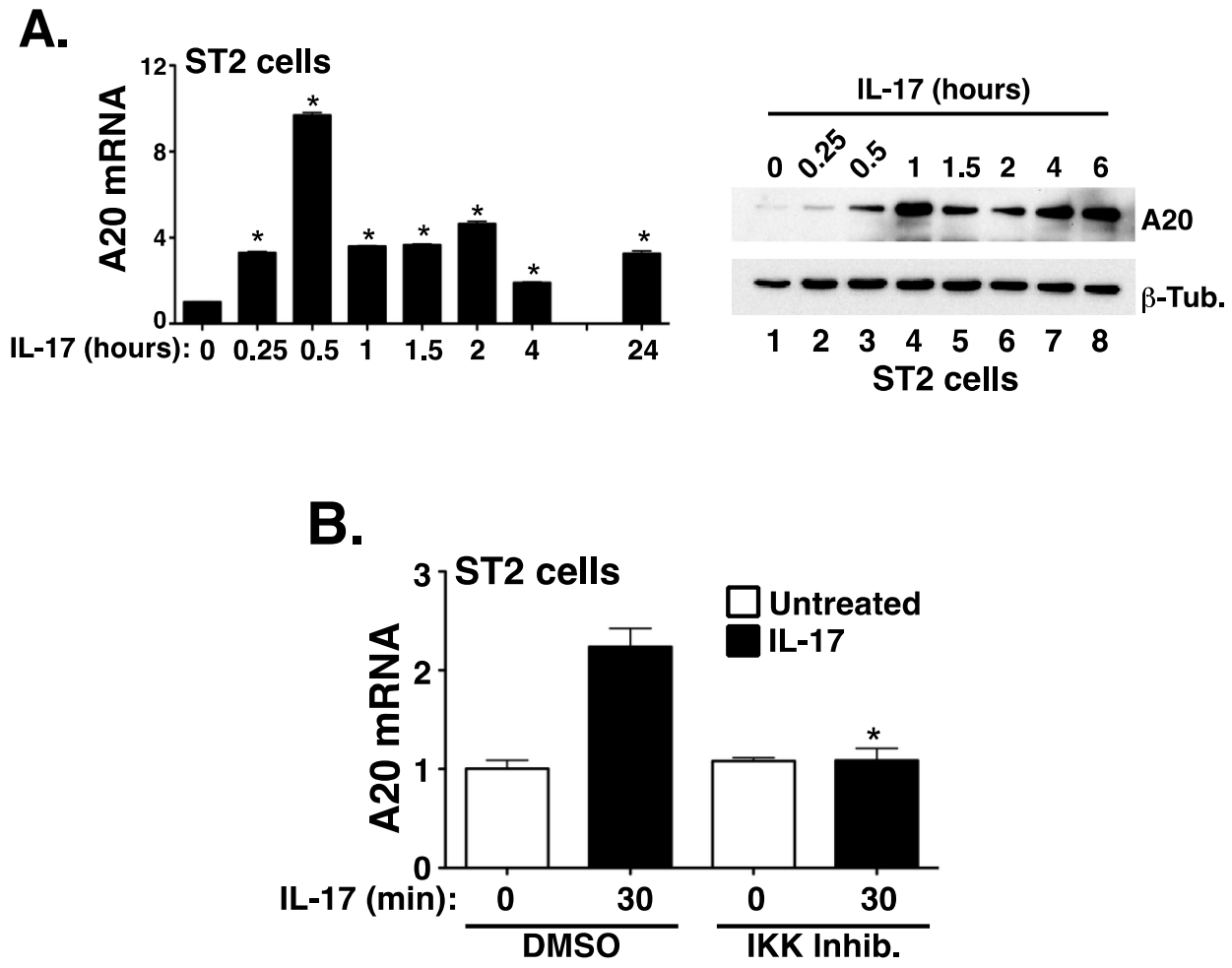


Figure 3.2: IL-17 induces *TNFAIP3* expression and production of A20, which is dependent on NF-κB.

(A) Left: Rapid induction of endogenous *TNFAIP3* mRNA expression by IL-17. ST2 cells were treated with IL-17 (200 ng/ml) for the indicated times and A20 mRNA abundance was assessed by qPCR in triplicate assays. Data are presented as the fold-induction in *TNFAIP3* (A20) mRNA abundance compared to untreated cells. Right: Endogenous A20 or β-Tubulin assessed by immunoblotting of ST2 cell whole cell lysates treated with IL-17 for the indicated times. **(B)** IL-17 induction of endogenous A20 is mediated by the NF-κB pathway. ST2 cells were treated with IL-17 for 30 min in the presence of DMSO or an IKK inhibitor (0.1 μM), and *TNFAIP3* was assessed by qPCR in triplicate assays. *P < 0.05 by ANOVA with post-hoc Tukey's test, compared to untreated (at 0 min)**.**

3.2.2 A20 inhibits IL-17 signaling.

Because inhibitory molecules are often induced by the cytokines that they suppress, we hypothesized that A20 would inhibit IL-17–mediated signaling. We transfected ST2 cells with siRNA specific for A20, using a non-targeting scrambled siRNA as a negative control. As a positive control, we evaluated siRNA specific for Act1, which positively activates IL-17R signaling [66, 67]. Forty-eight hours after transfection, cells were stimulated with IL-17 for 1.5 or 3 hours, and IL-6 in culture supernatants was measured by ELISA **(Figure 3.3-A)**. As expected, IL-6 production increased even in untreated cells after transfection with A20-specific siRNA, indicating that A20 controls the tonic expression of pro-inflammatory genes [121]. Stimulation with IL-17 in combination with A20 knockdown led to a significant ($P < 0.001$) increase in IL-6 production compared to that by stimulation with IL-17 alone, revealing an inhibitory role for A20 in the IL-17 pathway **(Figure 3.3-A)**. We verified that A20 was efficiently knocked down in these experiments **(Figure 3.3-B,C)**. To determine whether induction of A20 by IL-17 leads to termination of IL-6 expression, we evaluated the kinetics of *Il6* mRNA induction following IL-17 treatment. There was a slight drop at the 1- and 1.5-hour time points coinciding with the time when A20 protein was increased, but generally *Il6* mRNA was maintained at steady state for at least 4 hours **(Figure 3.3-D)**. This finding supports a model in which A20 serves to keep IL-17 signaling in check, but does not cause complete signal termination.

To further evaluate the role of A20 in IL-17 signal transduction, we analyzed expression of a panel of well-defined IL-17 target genes in the context of A20 silencing [69, 72, 131]. Consistent with an increase in IL-6 protein abundance, transfection with A20-specific siRNA enhanced IL-17–dependent induction of *Il6* expression. Similarly, knockdown of A20 enhanced

IL-17–induced expression of *Ccl20, Lcn2* (which encodes lipocalin 2), *Csf3* (which encodes G-CSF), and *Cxcl1*, and *Cxcl5* **(Figure 3.4-A)**. Although the magnitude by which A20-specific siRNA enhanced the expression of IL-17 target genes was variable, the extent of expression of all of these genes was significantly and reproducibly increased. However, there was no change in the expression of *Ikbz* in response to A20 knockdown **(Figure 3.4-A)**, suggesting that there may be distinct modes of regulation for some IL-17-induced genes.

Increased amounts of IL-17 as well as an increase in the extent of expression of IL-17–induced genes are associated with pathology in RA [132]. Accordingly, we tested the effect of A20 knockdown in human primary fibroblast-like synoviocytes (FLSs), a cell type that mediates pathogenesis in the inflamed joint. Transfection of FLS cells with A20 siRNA led to enhanced IL-17–dependent IL-6 production compared to control transfected cells **(Figure 3.4-B)**. Therefore, we conclude that A20 mediates feedback inhibition of IL-17 signaling, and that this regulation has the potential to be relevant in a disease where IL-17 plays a well-documented role.

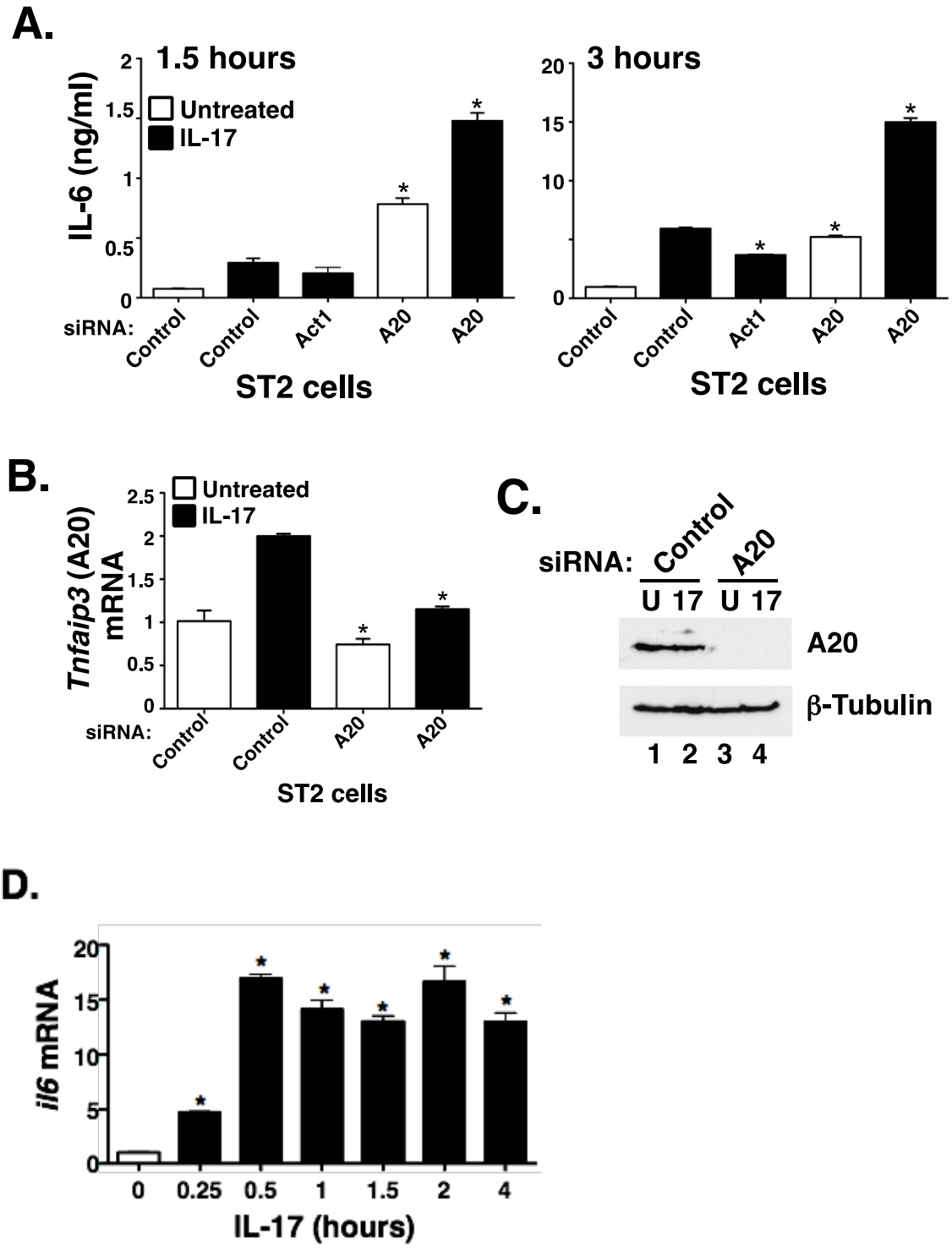


Figure 3.3: A20 inhibits IL-17 signaling.

(A) Knockdown enhances the production of IL-6 protein. ST2 cells were transfected with siRNA specific for Act1 or A20 or with a scrambled siRNA control, and the amount of IL-6 in

culture media was assessed in triplicate at 1.5 and 3 hours after treatment with IL-17 (200 ng/ml). Black bars indicate IL-17-treated samples, and white bars indicate untreated samples. **P* <0.05 by ANOVA with post-hoc Tukey's test compared to cells transfected with control siRNA and treated with IL-17. **(B, C)** Successful knockdown of *TNFAIP3* mRNA and protein. *TNFAIP3* mRNA in cells treated with siRNA was assessed by qPCR. After siRNA-mediated knockdown of *TNFAIP3* expression, the indicated cell lysates were analysed by western blotting for A20 protein (top) or β-tubulin (bottom) as a loading control. **(D)** Kinetics of IL-17-induced IL-6 production. ST2 cells were treated with IL-17 for the indicated times and IL-6 mRNA assessed by qPCR, normalized to the zero time point.

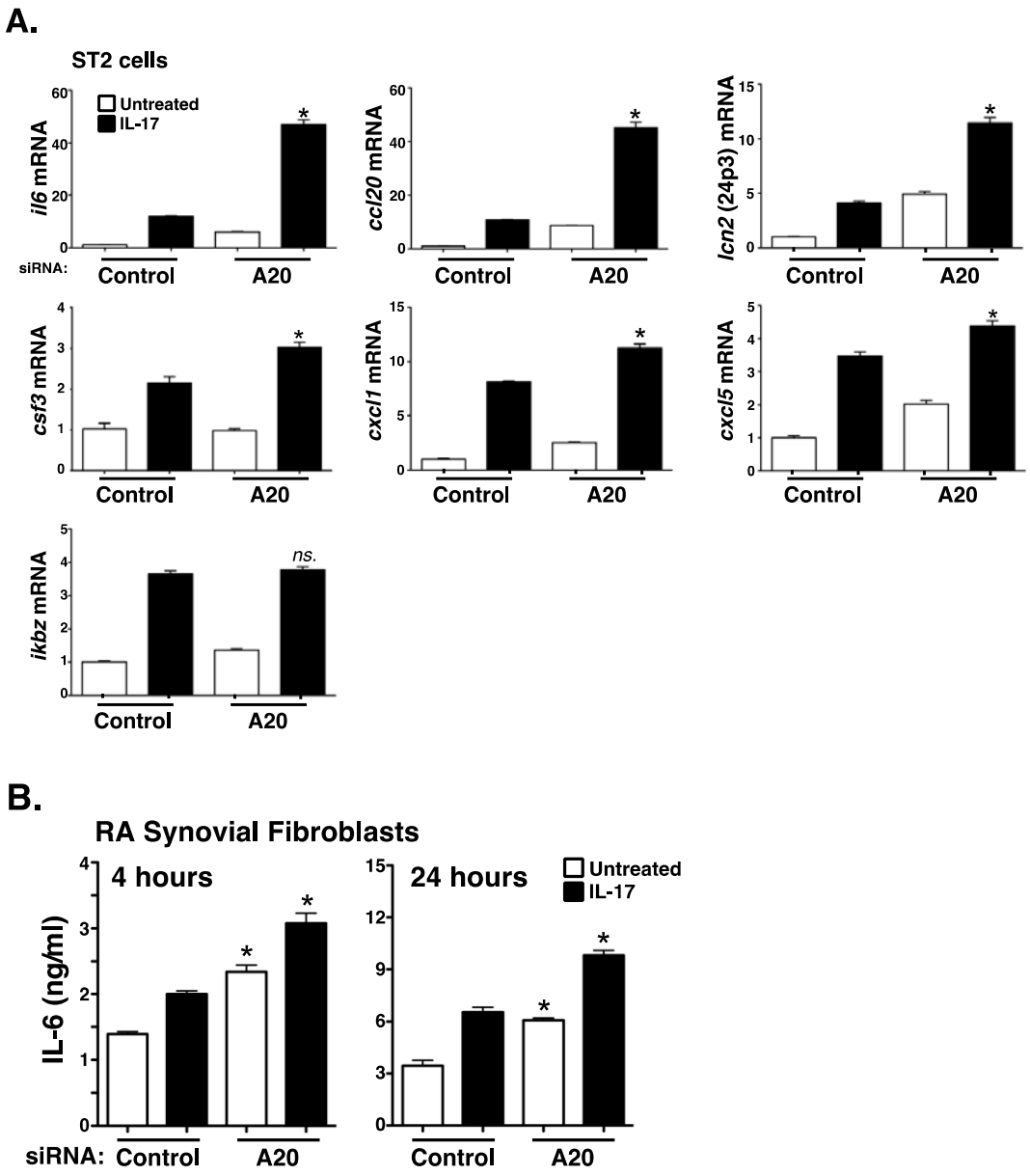


Figure 3.4: A20 knockdown suppresses IL-17-mediated gene expression.

(A) A20 knockdown enhances expression of most but not all IL-17 target genes. ST2 cells transfected with the indicated siRNAs and left untreated or treated with IL-17 for 3 hours were analyzed for the expression of the indicated genes by qPCR. **(B)** A20 knockdown enhances IL-17R signaling in human FLS cells. FLS cells were transfected in triplicate with the indicated siRNAs and IL-6 secretion after 4 or 24 hours of IL-17 stimulation was assessed by ELISA in triplicate. **P* <0.05 by ANOVA and post-hoc Tukey's test compared to cells transfected with control siRNA and treated with IL-17.

3.2.3 Overexpression of A20 inhibits IL-17 target promoter activation.

NF-κB is required for activation of many IL-17 target genes, including 24p3/lipocalin 2 and IL-6 [72, 73]. To determine whether A20 impacts NF-κB-dependent promoters regulated by IL-17, ST2 cells were transfected with luciferase (Luc) constructs driven by the 24p3/lipocalin 2 or IL-6 promoters in combination with increasing concentrations of A20 [72, 73]. IL-17 stimulation triggered a ~2.5-fold increase in 24p3-Luc activity over baseline **(Figure 3.5-A)**. Co-transfection with A20 resulted in a dose-dependent reduction in 243p-Luc, supporting a model in which A20 inhibits IL-17 activation of this gene at the level of the promoter. A mild suppression in luciferase activity was also seen in cells treated without cytokines, consistent with the effect of A20 on tonic signaling noted in Figure 3.3. Parallel results were obtained with the IL-6 promoter **(Figure 3.5-B)**. Further evidence for a role for A20 in suppressing target promoter activation came from the observation that IL-17-mediated induction of the 24p3 promoter was more strongly enhanced in A20-deficient cells compared to cells reconstituted with A20 **(Figure 3.5-C)**. Together, these data support the concept that A20 inhibits IL-17 by modulating NF-κB-regulated gene expression.

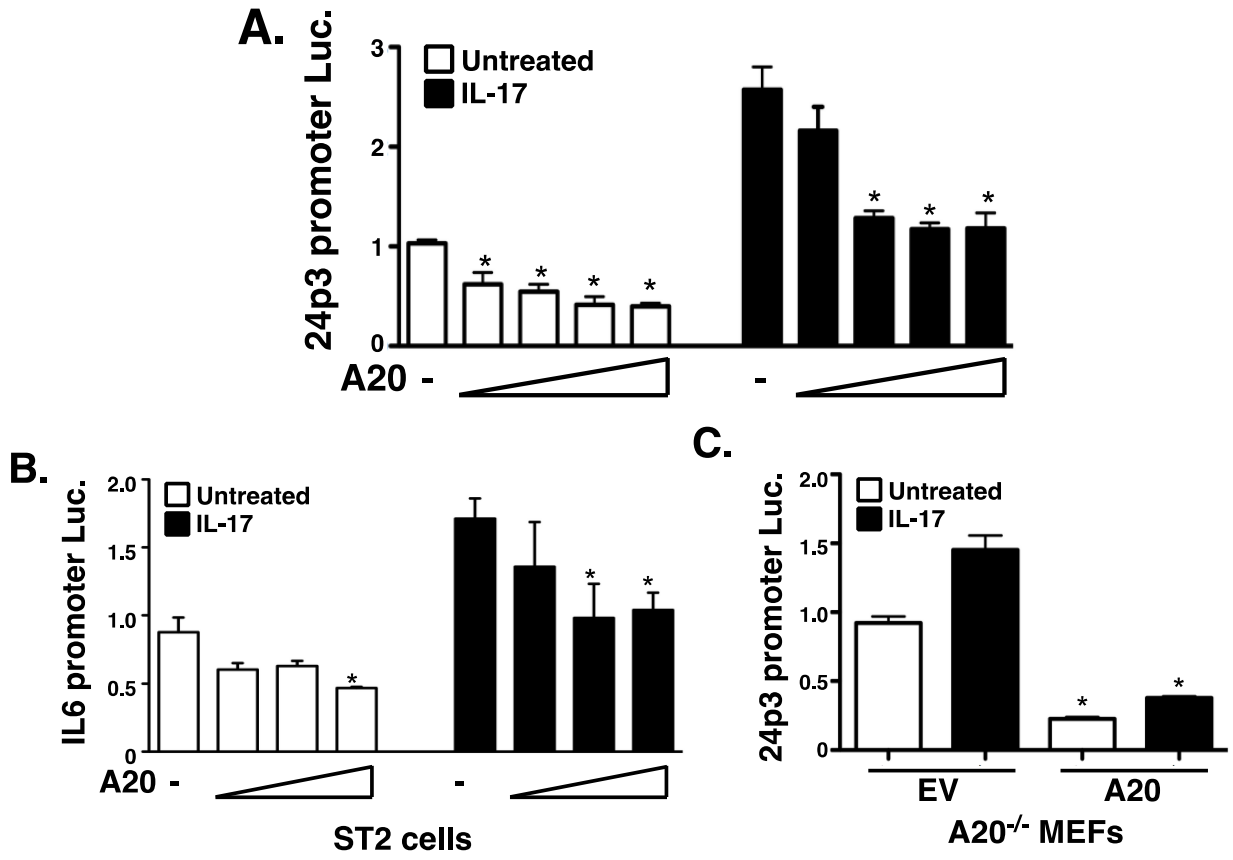


Figure 3.5: A20 suppresses IL-17-mediated activation of NF-κB-dependent gene promoters.

(A, B) Ectopic expression of A20 suppresses IL-17 activation of the 24p3 and IL-6 promoters. ST2 cells were transfected with luciferase constructs encoding the 24p3/lipocalin 2 promoter or the IL-6 promoter together with increasing concentrations of A20 plasmid DNA. After 8 h of IL-17 treatment, luciferase activity was assessed, normalized to samples transfected with control DNA and treated with no cytokines. **(C)** A20-deficency leads to enhanced IL-17 activation of the 24p3 promoter. $A20^{-/-}$ MEFs were transfected with the 24p3-Luc construct and either an empty vector (EV) or a plasmid encoding A20. After 8 hours of IL-17 treatment, luciferase activity was assessed, normalized to samples transfected with control DNA and treated with no cytokines. **P* <0.05 by ANOVA and post-hoc Tukey's test, compared to unstimulated EV sample.

3.2.4 Reconstitution of A20$^{-/-}$ MEFs reverses IL-17 induced target gene expression.

To verify independently that A20 inhibits the IL-17 pathway and rule out possible nonspecific or off-target effects of siRNA, A20$^{-/-}$ murine embryonic fibroblasts (MEFs) were transfected with either an empty vector (EV) or a plasmid encoding murine A20. Cells were stimulated with IL-17 for 4 hours, and IL-6 in culture supernatants was assessed. A20$^{-/-}$ MEFs reconstituted with EV showed an IL-17-dependent increase in IL-6 production, which was reduced upon reconstitution with A20 **(Figure 3.6-A)**. Production of CCL20 was similarly impacted by reconstitution with A20 **(Figure 3.6-A)**. We next determined the impact of reconstituting A20$^{-/-}$ MEFs on expression of a panel IL-17 target genes. IL-17-induced expression of *Il6*, *Ccl20*, *Cxcl1*, *Cxcl5*, *Csf3* and *lcn2* mRNA in A20$^{-/-}$ cells was repressed upon reconstitution with A20 **(Figure 3.6-B)**. Similar to the findings with siRNA (**Figure 3.4-A**), there was only a mild effect of A20 reconstitution on *Ikbz* expression **(Figure 3.6-B)**, indicating that A20 regulates most but not all IL-17 target genes.

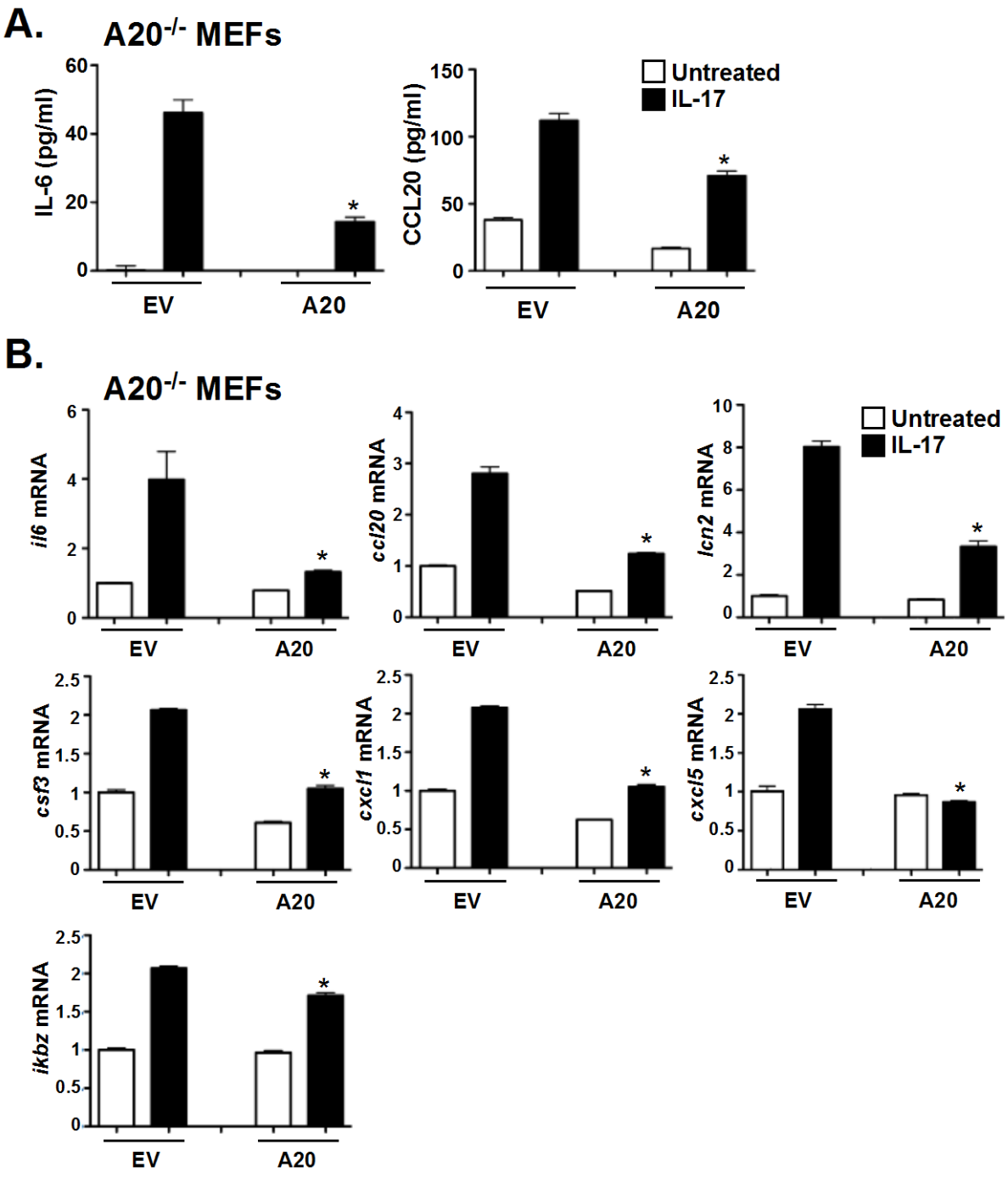


Figure 3.6: Reconstitution of A20$^{-/-}$ cells with A20 suppresses IL-17-dependent signaling.

(A-B) A20$^{-/-}$ MEFs were transfected in triplicate with EV or murine A20 and treated with IL-17 for 4 hours. Culture supernatants were evaluated in triplicate for IL-6 or CCL20 by ELISA **(A)**, and cell lysates were evaluated for the indicated mRNA transcripts by qPCR **(B).** *$P < 0.05$ by ANOVA and post-hoc Tukey's test compared to IL-17-treated samples transfected with EV.

3.2.5 A20 reverses IL-17-mediated TRAF6 activation.

Since TRAF6 is essential for IL-17-induced NF-κB activation [68], we asked whether IL-17 induces association between A20 and TRAF6. $A20^{-/-}$ MEFs were reconstituted with or without A20 and stimulated over a time course of 0 to 120 min. Cell lysates were immunoprecipitated for TRAF6 and immunoblotted for A20. There was no detectable baseline association between A20 and TRAF6 (**(Figure 3.7-A)**, lane 5). However, A20 associated with TRAF6 by 60 min. These kinetics are very similar to the association of A20 with TRAF6 and TRAF2, induced by IL-1β and TNFα, respectively [117, 133, 134] **(Figure 3.7-A)**.

A20 inhibits IL-1β and TNFα signaling by deubiquitinating K63-linked ubiquitin chains from TRAF6 and RIP1 [114, 117, 124]. To determine whether activation of A20 was associated with deubiquitination of TRAF6, reconstituted $A20^{-/-}$ cells were treated with IL-17, Act1 was immunoprecipitated from cell lysates, and the TRAF6 ubiquitination status was assessed by immunoblotting for TRAF6. Consistent with an association between A20 and TRAF6, TRAF6 exhibited sustained ubiquitination following IL-17 stimulation for 90-120 min in $A20^{-/-}$ cells, as evidenced by increased intensity of larger migrating forms reactive to the TRAF6 antibodies (**(Figure 3.7-B)**, lanes 1-3). In contrast, reconstitution of $A20^{-/-}$ cells with A20 was associated with a reduced appearance of the larger migrating forms of TRAF6 (**(Figure 3.7-B)**, lanes 4-6). Although the signals were weak, they were reproducible and are consistent with a recent published report [92]. These data probably also reflect the fact that IL-17-mediated activation of NF-κB is typically far more modest than classical inflammatory cytokines such as IL-1β or TNFα [17, 72].

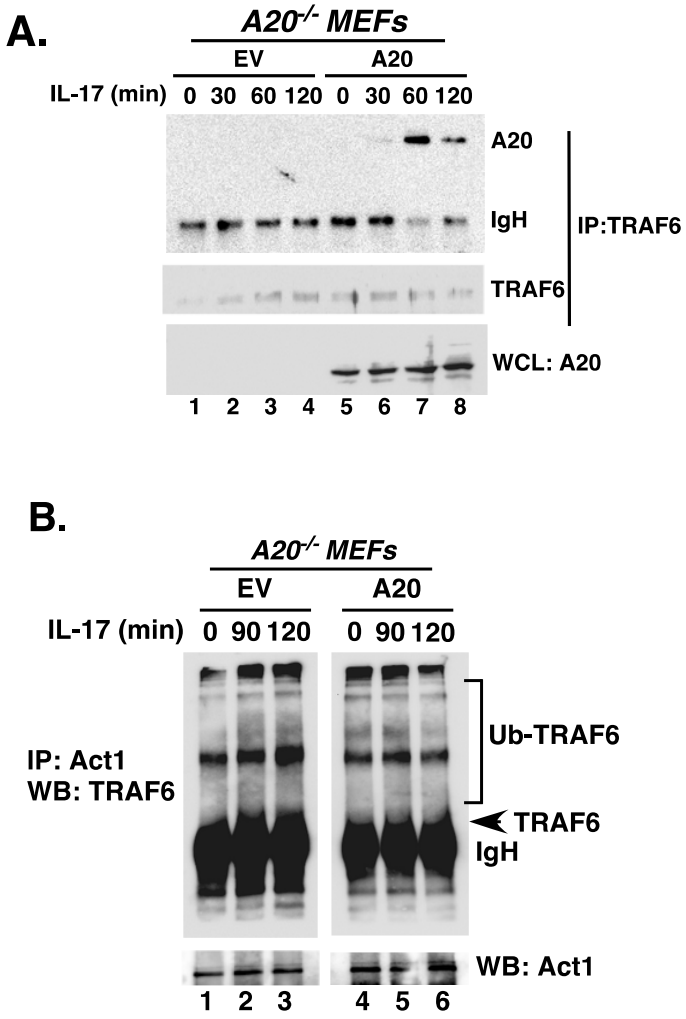


Figure 3.7: A20 reverses IL-17-mediated TRAF6 activation.

(A) A20 associates with TRAF6 in an IL-17-inducible manner. $A20^{-/-}$ cells were transfected with EV (lanes 1-4) or A20 (lanes 5-8) and lysates were immunoprecipitated with antibodies (Abs) against TRAF6. IP samples were immunoblotted with Abs to A20 (top) and TRAF6 (middle). Samples of whole cell lysates (WCL) were blotted for A20 (bottom) to verify transfection efficiency, **(B)** A20 mediates TRAF6 deubiquitination after IL-17 signaling. $A20^{-/-}$ cells were transfected with EV (lanes 1-3) or A20 (lanes 4-6) and lysates were immunoprecipitated with Abs against Act1. Lysates were blotted with α-TRAF6 Abs. Arrow indicates approximate migration of TRAF6 (band is obscured by IgH heavy chain). Note that lanes 1-3 and 4-6 are from the same gel, but the TRAF6 gel subjected to different exposure times to optimize visualization of larger, ubiquitinated TRAF6 bands.

3.2.6 A20 inhibits the IL-17-induced NF-κB/MAPK pathways.

NF-κB is activated when IκBα is phosphorylated, ubiquitinated and degraded, resulting in unmasking of the NF-κB nuclear localization signal and subsequent nuclear import. IκBα expression is, in turn, regulated by NF-κB in a feedback loop. To determine whether A20 inhibits IL-17-mediated induction of NF-κB, we monitored IκBα degradation in $A20^{-/-}$ MEFs reconstituted with or without A20 over 0-60 min of IL-17 treatment. Lack of A20 resulted in continued IL-17-induced degradation of IκBα, suggesting prolonged NF-κB activation. However, in cells reconstituted with A20, this degradation was inhibited very rapidly, suggesting regulation of NF-κB activity **(Figure 3.8-A)**. These data support a model in which A20 blocks the IL-17-induced NF-κB pathway.

In the IL-17 pathway, TRAF6 is required for activation of MAPK [18]. Although there is some variation among cell types, all 3 major pathways (ERK, p38 and JNK) have been shown to be induced by IL-17. To determine whether A20 suppresses MAPK activation, $A20^{-/-}$ cells were transfected with EV or A20, and IL-17-induced phosphorylation of JNK, ERK and p38 were assessed by immunoblotting. Transfection of A20 prevented prolonged phosphorylation of JNK, as indicated by the absence of p-JNK at the 30 and 60 min time points (**(Figure 3.8-B)**, lanes 7-8). A similar pattern was observed for p-ERK and p-p38 **(Figure 3.8-B)**. Therefore, A20 suppresses MAPK activation, particularly JNK.

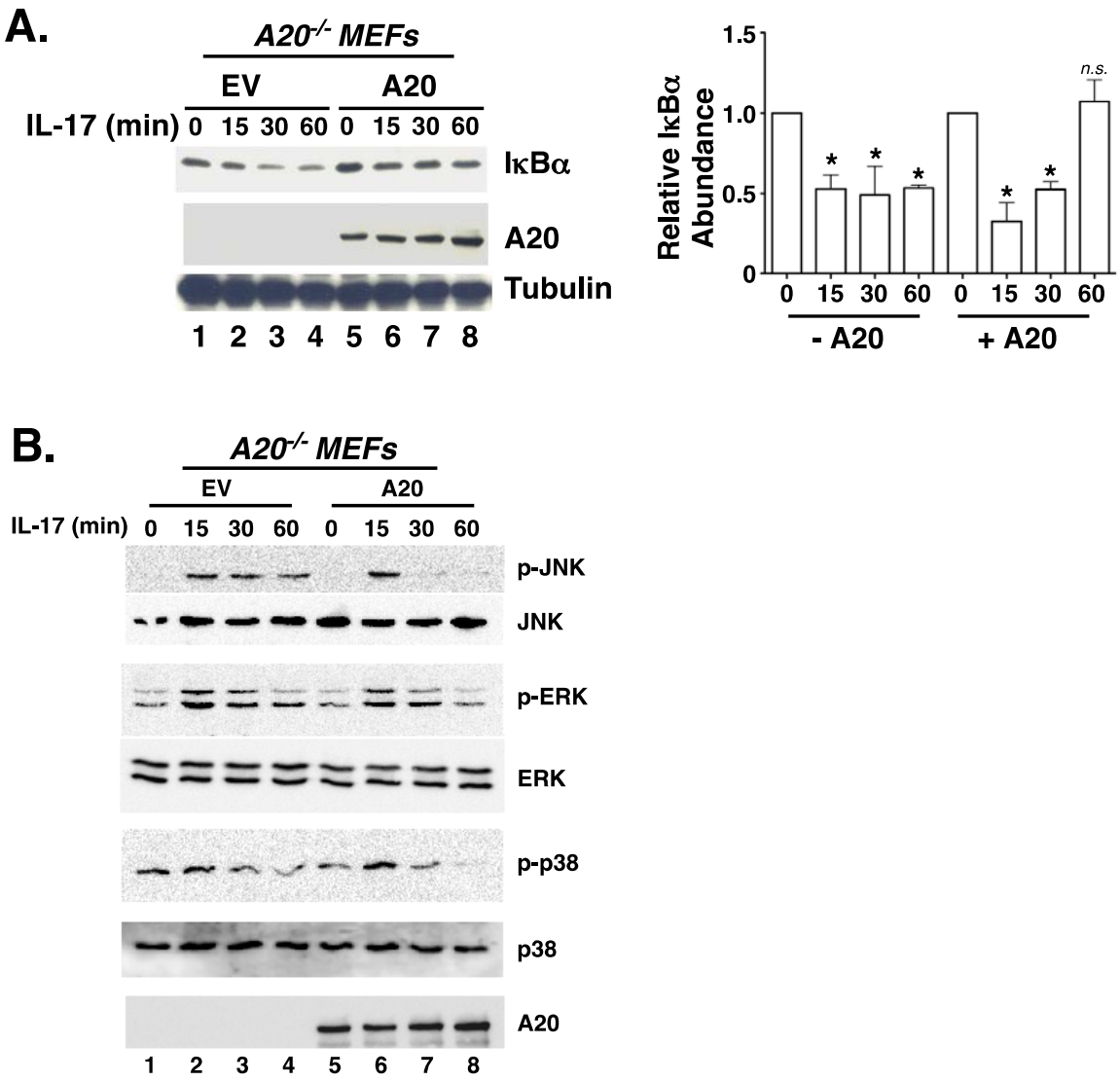
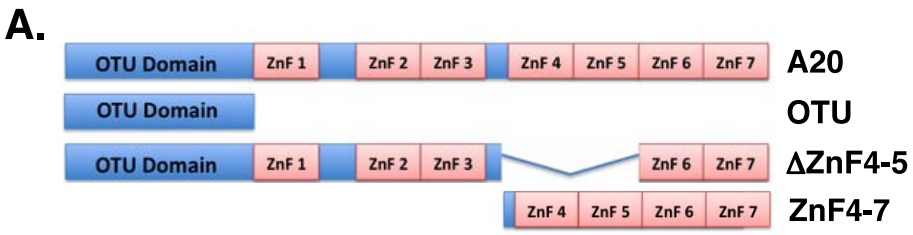


Figure 3.8: A20 inhibits the IL-17-induced NF-κB/MAPK pathway.

(A) A20 promotes IκBα degradation. Left: $A20^{-/-}$ cells were transfected with EV (lanes 1-4) or A20 (lanes 5-8), treated with IL-17 for the indicated times, and IκBα expression (top) was assessed by immunoblotting. Loading of A20 (middle) and β-tubulin (bottom) is shown. Right: Relative band intensity from two pooled independent experiments was determined by densitometry + SEM, **P* <0.05 by ANOVA and post-hoc Tukey's test compared to the unstimulated time point for each condition. **(B)** A20 suppresses IL-17-inducible MAPK activation. $A20^{-/-}$ cells were transfected with EV (lanes 1-4) or A20 (lanes 5-8), stimulated with IL-17 for the indicated times and lysates were immunoblotted for the indicated MAPK family members or A20.

3.2.7 Inhibition of IL-17 signaling is mediated through the OTU and Zinc Finger Domains of A20.

Multiple subdomains of A20 contribute to its inhibitory capacity **(Figure 3.9-A)** [117]. The best characterized is the N-terminal OTU (ovarian tumor) domain, which encodes deubiquitinase (DUB) activity and is proposed to be important for TRAF6 association [85, 135]. In addition, the seven C-terminal zinc-finger (ZnF) domains are important for A20 activity [117, 135-137]. In particular, ZnF4 and ZnF5 exhibit Ub-binding activity, which facilitates adaptor recruitment and substrate recognition [100, 138]. To determine the roles of these domains in IL-17 signaling, $A20^{-/-}$ MEFs were reconstituted with either WT A20, or the OTU domain and ΔZnF4-5 mutants **(Figure 3.9-A)**. Twenty-four hours later, cells were stimulated with IL-17 and supernatants evaluated for IL-6 by ELISA. Neither mutant inhibited IL-17-dependent IL-6 secretion as effectively as WT A20 **(Figure 3.9-B)**. Similarly, neither mutant exerted detectable suppression on *Il6* mRNA expression **(Figure 3.9-B)**. Although in some experiments the OTU mutant mediated a mild suppressive effect **(Figure 3.9-B)**, this was not always reproducible **(Figure 3.9-C)**, and may be due to a higher expression capacity of the mutants in comparison to WT A20 **(Figure 3.9-D)**. To further confirm the role of both OTU and ZnF domains, we reconstituted $A20^{-/-}$ MEFs with a construct encoding just the terminal four ZnF domains **(Figure 3.9-C)**; this mutant also failed to inhibit IL-17-induced IL-6 production. Collectively, we conclude that A20-mediated inhibition of IL-17 signaling involves both the catalytic OTU domain as well as the ZnF domains of this protein.


A.
OTU Domain
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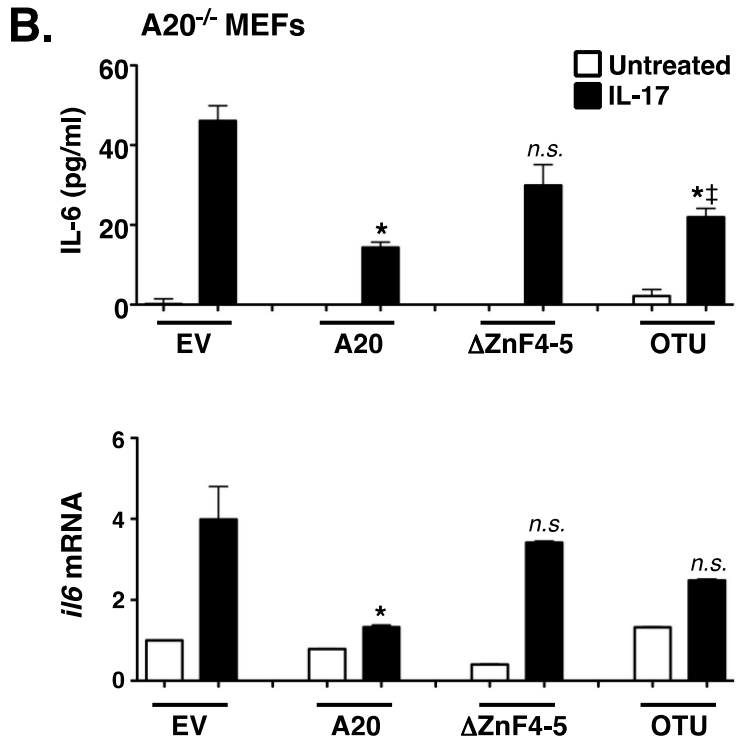
ZnF 2
ZnF 3
ZnF 4
ZnF 5
ZnF 6
ZnF 7
A20
OTU
ΔZnF4-5
ZnF4-7


B.
A20-/- MEFs
Untreated
IL-17
IL-6 (pg/ml)
60
40
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*
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EV
A20
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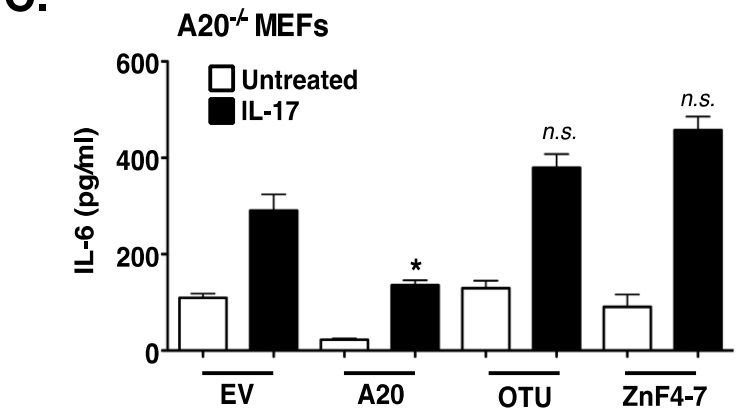
C.
A20-/- MEFs
Untreated
IL-17
IL-6 (pg/ml)
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ZnF4-7

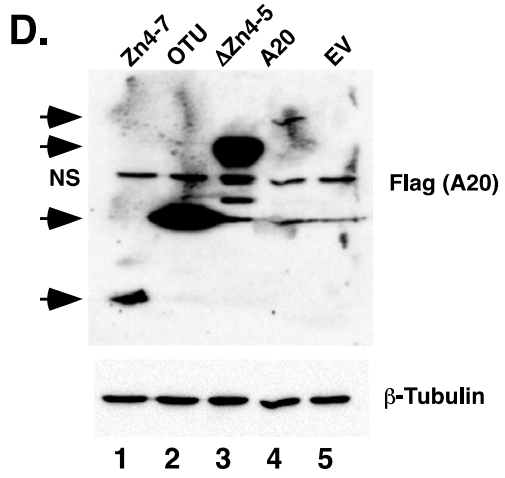

Figure 3.9: Inhibition of IL-17 signaling is mediated through the OTU and zinc finger domains of A20.

(A) Schematic diagram of A20 subdomains. OTU = DUB domain. Location of zinc fingers (ZnF) is shown. **(B)** ZnF mutants of A20 impair its ability to regulate IL-17 signaling. $A20^{-/-}$ cells were transfected in triplicate with EV, A20 or the indicated mutants and treated with IL-17 for 4 hours. IL-6 in culture supernatants was assessed by ELISA in triplicate (top), and expression of *Il6* mRNA was assessed by qPCR. * P <0.05 by ANOVA and post-hoc Tukey's test compared to samples transfected with EV and treated with IL-17. ‡ $P < 0.05$ by ANOVA and post-hoc Tukey's test compared to samples transfected with A20 and treated with IL-17. **(C)** $A20^{-/-}$ MEFs were transfected with the indicated A20 mutants, treated with IL-17 for 24 hours and IL-6 measured by ELISA in triplicate. *$P < 0.05$ by ANOVA and post-hoc Tukey's test. **(D)** Expression of A20 mutants. Lysates from A20-/- cells transfected with the indicated A20 mutants were blotted for Flag to detect A20 (top) or β-tubulin (bottom). Arrows indicate migration of individual A20 mutants. NS, nonspecific band.

3.2.8 A20 associates with IL-17RA independently of the receptor SEFIR domain.

IL-17RA contains several functional subdomains **(Figure 3.10-A)**. Therefore, we asked whether A20 interacts directly with IL-17RA, thereby inhibiting the signaling pathway. To determine if IL-17RA and A20 interact, HEK293T cells were co-transfected with A20 and murine IL-17RA (tagged at the C-terminus with Myc). Lysates were immunoprecipitated with anti-Myc Abs to pull down the IL-17 receptor, and association with A20 was determined by western blotting. As shown, A20 associated with IL-17RA in a dose-dependent manner **(Figure 3.10-B)**. Strikingly, this is to our knowledge the first time that A20 has been demonstrated to bind directly to an inducing receptor.

The SEFIR is conserved among IL-17 receptor family members and serves as a platform for association with Act1, which in turn interacts with TRAF6 [66]. A large, non-conserved extension of the SEFIR, termed a SEFEX, is also required for signaling in response to IL-17 [33, 57, 99]. A point mutation within the SEFEX (V553H) renders IL-17RA non-functional, and impairs activation of both the NF-κB and MAPK pathways [89, 99]. Because A20 restricts activation of both NF-κB and MAPK, we initially predicted that the SEFIR/SEFEX region would be the site of interaction with A20. Unexpectedly, A20 associated strongly with the IL-17RAΔSEFIR and IL-17RA.V553H mutants **(Figure 3.10-C)**, indicating that A20 binds to IL-17RA in a SEFIR/SEFEX-independent manner.

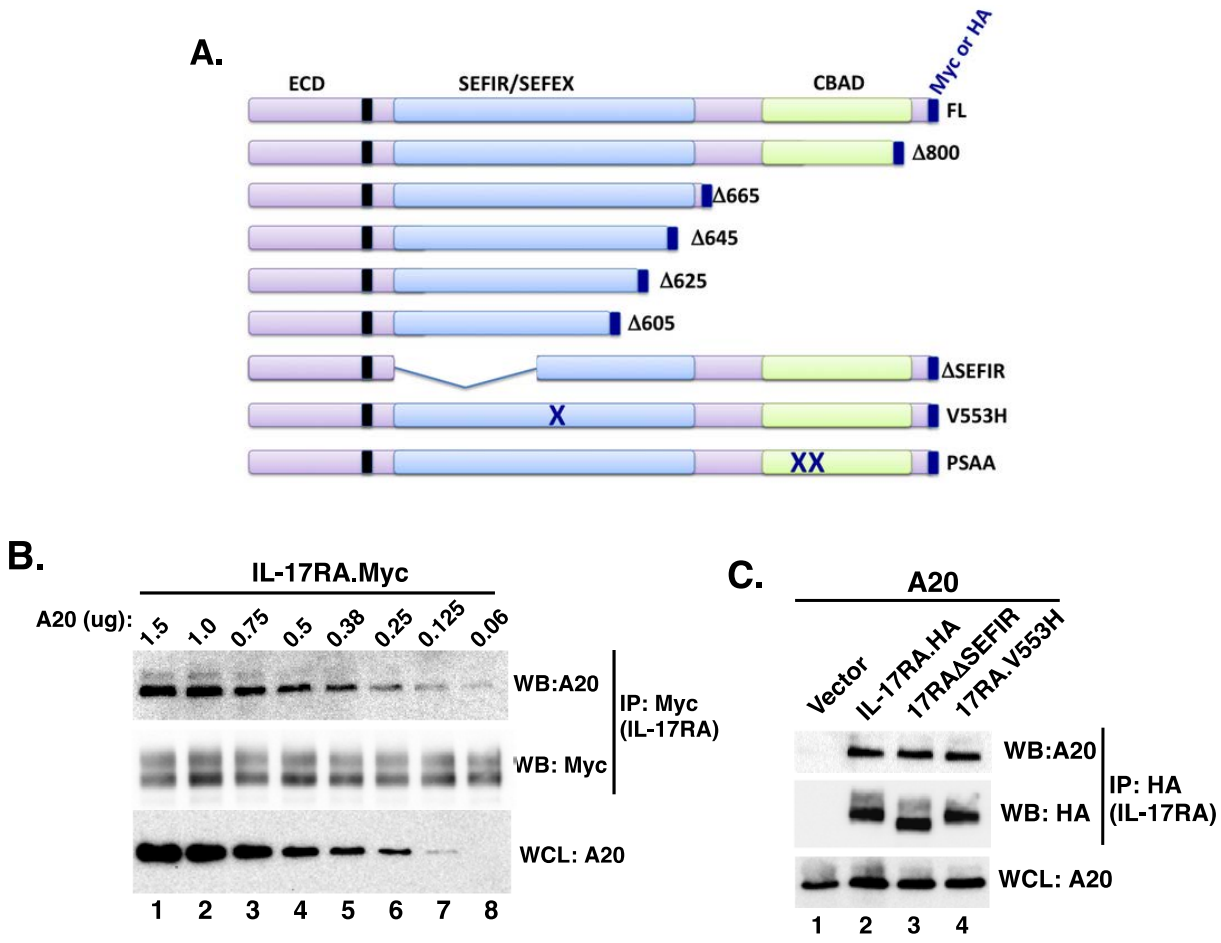

Figure 3.10: A20 binds IL-17RA independently of the SEFIR domain.

(A) IL-17RA binds to A20 in a dose-dependent manner. HEK293T cells were transfected with IL-17RA (tagged at the C-terminus with Myc) together with decreasing concentrations of A20 plasmid (1.5-0.06 µg). Anti-Myc Abs were used to pull down IL-17RA, and immunoprecipitates were blotted with A20 (top) or Myc to detect IL-17RA (middle). WCL from samples taken prior to IP were blotted for A20 (bottom). **(B)** Schematic diagram of IL-17RA mutants. ECD, extracellular domain; locations of SEFIR/SEFEX, point mutants (V553H, PSAA) and CBAD domains are indicated. **(C)** A20 binds to IL-17RA in a SEFIR/SEFEX-independent manner. HEK293T cells were transfected with IL-17RA (tagged at the C-terminus with HA) with A20. Anti-HA Abs were used to pull down IL-17RA, and IPs were blotted with A20 (top) or HA to detect IL-17RA (middle). Expression of A20 in whole cell lysates is indicated (bottom).

3.2.9 A20 binds to the IL-17RA at the C-terminal inhibitory domain.

In addition to the SEFIR/SEFEX, a C-terminal domain located downstream of residue 665 and non-overlapping with the SEFIR/SEFEX region has been described. This region is required for regulation of C/EBPβ, and hence was termed the "C/EBPβ activation domain" (CBAD) [99]. To determine whether A20 interacts with the CBAD, A20 was co-expressed with a panel of C-terminal truncations of IL-17RA. Binding of A20 to IL-17RAΔ800 was reduced compared to full length IL-17RA. IL-17RAΔ665 and all the larger truncations were also severely impaired in the ability to co-IP with IL-17RA **(Figure 3.11)**.

The CBAD contains a TRAF binding motif that was reported to be required for its interaction with TRAF3 [90]. Therefore, we tested the association of A20 with an IL-17RA in the absence of TRAF6 and TRAF3. A20 co-immunoprecipitated with IL-17RA in TRAF6-deficient cells **(Figure 3.12-A)**, consistent with the lack of a requirement for the SEFIR/SEFEX in its recruitment. To determine the role of TRAF3, we used an IL-17RA mutant lacking TRAF3 binding site (PSAA). A20 co-immunoprecipitated with the IL-17RA.PSAA mutant normally **(Figure 3.12-B)**. We and others have previously shown that the CBAD is an inhibitory domain, because IL-17RA truncation mutants lacking this region exhibit enhanced IL-17-dependent signaling [33, 89, 90, 99]. Its association with A20 may explain the underlying basis for how the CBAD inhibits IL-17 signal transduction.

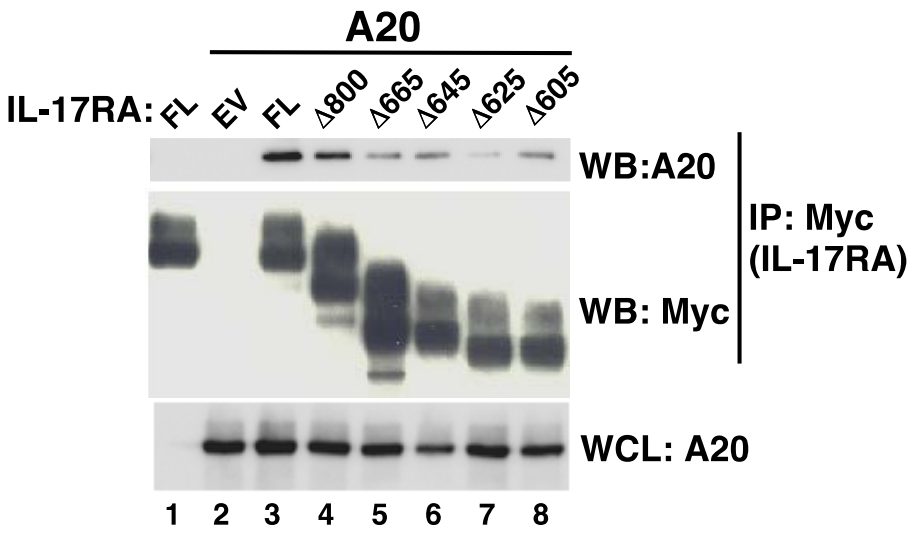

Figure 3.11: A20 binds to IL-17RA through the CBAD domain.

A20 binds to IL-17RA via the CBAD. HEK293T cells were transfected with IL-17RA (tagged at the C-terminus with Myc) with A20. Anti-Myc Abs were used to pull down IL-17RA, and IPs were blotted with A20 (top) or Myc to detect IL-17RA (middle). Expression of A20 in whole cell lysates is indicated (bottom).

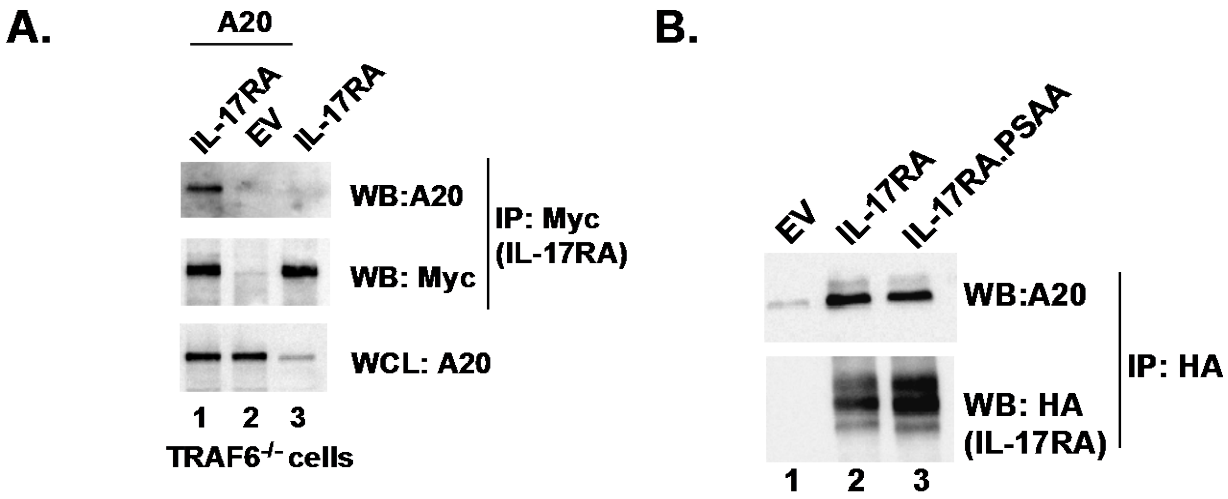

Figure 3.12: TRAF6 and TRAF3 are not required for association between IL-17RA and A20.

(A) A20 binds to IL-17RA in a TRAF6-independent manner. TRAF6$^{-/-}$ MEFs were co-transfected with A20 and Myc-tagged IL-17RA or empty vector (EV). Lysates were immunoprecipitated with Abs to Myc and blotted for A20 (top) or Myc (bottom). Whole cell lysates (WCL) prior to IP were blotted for A20 (top) or IL-17RA (Myc, bottom). **(B)** A20 does not bind to the CBAD TRAF consensus site. HEK293T cells were transfected with IL-17RA or IL-17RA.PSAA (tagged at the C-terminus with HA) together with A20. Anti-HA Abs were used to pull down IL-17RA, and IPs were blotted with A20 (top) or HA to detect IL-17RA (bottom).

3.3 DISCUSSION

The IL-17 family is the newest and least understood of the cytokine subclasses [18]. Composed of ligands IL-17A-IL-17F and receptors IL-17RA-IL-17RE, these cytokines have many unique structural and functional features. Since the discovery of the T_H17 subset in 2005, considerable attention has been paid to how T_H17 and other IL-17-producing cells are generated and maintained, but far less to how IL-17 mediates downstream signaling [23, 139].

Although IL-17 is vital for host defense against certain pathogens, it has high potential for inducing pathological damage to inflamed tissue. Hence, it is not surprising that there are numerous mechanisms in place to constrain IL-17/$T_H17$activity [140, 141]. To list a few examples, T_H1 and T_H2 cytokines (IFNγ and IL-4) and IL-2 block differentiation of Th17 cells [21, 22, 142]. Th17 cells often convert to Tregs or T_H1-like cells in vivo, tempering their inflammatory activity [143-145]. Immunoregulatory cytokines such as IL-25, IL-27 and IL-10 limit T_H17-mediated pathology [60, 146-148]. At the level of the IL-17R signaling pathway, TRAF3 was reported to inhibit IL-17 by binding to IL-17RA and displacing Act1 [90]. A recent report also implicates miR-23b in limiting IL-17 activity [98]. IL-17-inducible degradation of Act1 follows engagement of the receptor, mitigating the signaling response [96]. In studying activation of C/EBP transcription factors, we found that IL-17-induced phosphorylation of C/EBPβ by GSK-3β exerts a suppressive effect on IL-17 target gene expression [89]. Additionally, IL-17A can exist either as a homodimer or as a heterodimer with IL-17F, and in the latter state has a reduced signaling capacity that probably moderates its activity in vivo [56, 149, 150]. A new report identifies the DUB USP25 in targeting TRAF5 and TRAF6 and limiting IL-17 signaling [92].

Like other inflammatory effectors, IL-17 activates the canonical NF-κB pathway, albeit far more modestly than TNFα or TLR ligands [18]. The NF-κB pathway is intricately regulated by ubiquitination and deubiquitination [114]. A20 dampens TNFR-induced signaling [124] and is the gene product of a well-known susceptibility locus for autoimmunity. The primary enzymatic function of A20 is to serve as a DUB, though it can also act as a Ub-ligase and inhibit signaling independently of catalytic activity [117, 121, 135, 151]. In the IL-17 pathway, both Act1 and TRAF6 are E3 Ub-ligases that target TAK1 and IKKα/β/γ, ultimately causing degradation of IκBα and nuclear import of NF-κB [65, 70]. In IL-1 and TLR systems, A20 limits MAPK activation by targeting TRAF6 [124]. Our work reveals for the first time that A20 is both a gene target and a potent feedback inhibitor of IL-17 signaling, acting via TRAF6, NF-κB and MAPK. Therefore, regulation of ubiquitination is emerging as a central feature of IL-17 signal transduction **(Figure 3.13)**.

Using RNA silencing and $A20^{-/-}$ cells, we show that a variety of IL-17-induced target genes are negatively regulated by A20 **(Figure 3.2-3.6)**. As expected, nearly all these genes are regulated by NF-κB, including genes encoding IL-6 and 24p3 where the proximal promoters require an intact NF-κB element for their induction by IL-17 [72, 73]. Interestingly, there are certain exceptions. For example, *Ikbz* expression was largely unaffected by A20 **(Figure 3.4 and 3.6)**. The basis for this difference is not clear; however, IL-17 can also regulate the stability of many target mRNAs [152]. Best-studied in the context of the chemokine *Cxcl1* (KC, Groα), this event is mediated in a non-canonical fashion by activation of the splicing factor (SF) 2 through TRAF2 and TRAF5 [77]. A20 does not appear to influence mRNA stability, perhaps explaining its modest effect on some genes but not others **(Figure 3.3 and 3.4)**. Similar to the TNF pathway, A20 also inhibits IL-17-mediated activation of MAPK pathways, particularly Jun

kinase (JNK) (**Figure 3.8-B)**. MAPK activation leads to AP-1 activation, and AP-1 DNA binding sites are statistically overrepresented in IL-17 target gene promoters [73]; however, at least for the *Il6* gene promoter, the AP-1 site is dispensable for IL-17 activation [72]. In addition, MAPK signaling may participate in regulation of mRNA stability, although it is not clear to what extent this is the case for IL-17-dependent genes.

We found that A20 binds directly to IL-17RA, which is the first time A20 has been demonstrated to interact with a cytokine receptor, and supports a direct model of inhibition of IL-17 (**Figure 3.10).** Like most receptors, IL-17RA contains discrete functional subdomains [61, 99]. Intriguingly, A20 binds to the distal domain of IL-17RA, not the SEFIR/SEFEX region that is the site of engagement for Act1 and TRAF6 [33, 66]. This distal domain was initially identified by its ability to regulate C/EBPβ alternative translation and phosphorylation, and hence was termed a C/EBPβ activation domain, CBAD [89, 99]. Phosphorylation of C/EBPβ via glycogen synthesis kinase (GSK)-3β is mediated through the CBAD and is associated with dampened IL-17R signaling [89]. A TRAF consensus site within the CBAD is an interaction site for TRAF3 (but not TRAF6), which was reported to inhibit IL-17 by competing with Act1. Here, we show that A20 also binds the CBAD, although not via the TRAF consensus site **(Figure 3.11 and 3.12)**, indicating that the CBAD may serve as a platform for binding of multiple inhibitory proteins.

There are still many open questions regarding the details of how A20 restrains IL-17 signaling. In other systems, A20 cooperates with Itch, TAX 1 binding protein 1 (TAX1BP1) and RNF11 to form a functional ubiquitin-editing complex [120], but it is not known whether any/all of these factors are required to inhibit IL-17R signaling. It is also not known whether A20 blocks other IL-17 family ligands that signal via IL-17RA, such as IL-17E (IL-25) or IL-

17C. Since IL-17 has repeatedly been shown to use non-canonical pathways and signaling intermediates, it is likely that surprises will emerge regarding how this pathway is controlled.

The human A20 gene locus, *TNFAIP3*, is strongly associated with many autoimmune diseases. Clearly this is related in part through A20 inhibition of TNF [153]. However, A20 also constrains TLR and NLR signaling, which are increasingly recognized as important in mediating autoimmunity [154, 155]. We now add IL-17 to the list of pro-inflammatory pathways governed by A20. This finding dovetails well with the role for IL-17 in mediating at least some forms of autoimmune disease, as illustrated by the clinical success of biologic therapies targeting this cytokine [27]. Consistently, silencing of A20 led to enhanced IL-17 signaling in human rheumatoid arthritis FLS cells **(Figure 3.4-B)**, supporting the potential relevance of A20 in human cells. Nonetheless, more work is certainly needed to determine how much of A20's impact on IL-17 contributes to disease in vivo compared to other inflammatory stimuli. Defining molecular signaling intermediates, especially enzymes such as A20, has the potential to reveal strategies for developing small molecule therapeutics that target IL-17 [156, 157]. Drugs that enhance A20 function could theoretically help restrain IL-17 and other inflammatory cytokines that promote autoimmunity (**discussed in Chapter 6**); alternatively, blocking A20 might be useful in stimulating host defenses to settings where IL-17 activity is beneficial, such as fungal and bacterial infections [31].

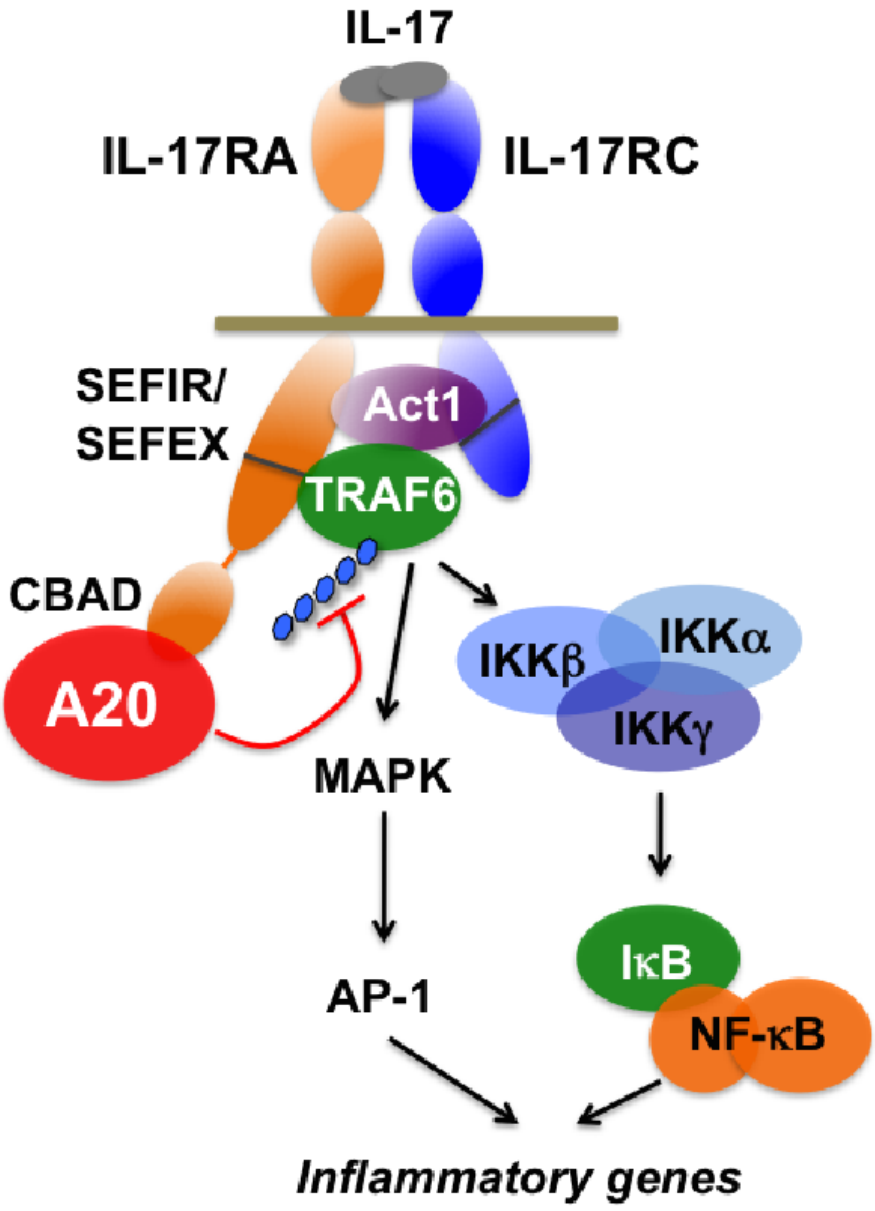

Figure 3.13: Schematic diagram of IL-17RA-mediated signaling, and role of A20 in restricting this process.

A20 serves as a feedback inhibitor of IL-17 signaling by restricting TRAF6 ubiquitination and subsequent NF-κB and MAPK activation. A20 directly associates with the IL-17RA CBAD domain, suggesting a model where IL-17RA recruits inhibitory proteins to the distal C-terminal domain.

4.0 CHAPTER FOUR: THE ANAPHASE-PROMOTING COMPLEX PROTEIN 5 (ANAPC5) ASSOCIATES WITH A20 AND INHIBITS IL-17 SIGNALING

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'The Anaphase-Promoting Complex Protein 5 (AnapC5) Associates with A20 and Inhibits IL-17-Mediated Signal Transduction'

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(* Equal Contribution)

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4.1 BACKGROUND

Multiple proteins are involved in the regulation of IL-17 signal transduction and have been described in detail earlier **(Chapter 1)**. Here we focus our attention on the inhibition of IL-17 receptor signaling. The IL-17 signaling pathway is kept in check by a variety of downstream inhibitors, which help limit collateral damage in settings of acute infection. To list a few examples, Act1 is inducibly degraded following receptor stimulation by the Skp-Cullin1-F box (SCF)-βTrCP complex [155]. MiR-23b blocks IL-17 signaling by targeting the NF-κB pathway and IκBζ, and is associated with several autoimmune diseases [98]. NF-κB activation is dampened by reversing ubiquitination of TRAFs, and in this regard, a DUB, USP25, was recently implicated in restricting the IL-17 pathway [92, 158]. Additionally, we described another deubiquitinase, A20, which acts by removing ubiquitin chains from TRAF6 (**Chapter 3**, [158]). While USP25 activity appears thus far to be limited to the TLR and IL-17 pathways [92, 159], A20 restricts NF-κB activation by TNFR, IL-1R, Nod-like receptors and TLRs as well as IL-17R [85].

In the TNFα, LPS and IL-1β signaling pathways, adaptor proteins such as TAX1BP1, Itch and RNF11, provide essential assistance to A20 [85, 117, 160]. In response to these inflammatory signals, TAX1BP1, Itch and RNF11 inducibly interact to form the A20 ubiquitin-editing complex [160]. Similar to $A20^{-/-}$ cells, cells lacking TAX1BP1, Itch or RNF11 exhibit persistent activation of NF-κB in response to inflammatory stimuli [161]. Although it is unclear what regulates the inducible interactions among A20, TAX1BP1, Itch and RNF11, the association of these proteins is critical for the termination of NF-κB signaling. Recent reports have shed some light into the individual roles of these proteins. For example, TAX1BP1 is

considered a ubiquitin-binding adaptor molecule for A20 [162]. This finding suggests a model where TAX1BP1 recruits K63-ubiquitinated proteins (TRAF6, RIP1) to the A20-TAX1BP1 complex and aids the deubiquitination function of A20 [163]. Similarly, in response to TNFα and LPS stimulation, Itch and RNF11 bind to the A20-TAX1BP1 complex and assist in A20-mediated NF-κB and MAPK inhibition [160, 164]. In addition to TNFα, TLR and IL-1β, A20 also inhibits IL-17 signaling **(Chapter 3)**. Therefore, we hypothesized that TAX1BP1 and Itch were required for A20-mediated inhibition of IL-17 receptor signaling.

Efforts to decipher signaling events downstream of the IL-17 receptor have largely focused on the IL-17RA subunit. However, IL-17RC also confers essential, but poorly understood, signaling properties [58]. Mutants of IL-17RC lacking the cytoplasmic tail, especially the SEFIR domain, fail to rescue signaling in an IL-17RC-deficient setting [55, 57]. Accordingly, a previous graduate student in the Gaffen laboratory, Allen Ho, performed a yeast-two hybrid screen to identify novel signaling intermediates that bind to IL-17RC [165]. The murine IL-17RC cytoplasmic domain was used as bait in order to screen for proteins that associated with this domain. One of the most frequent binding candidates was the anaphase promoting complex protein 7 (AnapC7, also known as APC7). Intriguingly, AnapC7 has never been previously associated with an inflammatory signaling pathway. In fact, the only described role of AnapC7 is in the Anaphase Promoting Complex (APC), as an adaptor protein. APC is a multi-protein E3 ubiquitin ligase that triggers the transition from metaphase to anaphase. APC regulates eukaryotic mitotic progression by targeting proteasomal degradation of specific proteins such as securin [166]. AnapC7 is a vertebrate-specific subunit of the APC [167]. Although AnapC7 itself does not possess enzymatic function, it is essential for APC activity. In addition to regulating APC progression, AnapC7 been shown to interact with the transcriptional

coactivators CBP and p300 and stimulate their transcriptional activity [168]. AnapC7 and its structurally and functionally related APC complex proteins, AnapC5 and AnapC3, contain multiple tetratrico peptide repeat (TPR) motifs that mediate the binding of adaptor protein CDH1 [167, 169-171]. Recruitment of CDH1 and other AnapC complex proteins is essential for the proper functioning of the APC [166]. The role of AnapC7 in the recruitment of APC subunits indicates that AnapC7 could potentially act as an adaptor protein in inflammatory pathways such as IL-17. Therefore, I chose to further analyze AnapC7 as a potential adaptor in the IL-17R signaling pathway.

Surprisingly, we found that neither TAX1BP1 nor Itch appear to participate in the IL-17 receptor pathway. Moreover, AnapC7 showed no detectable impact on IL-17 receptor signaling. However, we detected association of AnapC7 with both IL-17 receptor subunits (IL-17RA and IL-17RC). Therefore, we tested a closely related APC subunit, AnapC5 for a potential role in IL-17 signaling. AnapC5 was also able to bind IL-17RA and IL-17RC, and AnapC5 silencing enhanced IL-17-induced gene expression, suggesting an inhibitory activity. Strikingly, AnapC5 could also bind to A20 and interact with IL-17RA in the same receptor subdomain that binds to A20 [158]. Therefore, these data suggest a new model in which AnapC5 participates in IL-17 receptor signal transduction by forming a complex with the inhibitor A20.

4.2 RESULTS

4.2.1 The A20 accessory molecules Itch and TAX1BP1 are not involved in inhibitory IL-17 signaling.

Accessory molecules such as TAX1BP1 and Itch associate with A20 to form an active inhibitory complex in the TNFR and IL-1/TLR signaling cascades [120]. Given the negative regulatory role of A20 in the IL-17R signaling pathway, we investigated whether TAX1BP1 and Itch were required for IL-17-mediated inhibitory signaling. Surprisingly, knockdown of TAX1BP1 and Itch expression with siRNA revealed no significant modulation in IL-17-dependent expression of *Il6* or *Ccl20* mRNA levels, as measured by qPCR**.** Similar findings were made for additional IL-17 target genes, including *Ikbz* and *cxcl1*, though we observed very modest enhancements in these genes upon knockdown of TAX1BP1 and Itch **(Figure 4.1-A)**. The efficiency of TAX1BP1 and Itch knockdown was high, as confirmed by qPCR **(Figure 4.1-B)**. These data suggest that the A20 accessory molecules TAX1BP1 and Itch are dispensable for the negative regulatory functions of A20 downstream of IL-17.

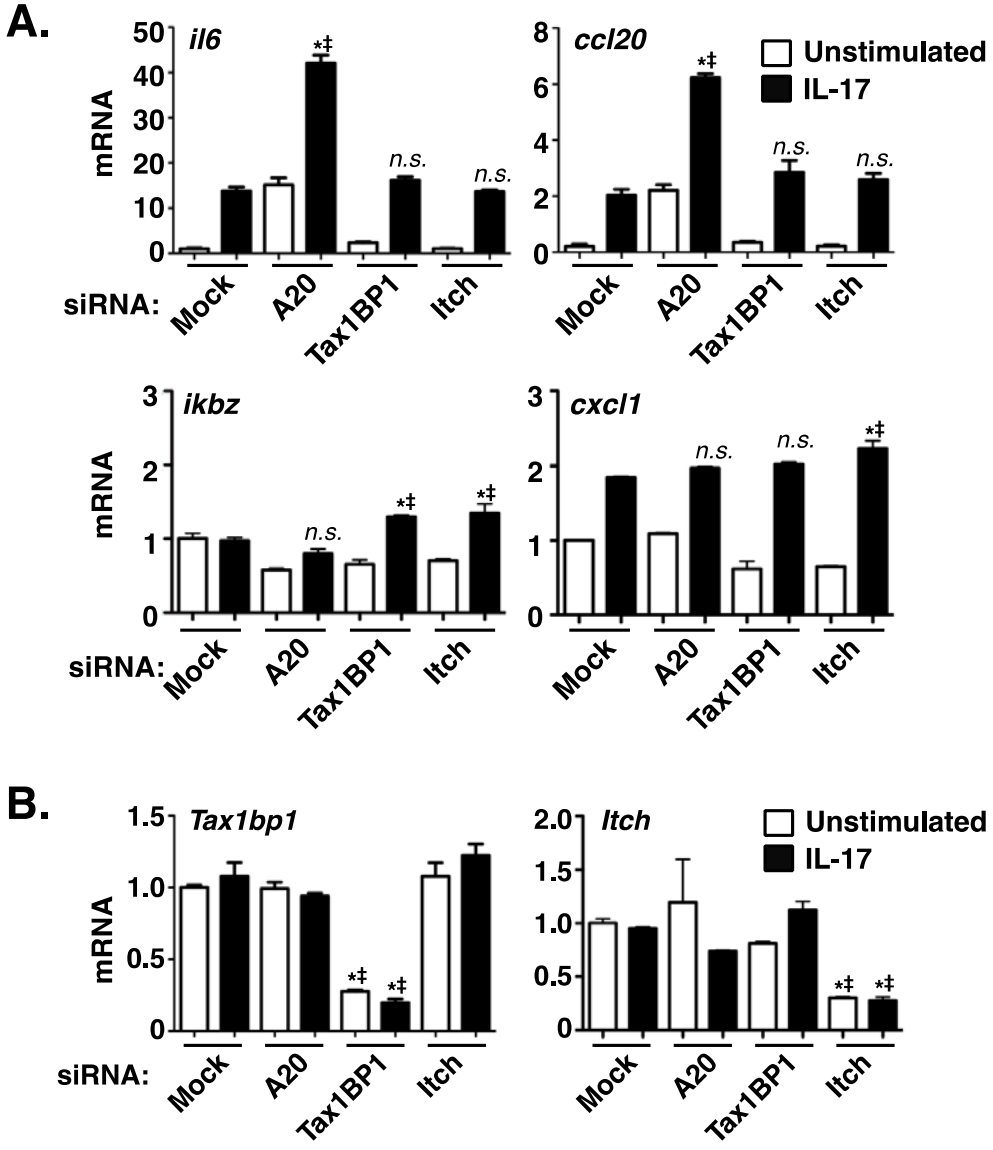


Figure 4.1: The A20 accessory molecules Itch and TAX1BP1 are not involved in inhibitory IL-17 signaling.

(A) Knockdown of Itch and TAX1BP1 does not impact IL-17R mediated signaling. ST2 cells were transfected with the indicated siRNAs, stimulated with IL-17 for 24 h, and mRNA expression was assessed by qPCR. **(B)** Efficient knockdown of Itch and TAX1BP1. mRNA from the samples in panel B were assessed for Itch and TAX1BP1 expression by qPCR. *n.s.*, not significant. *p<0.05 by ANOVA and post-hoc Tukey's test compared to mock-transfected controls. ‡ p<0.05 by Chi Square comparing experimental replicates. These experiments were conducted in collaboration with Lauren Kinner.

4.2.2 AnapC7 associates with the IL-17 receptor subunits but is dispensable for IL-17-dependent signaling.

To determine whether AnapC7 associated with IL-17RC or other IL-17R subunits, HEK293T cells were co-transfected with AnapC7 (HA-tagged) together with full length IL-17RA and IL-17RC (Myc-tagged) or a panel of receptor truncation mutants, and their ability to interact was evaluated by co-immunoprecipitation. AnapC7 associated with IL-17RC, confirming the yeast-two hybrid result. AnapC7 associated with IL-17RC mutants truncated at the C-terminal end of the SEFIR domain **(Figure 4.2-A,B)**, suggesting that the association of AnapC7 with IL-17RC occurs somewhere within the conserved SEFIR domain. AnapC7 also associated with full-length IL-17RA. However, a deletion of IL-17RA lacking the C-terminal "CBAD" domain abrogated this association **(Figure 4.2-A,B)**. Thus, AnapC7 interacts with both IL-17RA and IL-17RC.

There are no available AnapC7 knockout mice. To determine whether AnapC7 plays a role in IL-17R signaling, we silenced AnapC7 expression via siRNA knockdown in ST2 cells. Knockdown of AnapC7 had no impact on IL-17-dependent IL-6 production at the mRNA or protein levels **(Figure 4.2-C)**. As a control, we verified that siRNA silencing of Act1, a positive mediator of IL-17 signaling, reduced IL-17 signaling, as measured by IL-6 production. As expected, silencing of the DUBs A20 or USP25, recently shown to be inhibitors of IL-17 signaling [92, 158], resulted in enhanced IL-17-mediated IL-6 production **(Figure 4.2-C)**. These results demonstrated that, while AnapC7 associates efficiently with the IL-17 receptor complex, its activity is dispensable for functional IL-17-mediated signaling, either positively or negatively.

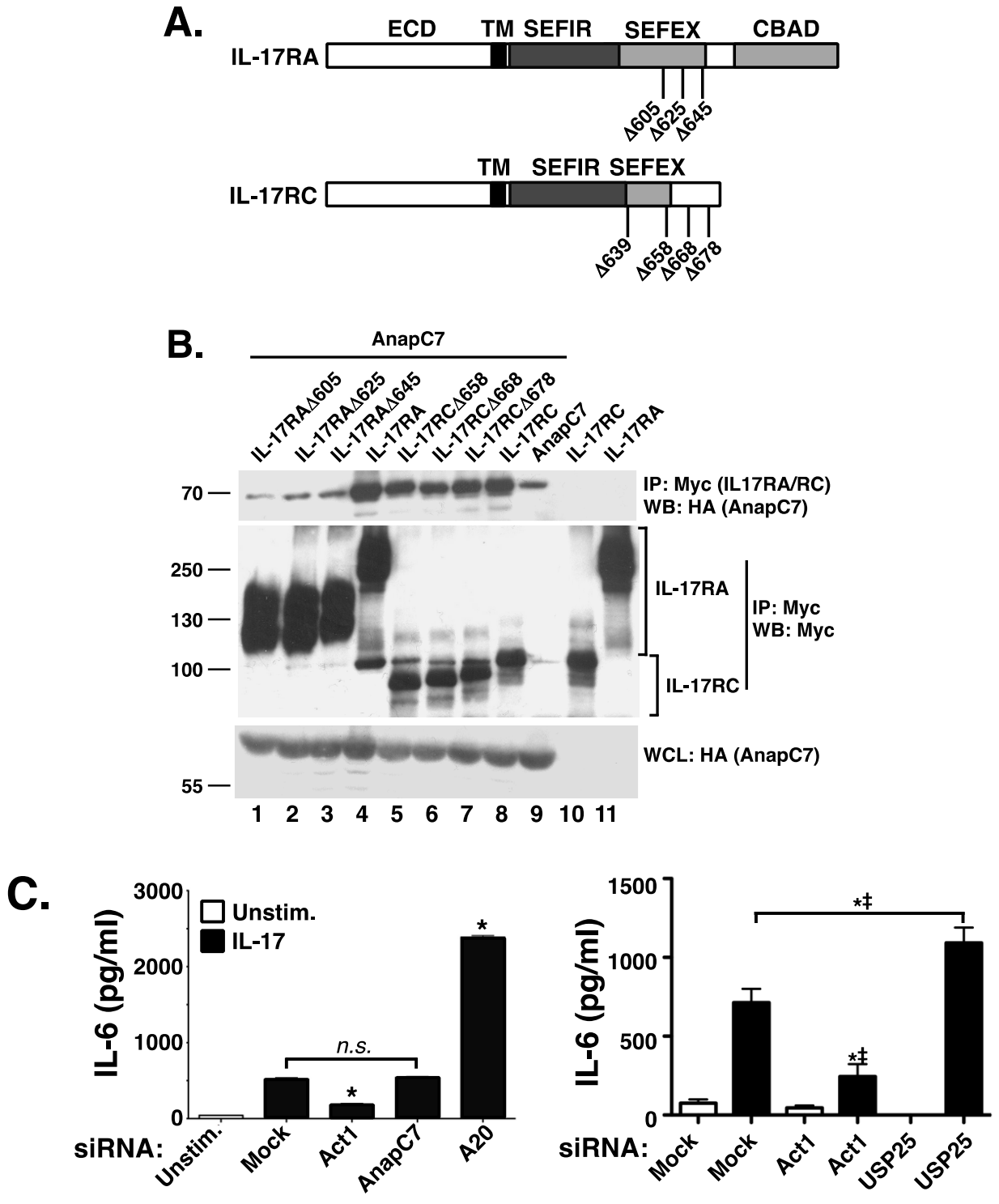


Figure 4.2: AnapC7 binds to IL-17R but does not impact IL-17 signaling.

(A) Schematic diagram of IL-17RA and IL-17RC mutants. ECD, extracellular domain. SEFIR and SEFEX domain approximate boundaries are indicated [61, 99]. CBAD, C/EBPβ activation

domain. (**B)** AnapC7 associates with IL-17RA and IL-17RC. HEK293T cells were transfected with AnapC7 tagged with HA and IL-17RA or IL-17RC tagged with Myc, as indicated. Lysates were immunoprecipitated with anti-Myc Abs and immunoblotted for HA or Myc. Whole cell lysates (WCL) were verified for AnapC7 by staining with anti-HA. Migration of protein size markers is indicated. **(C)** RNA silencing of AnapC7 does not alter IL-17-dependent signaling. ST2 cells were transfected with the indicated siRNAs, treated with IL-17 (black bars) for 24 h and IL-6 in culture supernatants assessed by ELISA. *n.s.*, not significant. *$p<0.05$ by ANOVA and post-hoc Tukey's test relative to unstimulated controls. ‡ $p<0.05$ by Chi Square comparing experimental replicates.

4.2.3 AnapC5 associates with IL-17RA through the receptor CBAD domain and negatively regulates IL-17 receptor signaling.

AnapC5 is another structural component of the APC/C. Like AnapC7, AnapC5 also activates the cAMP response element-binding protein (CREB) binding protein (CBP)/p300 complex [168]. Because of the analogous roles played by AnapC5 and AnapC7 in CBP/p300 regulation and the presence of common structural motifs in both proteins, we hypothesized that AnapC5 might participate in IL-17R signaling. To determine whether AnapC5 associated with the IL-17 receptor, HEK293T cells were co-transfected with AnapC5 and IL-17RA or IL-17RC, and interactions between these factors were evaluated by co-IP. AnapC5 associated efficiently with IL-17RA and IL-17RC (**(Figure 4.3-A)**, lanes 5-6). We further verified that AnapC5 also associated with AnapC7 (**(Figure 4.3-A),** lane 8). Thus, both AnapC5 and AnapC7 form a complex with the IL-17 receptor.

We then sought to delineate motifs within the IL-17R required for interaction with AnapC5. HEK293T cells were co-transfected with AnapC5 and a panel of IL-17RA truncation mutants. AnapC5 associated only with full-length IL-17RA and to a lesser extent IL-17RAΔ800, but not with other deletion mutants despite equivalent expression of the receptor mutants **(Figure 4.3-B)**. Thus, AnapC5 associates with a C-terminal domain of IL-17RA. This domain corresponds to the CBAD [89, 99], which has been previously linked to inhibitory signaling [89, 90, 99, 158]. We also attempted to define regions within the IL-17RC cytoplasmic tail necessary for co-association using a panel of IL-17RC truncation mutants. AnapC5 co-immunoprecipitated with all IL-17RC deletion mutants tested, suggesting that the site of interaction is within the SEFIR domain **(Figure 4.3-C)**.

To determine whether the association of AnapC5 with IL-17RA/C impacted IL-17 signaling, we employed siRNA to silence AnapC5 in ST2 cells **(Figure 4.4-A)**. When compared to cells transfected with a control siRNA, knockdown of AnapC5 resulted in significantly increased IL-17-dependent IL-6 expression **(Figure 4.4-A)**, suggesting that AnapC5 exerts an inhibitory function. Similar findings were made for *Il6* mRNA (data not shown). Silencing efficiency was verified by qPCR **(Figure 4.4-B)**. These findings indicated that AnapC5 binds to the IL-17R and dampens IL-17-mediated signal transduction.

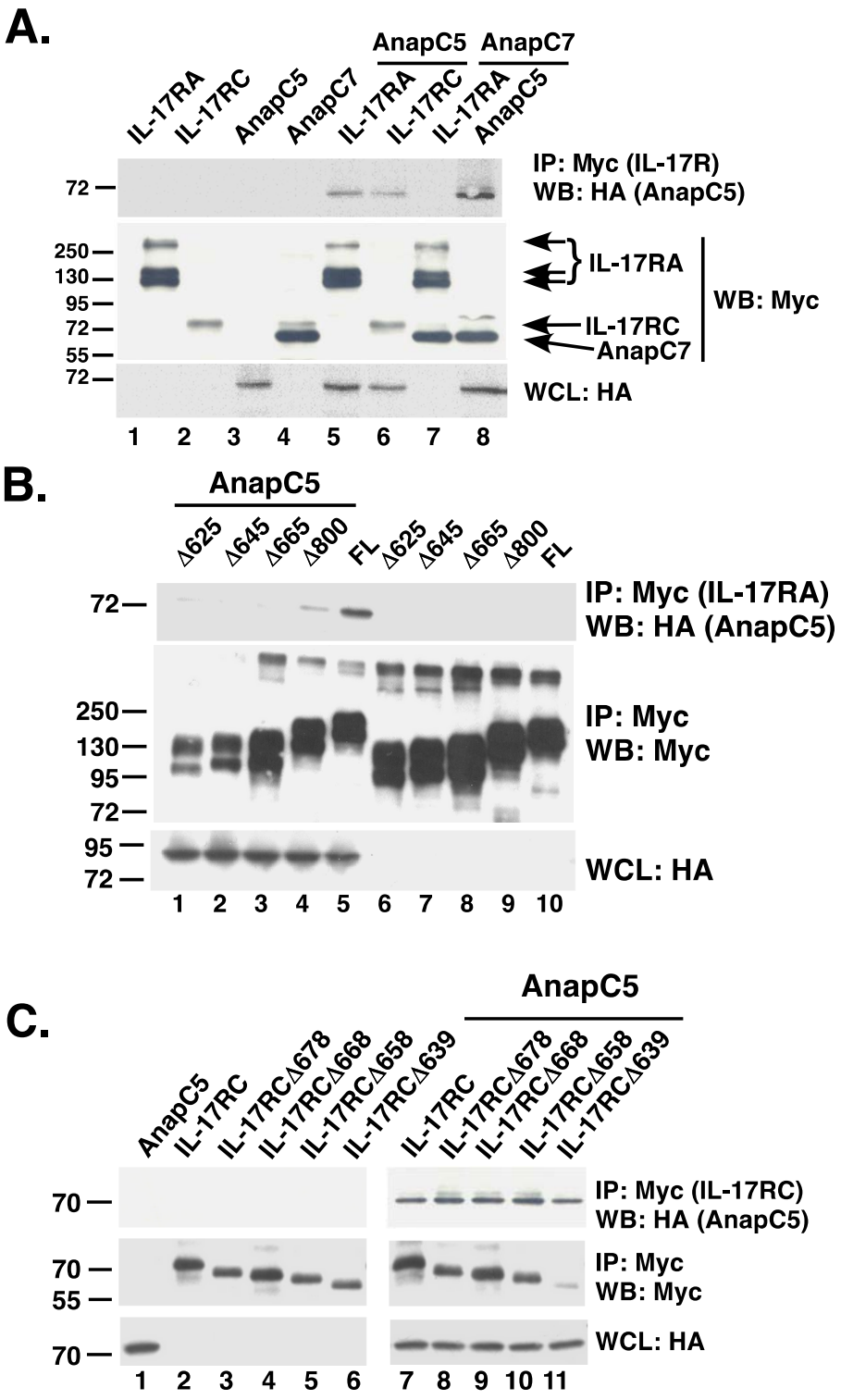


Figure 4.3: AnapC5 binds to the IL-17RA CBAD domain.

(A) AnapC5 associates with IL-17RA, IL-17RC and AnapC7. HEK293T cells were transfected with AnapC5 (tagged with HA) together with IL-17RA, IL-17RC or AnapC7 (tagged with

Myc), as indicated. Lysates were subjected to IP with anti-Myc Abs and immunoblotted with anti-HA or anti-Myc. Arrows indicate identity of each Myc-tagged protein. Whole cell lysates (WCL) were verified for AnapC7 by staining with anti-HA. Migration of protein size markers is indicated. **(B)** AnapC5 associates with IL-17RA through the inhibitory CBAD domain. HEK293T cells were transfected with AnapC5 and the indicated IL-17RA deletion constructs. Lysates were subjected to co-IP with anti-Myc and blotted for HA or Myc. Whole cell lysates (WCL) were verified for AnapC7 by staining with anti-Myc. Migration of protein size markers is indicated. **(C)** AnapC5 associates with IL-17RC in the SEFIR domain. HEK293T cells were transfected with AnapC5 and the indicated IL-17RC deletion constructs. Lysates were subjected to co-IP with anti-Myc and blotted for HA or Myc. Whole cell lysates (WCL) were verified for AnapC7 by staining with anti-HA. Migration of protein size markers is indicated. These experiments were conducted in collaboration with Leticia Monin.

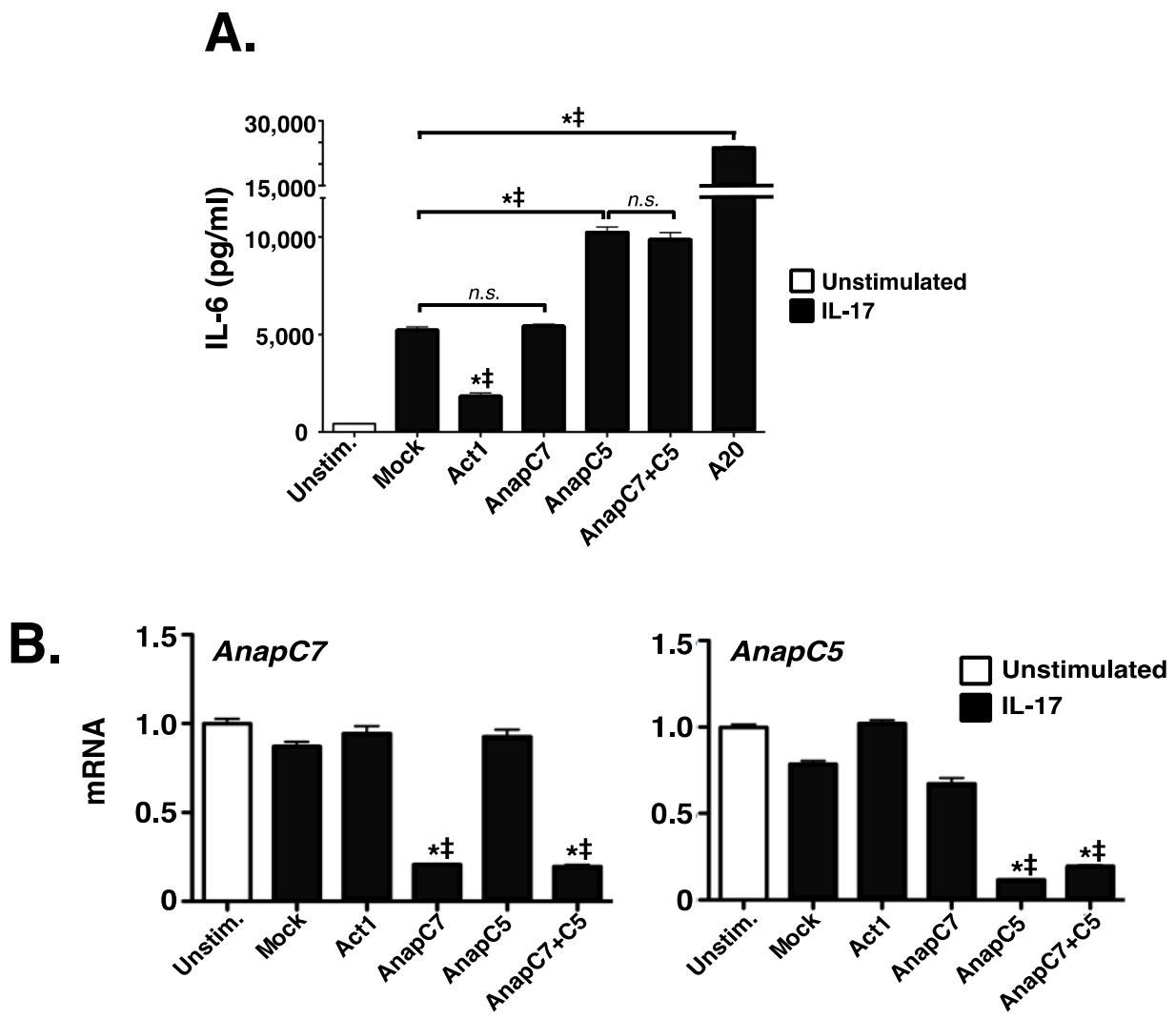


Figure 4.4: AnapC5 restricts IL-17-mediated signal transduction.

(A) Knockdown of AnapC5 enhances IL-17 signaling. ST2 cells were transfected with the indicated siRNAs, stimulated with IL-17 for 24 h, and IL-6 in culture supernatants assessed by ELISA. **(B)** Efficient knockdown of AnapC5 and AnapC7. mRNA from the samples in panel D were assessed for AnapC5 and AnapC7 expression by qPCR. *n.s.,* not significant. **p*<0.05 by ANOVA and post-hoc Tukey's test compared to unstimulated controls. ‡ *p*<0.05 by Chi Square comparing experimental replicates.

4.2.4 AnapC5 directly associates with A20.

Our recent data implicated the DUB A20 as a negative regulator of IL-17-dependent signaling, which binds to the CBAD within IL-17RA [33, 89, 90, 99]. Since AnapC5 associated with the CBAD and knockdown of AnapC5 enhanced IL-17 signaling, we hypothesized that AnapC5 might interact with A20. To test this hypothesis, A20 was co-transfected with AnapC5 (both the long and short forms of the gene) and AnapC7. As shown, A20 indeed co-immunoprecipitated with AnapC5, but not with AnapC7 **(Figure 4.5-A)**, potentially explaining the failure of AnapC7 knockdown to impact IL-17 signaling. These data are also consistent with the binding of AnapC5 to the CBAD, which is associated with negative regulation of IL-17 signaling **(Chapter 3, Section 4.3)**. To verify this finding in a more physiological setting, we transfected HEK293T cells with AnapC5 constructs and saw co-immunoprecipitation of endogenous A20, while there was no precipitation with a control plasmid **(Figure 4.5-B)**. Thus, AnapC5 associates with A20 and may facilitate the inhibitory functions of A20 downstream of the IL-17R **(Figure 4.6)**. Cumulatively, these findings suggest that AnapC5, but not TAX1BP1 or Itch, may serve as an accessory protein for A20 in IL-17 signal transduction.

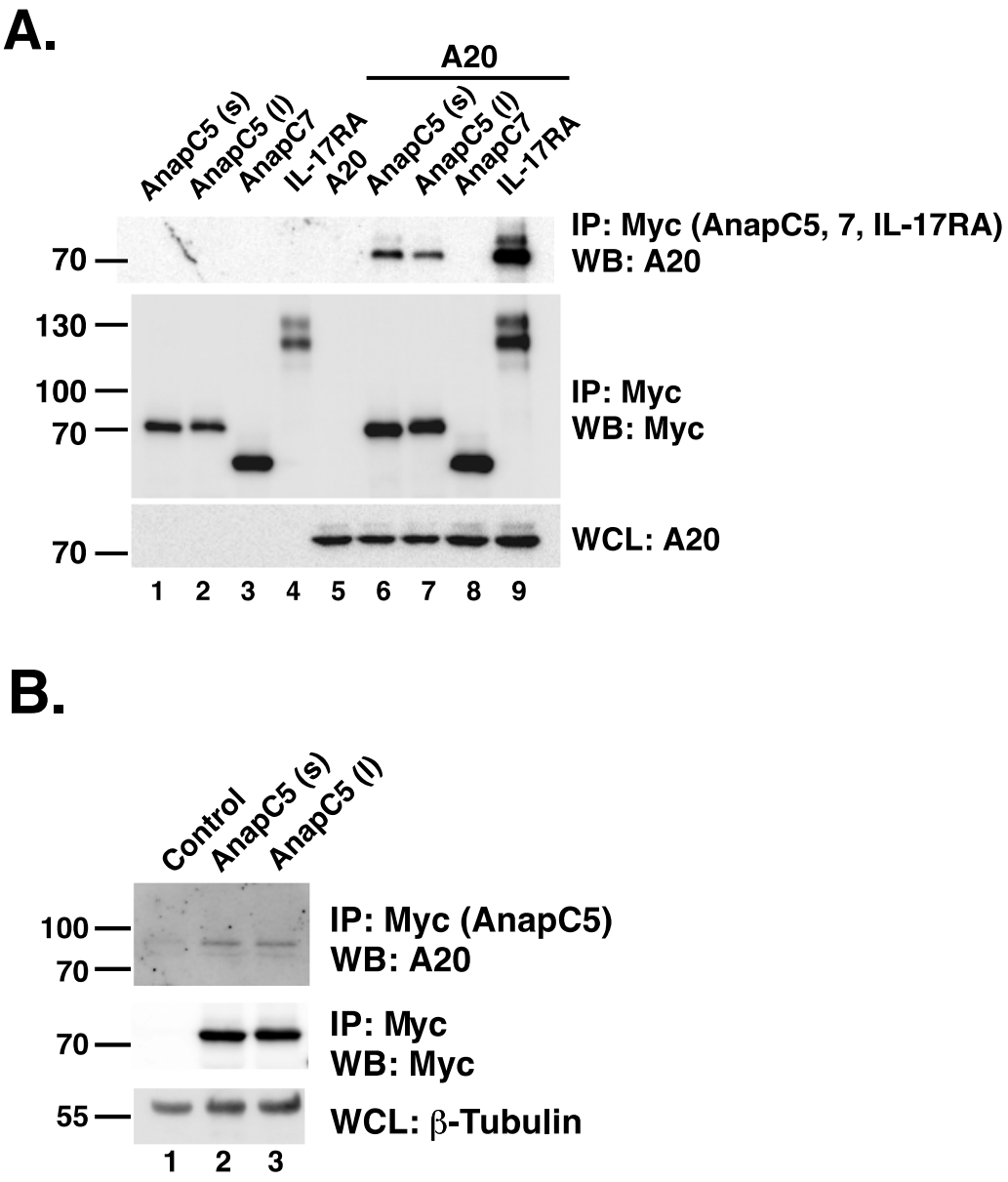


Figure 4.5: AnapC5 directly associates with A20.

(A) AnapC5 but not AnapC7 associates with A20. HEK293T cells were co-transfected with AnapC5 [short (s) and long (l) forms] or AnapC7 (both Myc-tagged) together with A20. Lysates were subjected to IP with anti-Myc Abs and blotted for A20 or Myc. WCL were blotted for A20. Migration of protein size markers is indicated. (**B)** AnapC5 associates with endogenous A20. HEK293T cells were transfected with Myc-tagged AnapC5. Lysates were subjected to IP with anti-Myc Abs and blotted for A20 or Myc. WCL were blotted for β-tubulin as a loading control. These experiments were conducted by Allen Ho.

4.3 DISCUSSION

IL-17 has emerged as a critical inflammatory cytokine involved in both host defense and autoimmune pathology. IL-17 promotes disease in a variety of mouse models of disease including multiple sclerosis, RA and IBD [20, 172-174]. Consequently, the IL-17 family is an appealing biological target that shows efficacy in early clinical trials [175]. On the host side, IL-17 is important for immunity to extracellular pathogens, most strikingly those of fungal origin such as *Candida albicans* [108, 176].

Efforts to understand the IL-17 signaling pathway have lagged behind other cytokine families. Superficially, IL-17 appears to share many of the same pathways and gene targets as other pro-inflammatory cytokines; for example, engaging the NF-κB and MAPK pathways and inducing expression of canonical inflammatory mediators such as IL-6 and CXC chemokines [18, 65]. However, IL-17R structure and signaling are atypical in multiple ways. The three-dimensional architecture of IL-17 family receptor and ligands are distinct from other cytokines [15, 177, 178]. The proximal adaptor Act1, required for NF-κB signaling, is not employed as a positive signaling intermediate by any other known inflammatory signaling pathway [115]. Similarly, the means by which IL-17 promotes stability of target mRNA transcripts is unusual, involving the Splicing Factor 2 (SF2) RNA binding protein rather than the more classical tristetraprolin protein [53, 152].

Polymorphisms in the gene encoding A20 (*TNFAIP3*) are implicated in various forms of autoimmunity and some cancers [85, 179]. A20 is a deubiquitinase (DUB) that serves as a negative feedback inhibitor of TNFα, IL-1, and IL-17 [85, 158]. A20 acts on the IL-17 signaling pathway by down-modulating the NF-κB and MAPK pathways through

deubiquitination of TRAF6 [119, 158]. In addition to its activity as a DUB, A20 also exhibits Ub ligase activity [135], which helps dictate the types of Ub linkages used to modulate signaling. In TNFα, IL-1β and TLR signaling, TAX1BP1, Itch and RNF11 are vital for A20 activity [160, 161]. However, the A20-accessory proteins Itch and TAX1BP1 had no apparent role in inhibiting IL-17-mediated signaling in my studies **(Figure 4.1).** Although this does not absolutely rule out a role for either factor in the IL-17 signaling pathway, they do not seem to be critical players. Moreover, we did not analyze whether RNF11 is involved in the regulation of IL-17 signaling, which would be important to test in the future. Finally, as IL-17 synergizes with TNFα and IL-1β, TAX1BP1, Itch and RNF11 might still have an effect on the synergistic target gene expression by these other cytokines.

We also examined the role of AnapC7, one of the most frequent "hits" yielded in the two hybrid screen, in regulating IL-17 signal transduction. AnapC7 is best understood as a component of a macromolecular E3 ubiquitin ligase complex required for mitotic progression [167]. AnapC7 is also implicated in regulating gene transcription by interacting with the CBP/p300 coactivator and stimulating its acetyl transferase activity [168]. Although AnapC7 interacted with IL-17RA and IL-17RC, knockdown of AnapC7 exerted no apparent impact on IL-17 signaling, either positively or negatively **(Figure 4.2)**. On the other hand, knockdown of AnapC5 enhanced IL-17 dependent signaling, suggesting that it might function as an inhibitor of the pathway **(Figure 4.4)**. AnapC7 and AnapC5 share sequence homology with E1A, an adenoviral protein that drives cellular transformation via CPB/p300 complexes. The present work is to our knowledge the first evidence implicating AnapC5 as a signaling intermediate for cytokine receptor signaling.

A20 binds to IL-17RA through a distal inhibitory domain in the receptor known as the "CBAD," a domain originally identified based on its role in regulation of the C/EBPβ transcription factor [89, 99]. Since AnapC5 is part of a multi-protein E3 Ub ligase complex and also binds to the CBAD **(Figure 4.3)**, we speculated that its ability to inhibit IL-17 signaling might be regulated through A20. Indeed, A20 interacted strongly with AnapC5, but not with AnapC7 **(Figure 4.5)**. Together, these data raised the possibility that the AnapC5 is part of a novel inhibitory complex that includes A20 and that assembles on the IL-17R CBAD domain **(Figure 4.6)**.

It is unclear what role, if any, is played by AnapC7 in IL-17 signaling. Unlike AnapC5, silencing of AnapC7 did not impact IL-17 induction of genes such as *Il6*. One possibility is that AnapC7 acts as a scaffold protein for as-yet unidentified factors that mediate signal transduction specificity by IL-17RC **(Figure 4.6)**. In this regard, the role of the IL-17RC cytoplasmic tail in IL-17R signaling is still unresolved. Deletion studies indicate that the IL-17RC tail is essential [55, 57], so the receptor is evidently needed for more than just engaging the ligand. We previously showed that a dimer of the IL-17RC tail is insufficient to mediate signaling; namely, a chimeric receptor composed of the extracellular and transmembrane domain of IL-17RA fused to the cytoplasmic tail of IL-17RC could not rescue signaling in IL-17RA-deficient cells [33]. However, it is not known whether IL-17RC recruits novel proteins into the IL-17R signaling complex, or whether this subunit is simply needed to multimerize or stabilize existing signaling complexes by providing additional binding sites for proteins such as Act1 or the AnapCs.

There are important questions raised by this study that will need to be explored in future studies. For example, what are the relative contributions of A20, βTrCP1 and USP25 on inhibition of IL-17 signaling? Are they redundant, cooperative or do they play tissue-specific

roles? Does AnapC5 serve as a “switch” to favor one inhibitory pathway over the other? How important is the interaction of AnapC5 in binding to IL-17RC versus IL-17RA? Ultimately, defining the molecular events in the IL-17R pathway may yield new therapeutic targets, and will certainly provide new insights into how this intriguing cytokine receptor family operates.

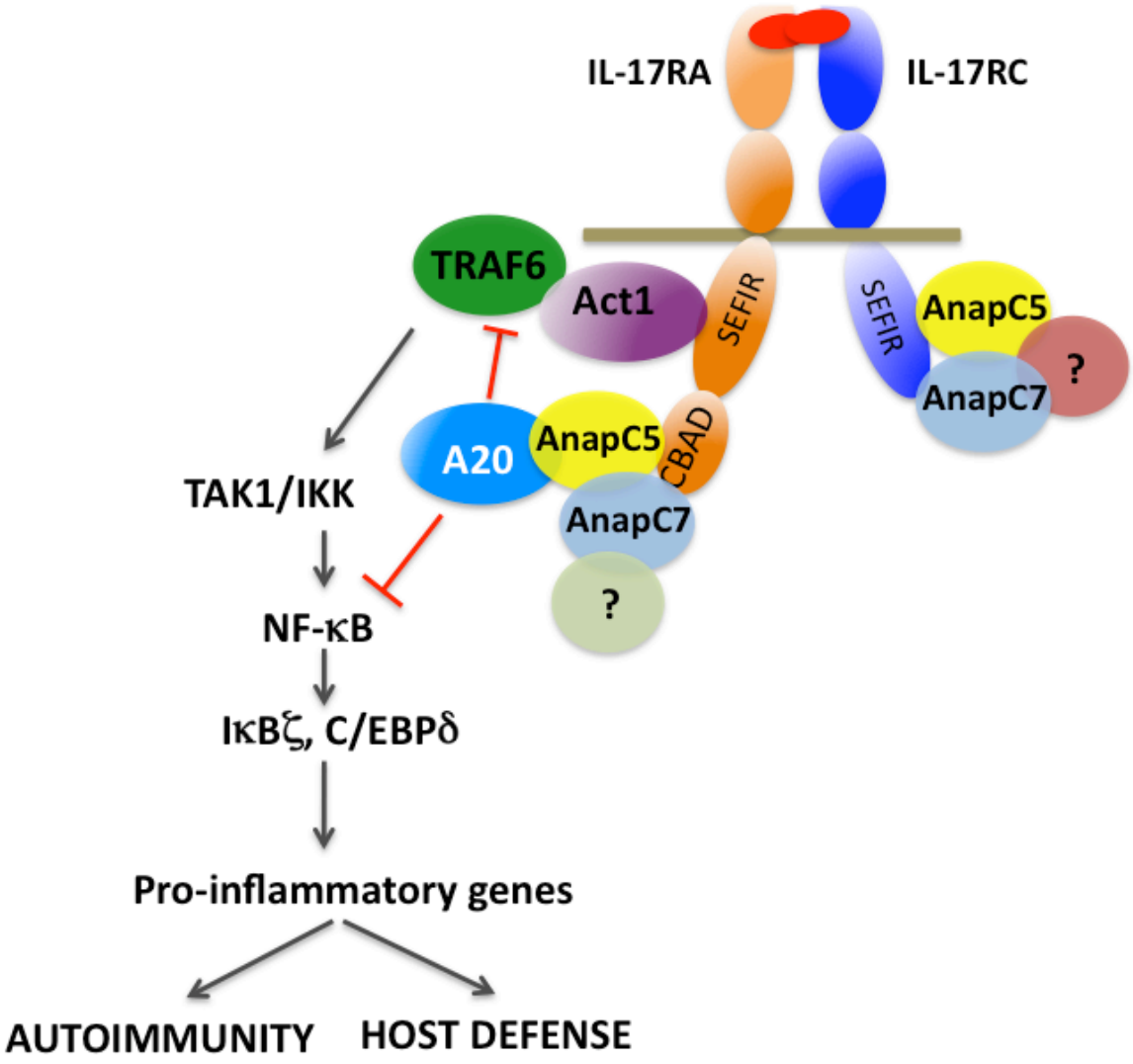


Figure 4.6: Schematic model of AnapC5 and AnapC7 in IL-17R signaling.

The data discussed above support a model in which AnapC5 serves an adaptor or scaffold protein to facilitate A20 recruitment to the CBAD domain of IL-17RA. Although AnapC7 binds to both IL-17RA and IL-17RC, its functional role is still unclear.

5.0 CHAPTER FIVE: MCPIP1 (REGNASE-1) INHIBITS IL-17 SIGNALING BY MEDIATING DEGRADATION OF IL-17 RECEPTOR TRANSCRIPT

5.1 BACKGROUND

Proinflammatory cytokines such as IL-17 exert their function by inducing expression of inflammatory cytokines and chemokines. Additionally, inflammatory signaling pathways switch on expression of proteins that act as feedback inhibitors of these pathways. For example, A20 and miR-23b expression are regulated by IL-17, and these molecules act as feedback inhibitors of IL-17 pathway [98, 158]. Therefore, in line with our interest in elucidating inhibitory mechanisms of IL-17 pathway, we aimed to identify novel physiologically relevant feedback inhibitors of IL-17 signaling. To this end, we mined previously published microarray data comparing the gene expression profiles in WT and IL-17RA$^{-/-}$ mice infected orally with *C. albicans*, a setting in which IL-17RA$^{-/-}$ mice are susceptible to disease [28]. We screened for the genes that were induced more strongly in WT mice than in IL-17RA$^{-/-}$ mice. Through this screen, we identified *Zc3h12a* (Zinc Finger CCCH-type containing 12A) as an IL-17-induced gene. *Zc3h12a* encodes a protein called MCPIP1 (Monocyte Chemotactic Protein-1 Induced Protein-1, also known as Regnase-1) that had previously shown to inhibit TLR and IL-1R signaling [101, 180]. Therefore, we decided to assess whether MCPIP1 is involved in the regulation of IL-17R signaling.

MCPIP1 is expressed constitutively in most cells types, including both immune cells (particularly T cells) and non-immune cells. Furthermore, MCPIP1 expression is upregulated by several cytokines and inflammatory stimuli, including MCP-1, IL-1β and LPS [181]. MCPIP1 belongs to a family of proteins (MCPIP1 through MCPIP4) that contain a highly conserved Nedd4-BP1, YacP Nuclease (NYN) domain with RNase activity at the N-terminus and a single CCCH-type zinc finger domain with RNA-binding potential in the intervening region [182-184]. Other examples of CCCH-type zinc finger proteins include tristetraprolin (TTP). TTP was

the first identified CCCH-type zinc finger protein and acts as an RNA destabilizing agent by binding to the adenylate-uridylate-rich (AU-rich) elements (AREs) in mRNA to increase RNA turnover [185, 186]. Through this activity, TTP regulates the expression of multiple cytokines and chemokines such as TNFα, IL-1 and CXCL1. However, studies so far suggest that IL-17 signaling employs TTP-independent mechanisms to regulate mRNA stability [77].

Like other CCCH-type zinc finger proteins, MCPIP1 is an RNase that inhibits inflammatory cytokine expression by degrading *Il6*, *Il1b* and *Il12b* (p40) mRNA [183]. The RNase activity of MCPIP1 is dependent on its CCCH-type zinc finger domain of MCPIP1, which includes three cysteine residues and one histidine residue. The zinc finger coordinates zinc ion binding, which is necessary for RNA-binding capacity of MCPIP1. MCPIP1 catalytic function also depends on Mg^{2+} ion binding. The crystal structure of the N-terminal RNase domain of human MCPIP1 as well as MCPIP1 point mutant studies revealed a catalytic pocket composed of several conserved acidic amino acids, including several aspartate residues that are involved in Mg^{2+} ion binding [101, 183, 184, 187].

MCPIP1 inhibits LPS, IL-1β and TNFα signaling by degrading *Il6* and *Il12b* mRNA and restricting ensuing inflammation [180, 183, 188]. MCPIP1 also suppresses miRNA biosynthesis and activity via cleavage of the terminal loops of precursor miRNAs (pre-miRNAs) and inhibiting DICER activity [187]. Additionally, MCPIP1 inhibits LPS, IL-1β and TNFα-mediated NF-κB and c-Jun N-terminal kinase (JNK) activation by deubiquitinating TRAFs, including TRAF2, TRAF3 and TRAF6 [101]. Thus, by regulating mRNA decay of inflammatory proteins as well as through its DUB activity, MCPIP1 potently suppresses aberrant activation of innate and adaptive immune cells.

Consistent with a critical role for MCPIP1 in regulating inflammation, mice lacking MCPIP1 (MCPIP1$^{-/-}$) display severe systemic inflammation characterized by abnormal T cell and B cell activation. Furthermore, these mice show hyperimmunoglobulinemia, autoantibodies, growth retardation, and die prematurely [101, 183]. Systemic inflammation in MCPIPI$^{-/-}$ mice has been attributed to the lack of regulation in both immune and non-immune compartments [189-191]. In T cells, MCPIP1 is expressed constitutively and prevents spontaneous activation of T cells. In addition to degrading *Il6* and *Il12b* mRNA, MCPIP1 also targets degradation of mRNA transcripts that encode key proteins involved in T-cell activation. For example, in T cells, MCPIP1 degrades mRNAs encoding c-Rel, IL-2, OX40 and ICOS [190]. As a result, mice lacking MCPIP1 specifically in T cells are unable to dampen spontaneous T-cell activation, therefore contributing to autoimmune symptoms. Additionally, MCPIP1-mediated regulation of TLR signaling is also relevant in controlling autoimmune inflammation. MCPIPI$^{-/-}$ mice treated with antibiotics to eradicate commensal microbial flora were found to have both better survival and reduced inflammation in spleen and lymph nodes [189]. As previously stated, we found that MCPIP1 expression is also induced by IL-17. Additional reports have confirmed induction of *Zc3h12a* expression by IL-17 in an Act1-dependent manner [75, 192]. However, whether MCPIP1 regulates IL-17R signaling has not been determined.

In this study, we demonstrate that MCPIP1 is a negative regulator of IL-17R signaling. We discovered that *Il17ra* and *Il17rc* mRNA are degraded by MCPIP1, revealing a novel target for MCPIP1. Furthermore, lack of MCPIP1 in mice resulted in enhanced IL-17 induced pulmonary inflammation, better control of disseminated *C. albicans* infection and increased severity of EAE. Therefore, we establish that MCPIP1 is a critical modulator of IL-17 induced inflammation.

5.2 RESULTS

5.2.1 MCPIP1 inhibits IL-17 signaling.

Multiple inflammatory stimuli including LPS, IL-1β, MCPI-1 and IL-17 induce expression of *Zc3h12a* (encoding MCPIP1) [192, 193]. To confirm that *Zc3h12a* is an IL-17 target gene, we stimulated ST2 cells with IL-17 over a time course of 24 hours, and determined relative *Zc3h12a* expression by qPCR. We detected an increased expression of *Zc3h12a* mRNA over baseline as early as 15 minutes after IL-17 stimulation. *Zc3h12a* mRNA expression peaked at approximately 7-fold within one hour and stayed elevated up to 24 hours **(Figure 5.1-A)**. The kinetics of *Zc3h12a* expression are different from *Tnfaip3* expression, which peaks within 30 minutes (10-15 fold over baseline) of IL-17 stimulation, drops rapidly and stabilizes at a lower level (4-5 fold over baseline) **(Figure 3.2A)** [158].

MCPIP1 is a feedback inhibitor of inflammatory receptor signaling pathways such as TLR and IL-1R [101, 181]. Therefore, we hypothesized that MCPIP1 is also an inhibitor of IL-17R signaling. To assess the function of MCPIP1 in IL-17 signaling, ST2 cells were transfected with siRNA targeting MCPIP1 or control non-targeting scrambled siRNA. siRNA targeting Act1, an essential adaptor protein in IL-17R signaling, was used as a positive control. Forty-eight hours after transfection, cells were stimulated with IL-17 for three hours and IL-6 protein levels and mRNA transcript expression were measured by ELISA and qPCR, respectively. As expected, cells transfected with control siRNA exhibited increased IL-6 protein and mRNA expression after IL-17 stimulation, whereas Act1-targeting siRNA led to a significant drop in IL-17-dependent IL-6 expression. Interestingly, we observed that the cells transfected with siRNA targeting MCPIP1 exhibited an increase in baseline IL-6 expression, pointing to an

important role of MCPIP1 in regulating basal inflammatory cytokine expression. However, IL-17 stimulation in these cells resulted in a further significant increase in IL-6 protein and mRNA expression **(Figure 5.1-B,C)**. To confirm the function of MCPIP1 in regulating IL-17 signaling, we tested the effect of MCPIP1 knockdown on other IL-17 target genes. As expected, MCPIP1 knockdown resulted in a significant increase in IL-17-dependent expression of *lcn2* and *csf3*. Finally, we verified that the siRNA targeting MCPIP1 efficiently reduced *Zc3h12a* expression **(Figure 5.2-C)**.

To rule out possible off-target effects of the siRNA, we reconstituted mouse MCPIP1 knockout tail tip fibroblasts with either empty vector (EV) or plasmid expressing MCPIP1. Twenty-four hours after transfection, the cells were stimulated with IL-17 for four hours. Consistent with the earlier observations, reconstitution of MCPIP1 knockout fibroblasts with MCPIP1 resulted in a reduced IL-17-dependent IL-6 protein production, compared to the cells reconstituted with EV. Taken together, these findings indicate that MCPIP1 is a feedback inhibitor of IL-17 signaling.

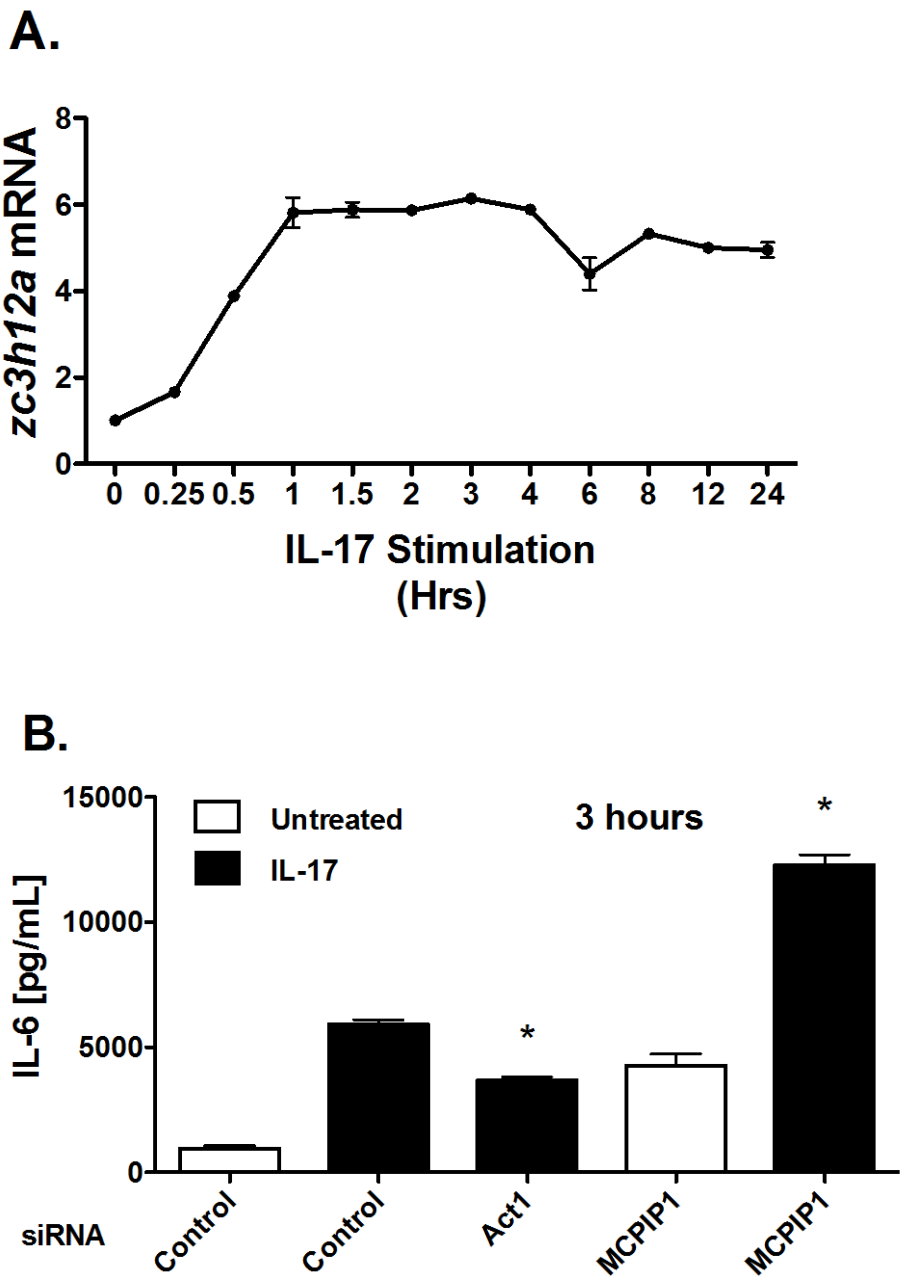

A.
zc3h12a mRNA
0
2
4
6
8
0 0.25 0.5 1 1.5 2 3 4 6 8 12 24
IL-17 Stimulation
(Hrs)
B.
IL-6 [pg/mL]
0
5000
10000
15000
Untreated
IL-17
3 hours
*
*
siRNA
Control
Control
Act1
MCPIP1
MCPIP1

Figure 5.1 Contd.

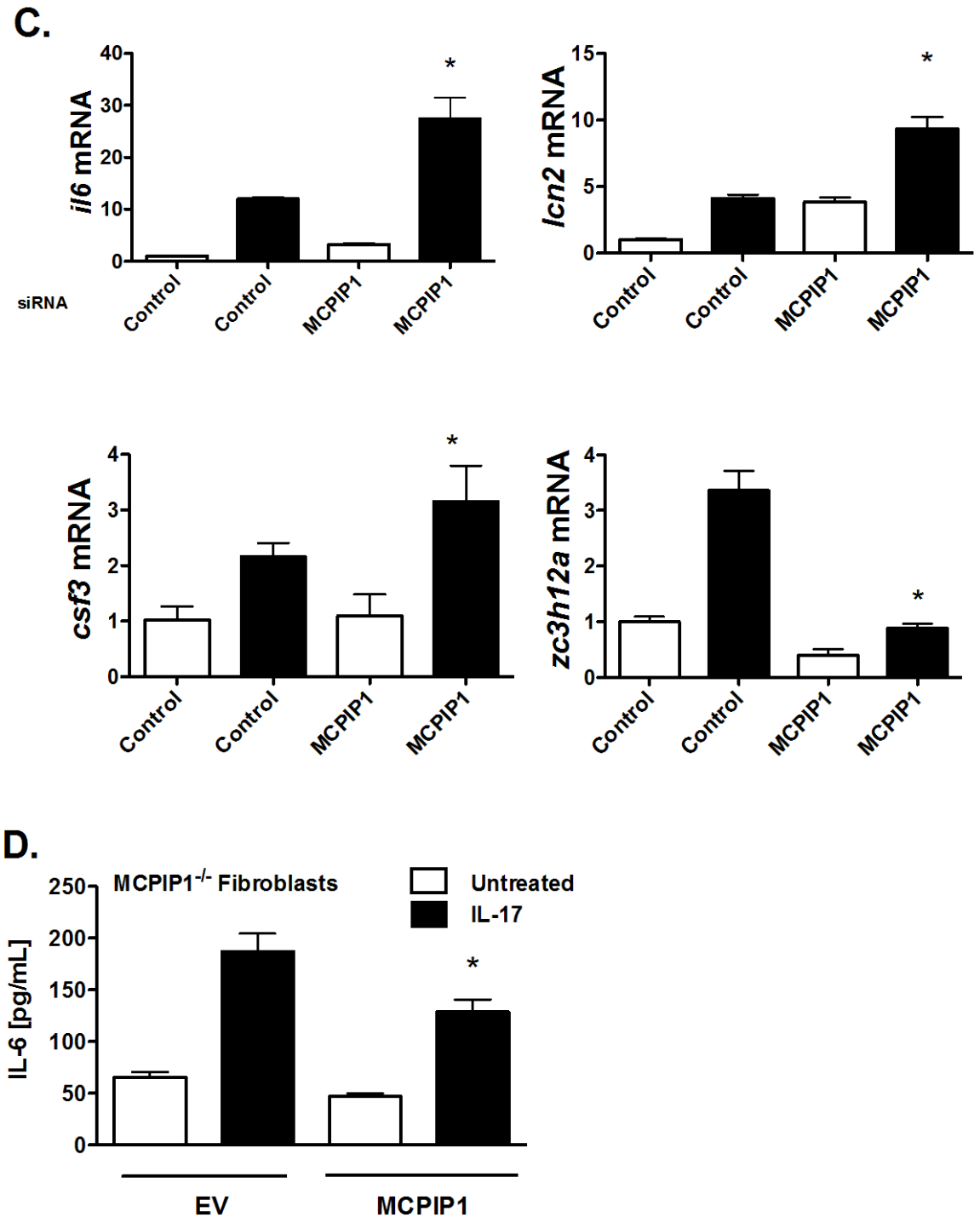


Figure 5.1: MCPIP1 inhibits IL-17 signaling.

(A) Induction of endogenous *Zc3h12a* mRNA expression by IL-17. ST2 cells were treated with IL-17 (200 ng/ml) for the indicated times and *Zc3h12a* mRNA abundance was assessed by qPCR in triplicate assays. Data are presented as the fold-induction in *Zc3h12a* mRNA abundance compared to untreated cells. **(B)** MCPIP1 knockdown enhances the production of IL-6 protein. ST2 cells were transfected with siRNA specific for Act1 or MCPIP1 or with a scrambled siRNA control, and the amount of IL-6 in culture media was assessed in triplicate at three hours after treatment with IL-17 (200 ng/ml). Black bars indicate IL-17-treated samples, and white bars indicate untreated samples. **(C)** MCPIP1 knockdown enhances multiple IL-17 target gene expression. ST2 cells transfected with the indicated siRNAs and left untreated or treated with IL-17 for 3 hours were analyzed for the expression of the indicated genes by qPCR. Successful knockdown of *Zc3h12a* was also confirmed by qPCR. **(D)** Reconstitution of MCPIP1$^{-/-}$ fibroblasts inhibits IL-17 signaling. MCPIP1$^{-/-}$ tail tip fibroblasts were transfected in triplicate with EV or murine A20 and either left untreated or treated with IL-17 for 4 hours. IL-6 protein in supernatants was assessed by ELISA. **P* <0.05 by ANOVA with post-hoc Tukey's test compared to cells transfected with control siRNA or EV and treated with IL-17.

5.2.2 Mechanism of MCPIP1-mediated IL-17 signaling is distinct from USP25 and A20.

DUBs such as USP25 and A20 inhibit TLR and IL-17R signaling pathways [85, 92, 158, 159]. Since MCPIP1 also functions as a DUB in the TLR pathway [101], we examined whether MCPIP1-mediated inhibition of IL-17 signaling is redundant with USP25 and A20. We transfected ST2 cells with siRNA targeting MCPIP1 alone or in combination with siRNA targeting A20 or Usp25. The cells were either left unstimulated or stimulated with IL-17 for three hours. As expected, knockdown of MCPIP1, A20 or USP25 resulted in a significant increase in IL-17-dependent IL-6 protein production (measured by ELISA), compared to control siRNA. Furthermore, double knockdown of MCPIP1 with either A20 or USP25 resulted in an additional increase in IL-17-dependent IL-6 production over individual knockdown of MCPIP1, A20 or USP25 **(Figure 5.2)**. Hence, the inhibitory role of MCPIP1 in IL-17R signaling is non-redundant to other known deubiquitinase inhibitors of this pathway.

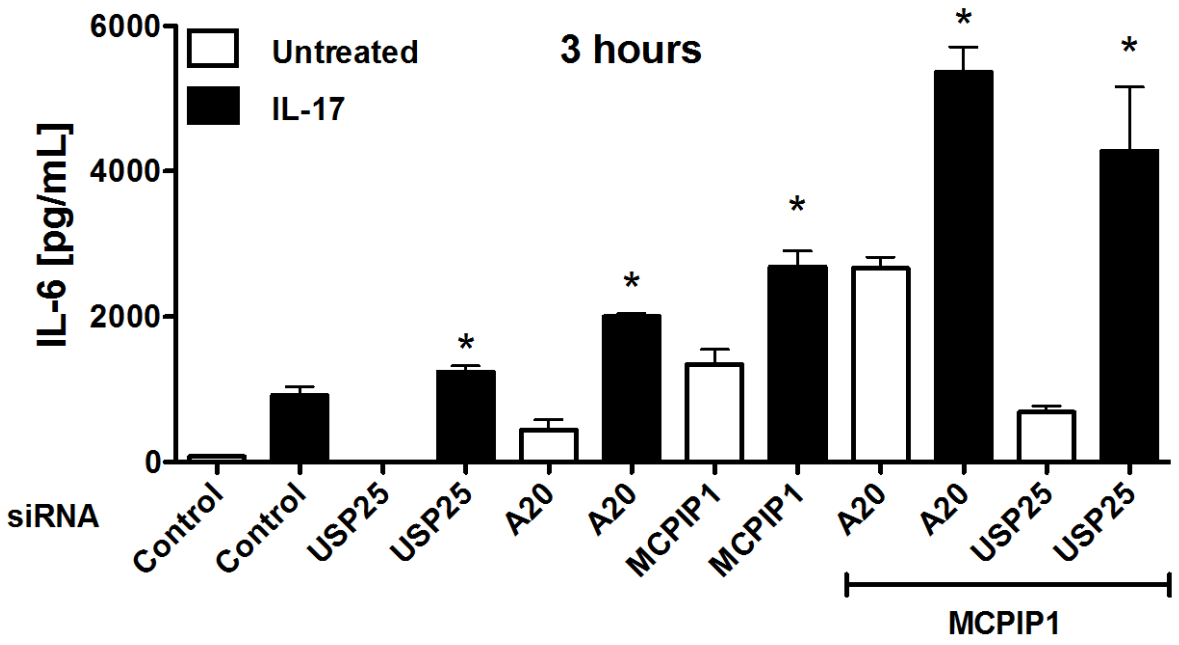


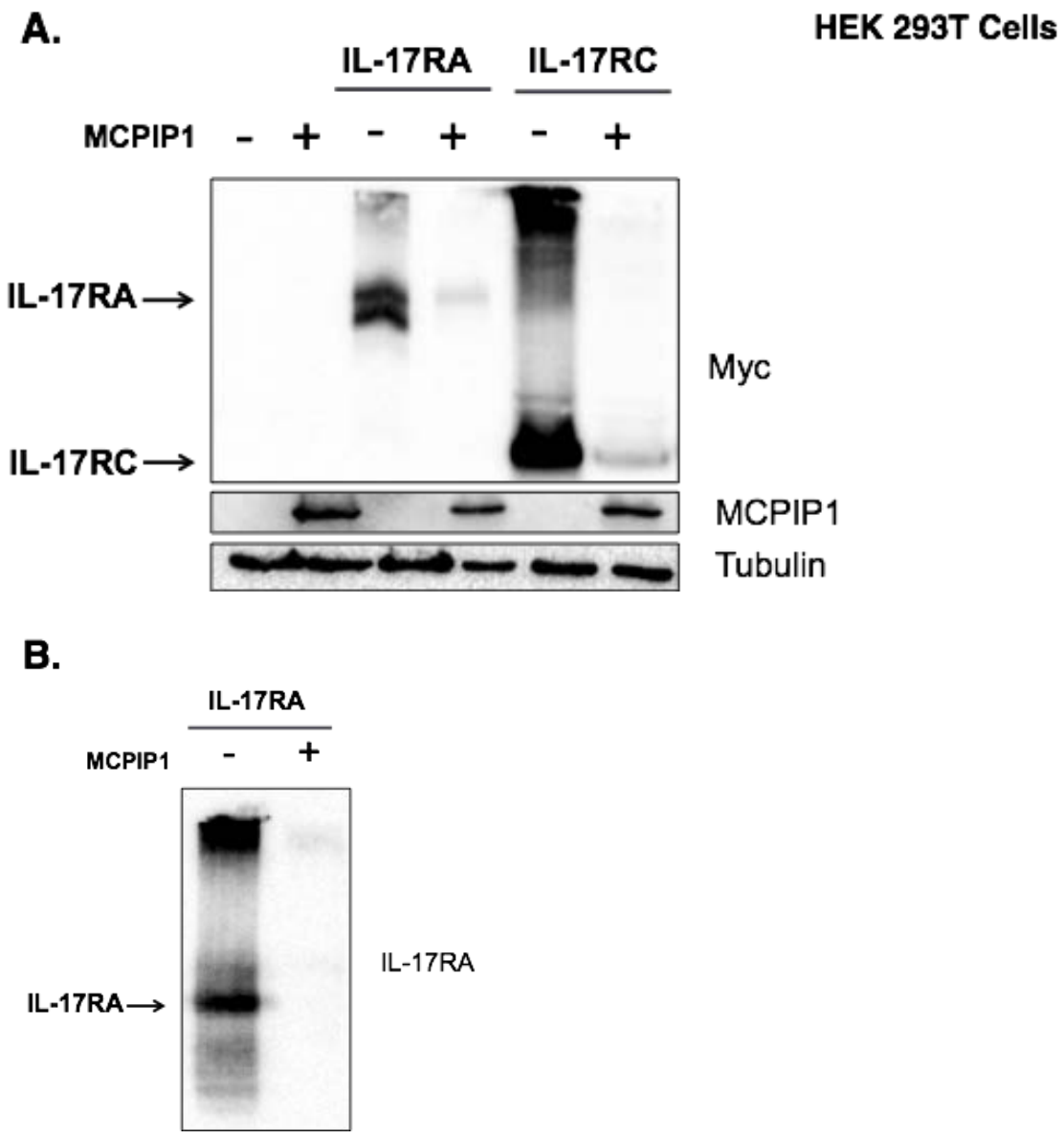
Figure 5.2: Mechanism of MCPIP1-mediated IL-17 signaling is distinct from USP25 and A20.

MCPIP1 knockdown in combination with USP25 or A20 further enhances IL-6 production. ST2 cells were transfected with the indicated siRNA and the amount of IL-6 in culture media was assessed in triplicate at three hours after treatment with IL-17. **P* <0.05 by ANOVA with post-hoc Tukey's test compared to cells transfected with control siRNA or EV and treated with IL-17.

5.2.3 Co-expression of MCPIP1 reduces IL-17RA and IL-17RC expression, but does not affect Act1 or TRAF6.

IL-17RA is involved in the activation as well as inhibition of IL-17 signaling [33, 89, 90, 158]. Therefore, we evaluated the potential association of IL-17 receptor subunits, IL-17RA and IL-17RC, with MCPIP1. Plasmids expressing murine IL-17RA or IL-17RC (Myc-tagged at the C-terminus) were co-transfected with a plasmid expressing murine MCPIP1 in HEK293T cells. Surprisingly, expression of MCPIP1 with either of the IL-17R subunits resulted in a striking reduction in the expression of IL-17RA and IL-17RC, as detected by western blotting with an anti-Myc antibody **(Figure 5.3-A).** We also confirmed the MCPIP1-mediated reduction in IL-17RA expression by immunoblotting with an antibody that detects IL-17RA directly **(Figure 5.3-B).**

Next, we asked whether MCPIP1 targeted other factors in the IL-17R signaling pathway. Act1 and TRAF6 are both essential for the activation of IL-17R signaling. Therefore, we co-transfected plasmids expressing Act1 and MCPIP1 in HEK293T cells and analyzed Act1 expression by western blotting. We did not observe any alteration in Act1 expression in the presence of MCPIP1 **(Figure 5.3-C)**. Similar to the full length Act1, MCPIP1 did not affect the expression of Act1 mutants that lack ubiquitin ligase activity (dUbox) or IL-17R binding ability (dSEFIR) **(Figure 5.3-C**). MCPIP1 also did not impact TRAF6 expression **(Figure 5.3-D).** Therefore, we conclude that MCPIP1 specifically targets IL-17RA and IL-17RC, but not receptor proximal proteins such as Act1 and TRAF6.


A.
HEK 293T Cells
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MCPIP1
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B.
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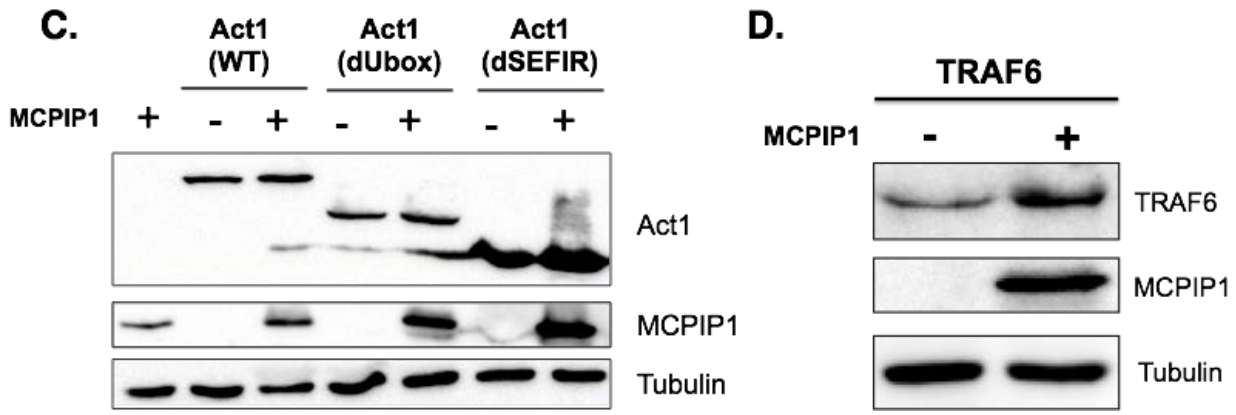


Figure 5.3: Co-expression of MCPIP1 reduces IL-17RA and IL-17RC expression, but does not affect Act1 or TRAF6.

(A) MCPIP1 targets IL-17RA and IL-17RC. HEK293T cells were transfected with murine IL-17RA and IL-17RC (Myc-tagged at the C-terminus) together with murine MCPIP1. WCL were blotted with anti-Myc to detect IL-17RA and IL-17RC. Blotting with anti-MCPIP1 Abs confirmed MCPIP1 expression and tubulin was used as a loading control. **(B)** Lysates from previous experiment were blotted with IL-17RA Abs to determine IL-17RA expression. **(C, D)** MCPIP1 does not degrade Act1 or TRAF6. HEK293T cells were transfected with plasmids expressing (Left) murine Act1 full length (Act1 WT), Act1 deletion mutants (Act1 dUbox, Act1 dSEFIR) or (Right) murine TRAF6 together with construct expressing murine MCPIP1. Act1 and TRAF6 expression was determined by blotting WCL with anti-Act1 and anti-TRAF6 antibodies. Expression of MCPIP1 and Tubulin in WCL is indicated (bottom).

5.2.4 MCPIP1-mediated decrease in total IL-17RA protein results in reduced receptor surface expression.

IL-17 signal transduction is activated upon IL-17 binding to IL-17RA and IL-17RC at the surface of IL-17 target cells. Therefore, we tested whether the decrease in total IL-17RA upon co-expression with MCPIP1 translates to a reduced surface expression of the receptor. To assess the effect of MCPIP1 on IL-17RA cell surface levels, we transfected HEK293T cells with plasmids expressing either IL-17RA alone or in combination with MCPIP1. Twenty-four hours later, these cells were stained with antibodies detecting IL-17RA. We observed that the co-expression of MCPIP1 with IL-17RA resulted in a reduced expression of IL-17RA at the surface of HEK293T cells, determined by a shift in the peak representing IL-17RA positive cells **(Figure 5.4-A)**.

To verify that the reduced IL-17RA surface expression was indeed due to reduced protein level and not an artifact of co-expressing plasmids, we co-transfected IL-17RA with either TRAF6 or A20. Both TRAF6 and A20 play an important role in the regulation of IL-17R signaling but have not been reported to directly impact IL-17RA expression. As expected, cells expressing IL-17RA alone or in combination with TRAF6 or A20 exhibited a similar IL-17RA surface expression pattern **(Figure 5.4-B)**. Altogether, these findings indicate that MCPIP1 targets IL-17 receptor subunits and results in a reduction of IL-17R expression.

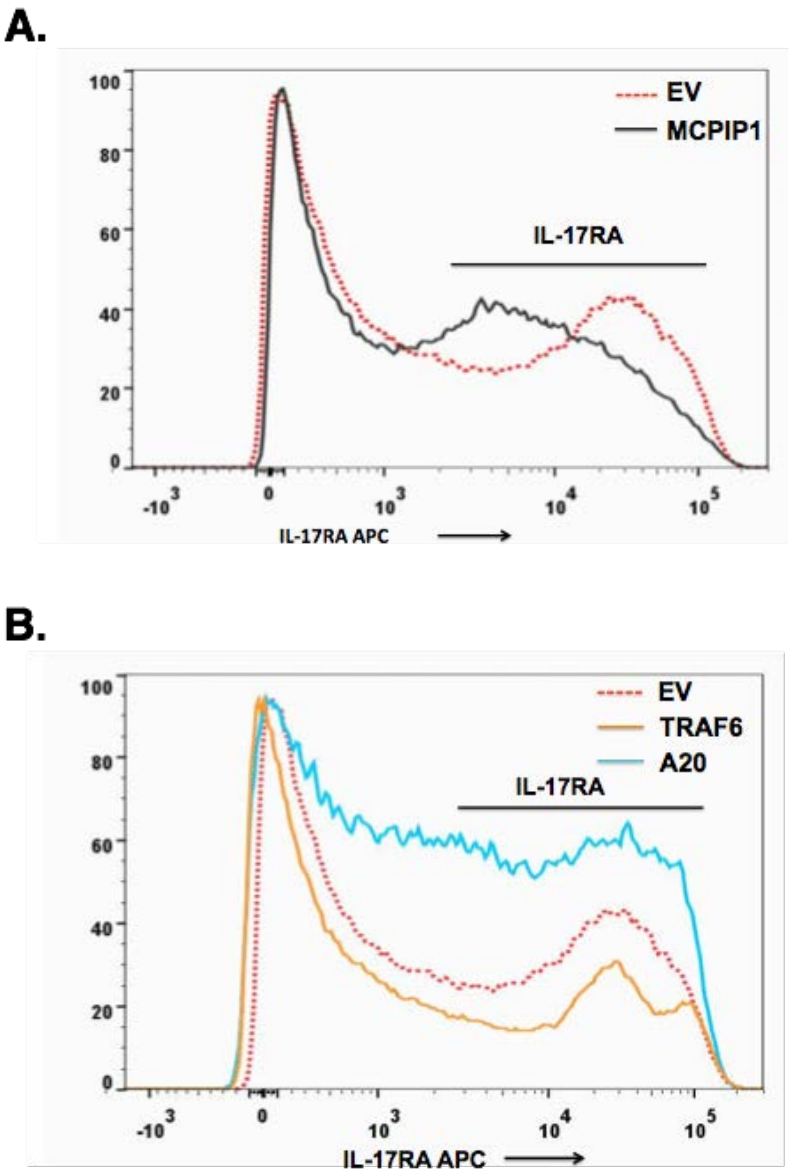


Figure 5.4: MCPIP1-mediated decrease in total IL-17RA protein results in reduced receptor surface expression.

(A) MCPIP1 regulates IL-17RA surface expression. HEK293T cells were transfected with murine IL-17RA with either EV or murine MCPIP1. Twenty-four hours later the cells were harvested, stained with anti-IL-17RA antibodies and counterstained with flourochrome conjugated anti-mouse secondary antibody. **(B)** HEK293T cells were transfected with murine IL-17RA with EV, murine TRAF6 or murine A20. Cells were stained as described in Section 2.2.9. These experiments were conducted by Juan Agustin Cruz.

5.2.5 MCPIP1 regulates TLR4 expression.

MCPIP1 has been previously described as an inhibitor of TLR signaling, activity that requires both DUB and RNase functions of MCPIP1 [101, 180, 188]. Based on our observation that MCPIP1 inhibits IL-17RA expression, we hypothesized that MCPIP1 has a similar effect on TLR4 expression. Therefore, we transfected HEK293T cells with plasmids expressing TLR4 and MCPIP1. Similar to IL-17RA, presence of MCPIP1 resulted in reduced total TLR4 expression, measured by western blotting **(Figure 5.5**). This is to our knowledge the first report of a MCPIP1-dependent reduction in inflammatory receptor expression by MCPIP1. Therefore, we propose that MCPIP1 inhibits TLR4 and IL-17R signaling by targeting receptor expression, in addition to destabilizing the mRNA of inflammatory genes such as *Il6* and *Il12b*.

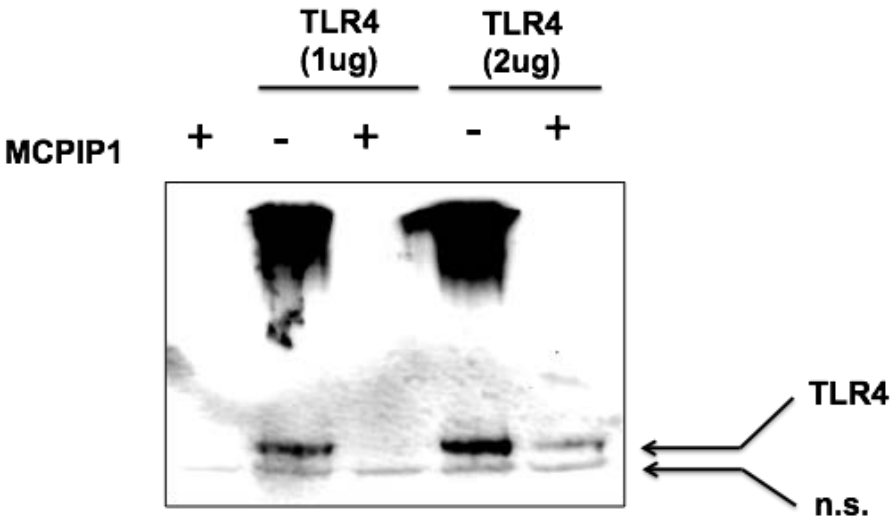


Figure 5.5: MCPIP1 regulates TLR4 expression.

MCPIP1 targets TLR4. HEK293T cells were transfected with murine TLR4- HA-tagged (1ug or 2ug) together with murine MCPIP1. TLR4 expression in WCL was detected by western blotting with anti-HA Abs.

5.2.6 MCPIP1 regulates IL-17RA and IL-17RC by mRNA degradation.

MCPIP1 is an RNase that degrades *Il6* and *Il12b* mRNA [183]. Therefore, we hypothesized that MCPIP1 regulates expression of IL-17RA by degrading its mRNA. To determine the effect of MCPIP1 on *Il17ra* mRNA expression, we transfected an IL-17RA-expressing construct together with an increasing amount of MCPIP1 expressing plasmid in HEK293T cells. Twenty-four hours later, these cells were harvested and *Il17ra* mRNA levels were determined by qPCR. Indeed, we detected that IL-17RA mRNA expression was abrogated in the presence of MCPIP1 **(Figure 5.6-A)**. Notably, even the smallest dose of MCPIP1 construct used in this assay resulted in a significant reduction in *Il17ra* transcript. We also observed a similar pattern of IL-17RC mRNA degradation when expressed along with MCPIP1 **(Figure 5.6-B).**

Consistent with earlier findings, we observed no effect of MCPIP1 on the expression of *traf3ip2* (encoding Act1) mRNA **(Figure 5.6-C)**. Therefore, we conclude that MCPIP1 impacts *Il17ra* and *Il17rc* mRNA expression, suggesting a mechanism of inhibition of IL-17R signaling.

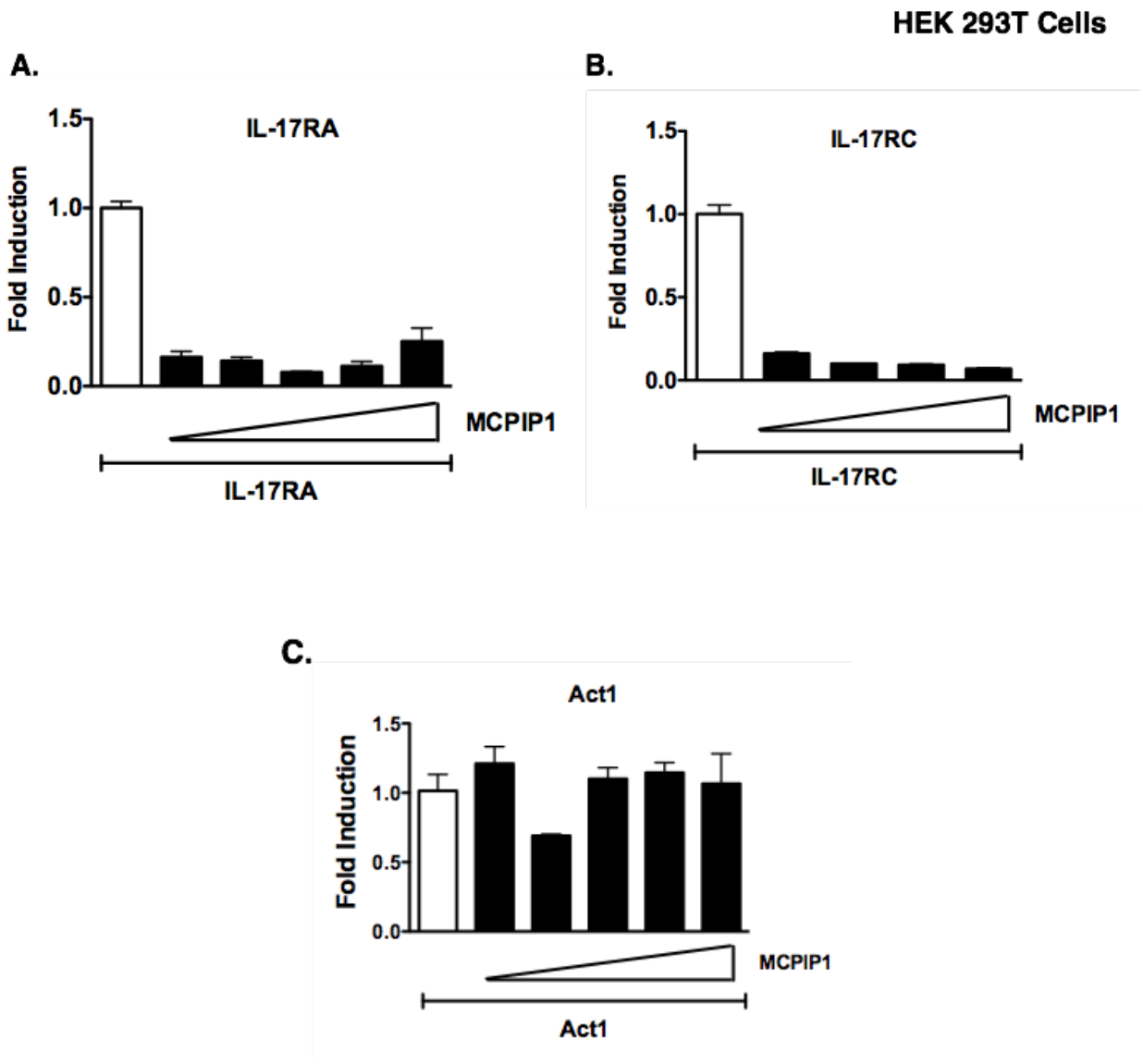


Figure 5.6: MCPIP1 regulates IL-17RA and IL-17RC by mRNA degradation.

(A) MCPIP1 degrades *Il17ra* mRNA. HEK293T cells were transfected with IL-17RA together with increasing concentrations of MCPIP1 expressing plasmid (0-0.75 µg). *Il17ra* mRNA expression by measured by qPCR and normalized to the cells transfected with IL-17RA in the absence of MCPIP1. **(B, C)** MCPIP1 degrades *Il17rc* mRNA but not Act1 mRNA. HEK293T cells were transfected with either IL-17RC **(B)** or Act1 **(C)** plasmids together with increasing concentrations of MCPIP1 (0-0.75 µg). *Il17ra* and *traf3ip2* mRNA expression by measured by qPCR and normalized to the cells transfected with constructs expressing only IL-17RC or Act1 respectively. These experiments were conducted in collaboration with Juan Agustin Cruz.

5.2.7 RNase activity is essential for MCPIP1-mediated IL-17 receptor degradation and IL-17 signaling inhibition.

MCPIP1 RNase function is dependent on the N-terminal nuclease domain as well as the CCCH zinc finger. MCPIP1 mutants lacking crucial charged aspartate residues in the RNase domain (D225/226A), lacking complete zinc finger domain (ΔZF) or a point mutant (C306R), lack the ability to degrade MCPIP1 target mRNA transcripts such as *Il6*. **(Figure 5.7-A)**. Because MCPIP1 functional domains are conserved across several species, we used constructs expressing human MCPIP1 (provided by our collaborator Dr. Pappachan E. Kolattukudy, University of Central Florida**).** To confirm the importance of MCPIP1 RNase activity in targeting IL-17RA, we transfected constructs expressing murine IL-17RA along with plasmids expressing WT MCPIP1 or one of the above-mentioned RNase mutants. Consistent with reduced IL-17RA protein levels when co-expressed with murine MCPIP1, human MCPIP1 also resulted in a similar decrease in IL-17RA expression, as determined by western blot analysis of the whole cell extract. However, we did not observe any decrease in IL-17RA protein levels in the presence of MCPIP1 mutants lacking RNase activity **(Figure 5.7-B)**, establishing the importance of MCPIP1 RNase function in regulating IL-17RA mRNA and subsequent protein expression.

Next, we examined whether MCPIP1 RNase activity is required for in the inhibition IL-17R signaling. Murine MCPIP1 knockout fibroblasts were reconstituted with plasmids expressing either wild type MCPIP1 or MCPIP1 mutant C306R. Twenty-four hours later, these cells were stimulated with IL-17 for four hours and supernatants were evaluated for IL-6 by ELISA. As expected, MCPIP1 expression resulted in reduced IL-17-dependent IL-6 production as compared to EV. However, MCPIP1 C306R did not suppress IL-6 production. We also

observed a comparable lack of inhibition of IL-17 dependent *Il6* mRNA expression upon reconstitution with MCPIP1 C306R **(Figure 5.7-C)**. Collectively, these data indicate that MCPIP1 facilitates *Il17ra*/*Il17rc* mRNA degradation and restricts IL-17 signaling through its RNase and zinc finger domains.

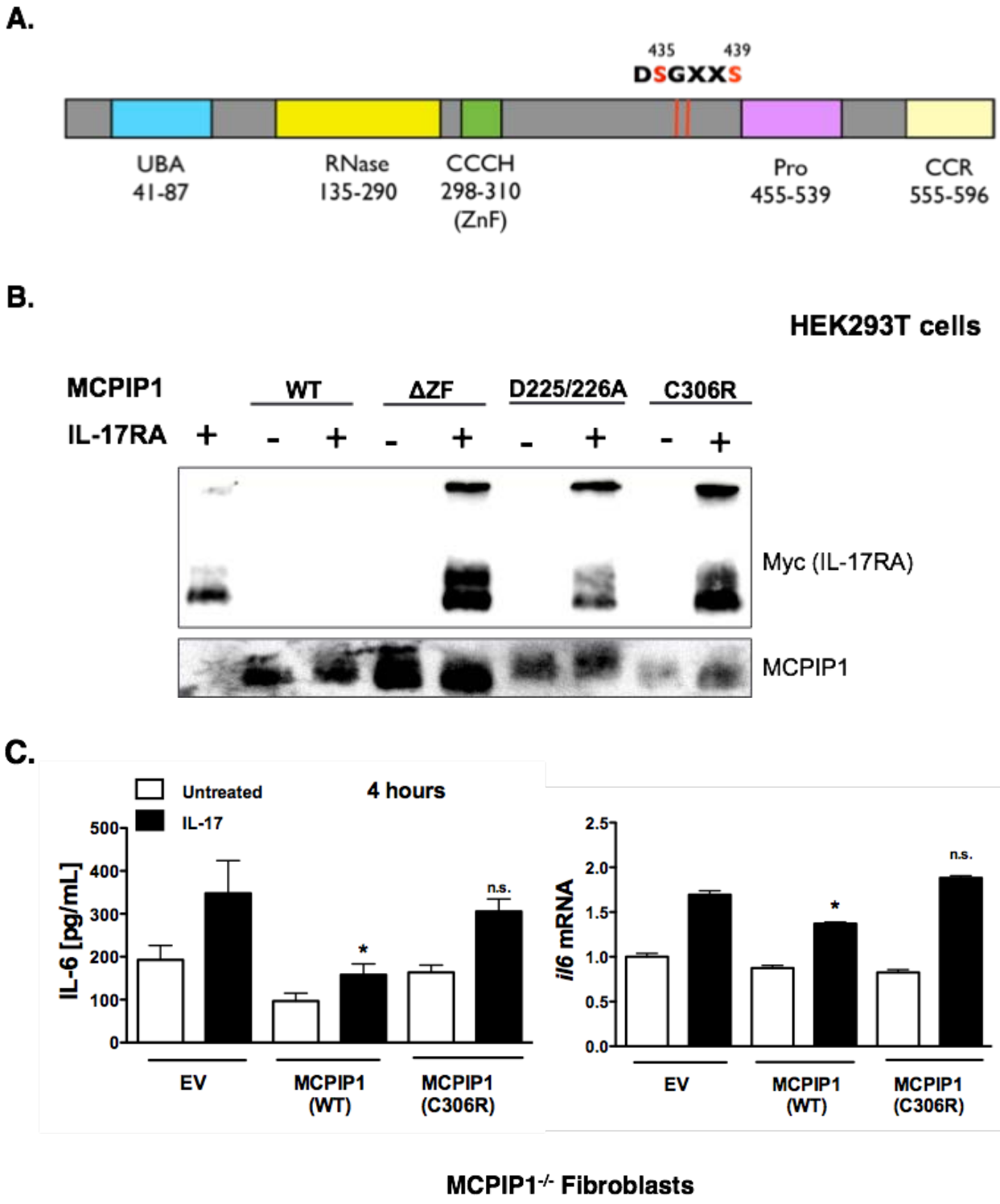


Figure 5.7: RNase activity is essential for MCPIP1-mediated IL-17 receptor degradation and IL-17 signaling inhibition.

(A) Schematic diagram of MCPIP1 domains. ZnF, Zinc Finger; locations of RNase, Proline rich region and MCPIP1 phosphorylation sites are indicated. **(B)** MCIP1 mutants of MCPIP1 impair its ability to target IL-17RA. HEK293T cells were transfected with murine IL-17RA (tagged at the C-terminus with Myc) together with constructs expressing human MCPIP1 or indicated mutants. IL-17RA expression was detected by western blotting with anti-Myc Abs whereas MCPIP1 and its mutants were detected with anti-MCPIP1 antibody. **(C)** MPCIP1 RNase function is important to inhibit IL-17 signaling. MCPIP1$^{-/-}$ tail tip fibroblasts were reconstituted with plasmids expressing EV, human MCPIP1 (WT) or human MCPIP1 (C306R) mutant. Twenty-four hours later, cells were either left untreated or stimulated with IL-17 for 4 hours and the amount of IL-6 in culture media was assessed by ELISA. n.s. Not significant, $*P < 0.05$ by ANOVA with post-hoc Tukey's test compared to cells transfected with EV and treated with IL-17.

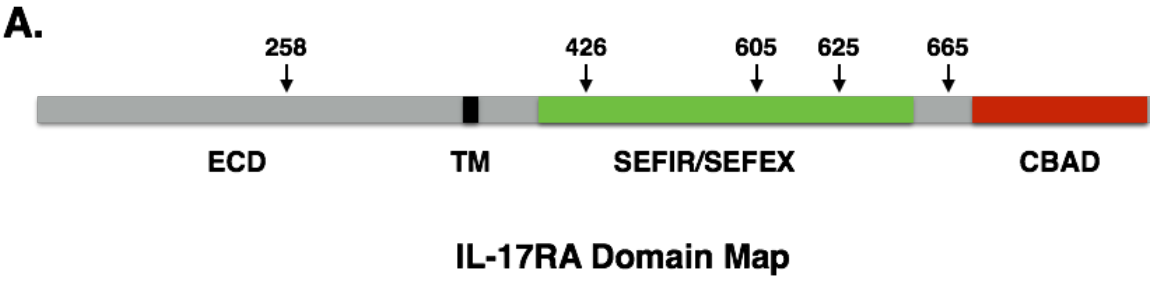
5.2.8 MCPIP1 targets IL-17RA through its N-terminal coding region.

MCPIP1 destabilizes its target mRNAs, such as *Il6* and *c-Rel*, by targeting stem-loop structures in their 3' untranslated region (UTR) [183, 190]. Although MCPIP1 also degrades *Il17ra* and *Il17rc* transcripts, the constructs expressing these IL-17R subunits in our system do not include the respective 3' UTR sequences. Therefore, we hypothesized that sequences within the receptor coding regions might be targets for mRNA degradation by MCPIP1.

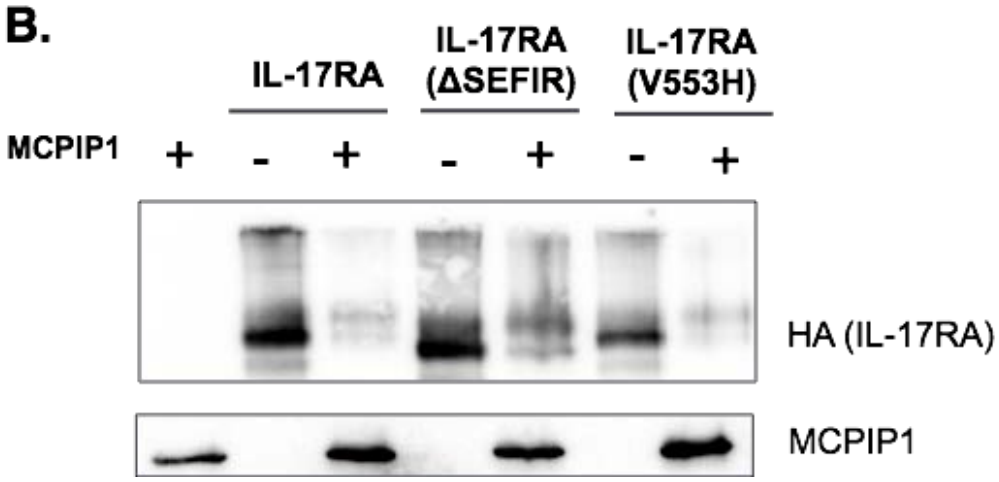
To identify the *Il17ra* sequence targeted by MCPIP1, we transfected HEK293T cells with constructs expressing MCPIP1, along with either full length (FL) IL-17RA, IL-17RAΔSEFIR or IL-17RA.V553H and assessed the total IL-17RA protein by western blotting **(Figure 3.10A**). Expression of IL-17RA.FL, IL-17RAΔSEFIR and IL-17RA.V553H was efficiently reduced upon co-expression with MCPIP1 **(Figure 5.8-B)**. Although we could detect some expression of IL-17RAΔSEFIR even in the presence of MCPIP1, we believe that is due to higher baseline expression of the IL-17RA.ΔSEFIR mutant. Next, we transfected plasmids expressing IL-17RA mutants lacking specific C-terminal residues. For example, IL-17RAΔ665 lacks amino acid residues downstream of position 665. The expression of truncation constructs and IL-17RA degradation in the presence of MCPIP1 were determined by western blotting. All the mutants tested were degraded by MCPIP1 **(Figure 5.8-A,C)**. Cumulatively, these data suggest that *Il17ra* sequence targeted by MCPIP1 is 5' of nucleotides at position 1276-1278 (encoding IL-17RA amino acid residue 426).

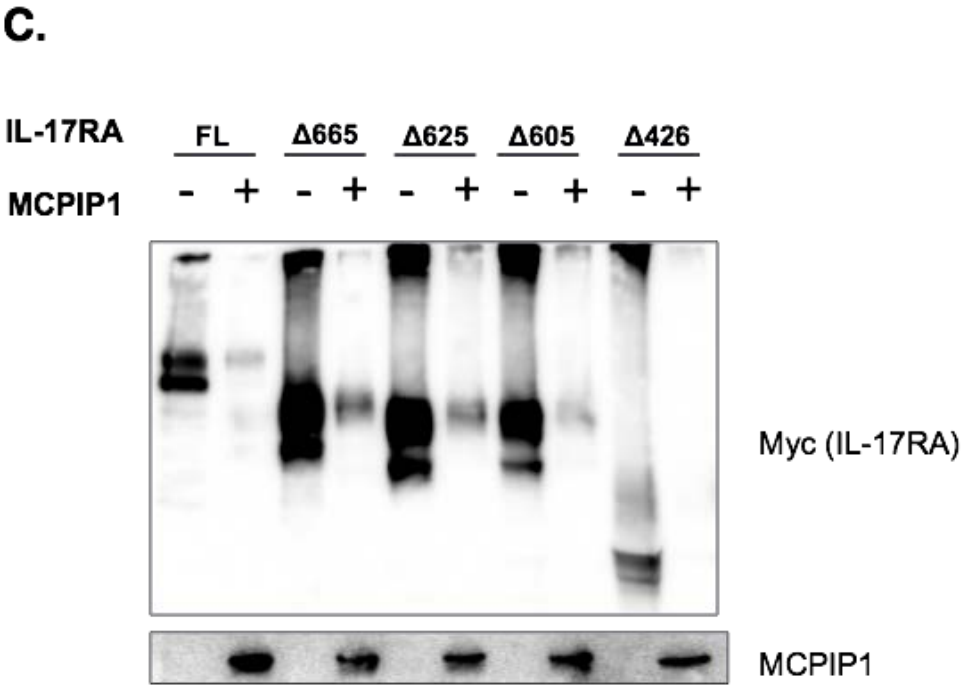
Because amino acid at position 426 is close to IL-17RA transmembrane domain, we questioned whether deleting the transmembrane domain would rescue *Il17ra* mRNA from MCPIP1-mediated degradation. Therefore, we transfected the MCPIP1-expressing plasmid in

combination with either IL-17RA.FL or IL-17RA constructs expressing mRNA from nucleotides at position 1 to 775 (IL-17RAΔ258). As IL-17RAΔ258 lacks the transmembrane domain, we directly determined *Il17ra* mRNA expression by qPCR. IL-17RA.FL and IL-17RAΔ258 mRNA were degraded to a similar level when expressed with MCPIP1 **(Figure 5.8-A,D)**. Therefore, we conclude that MCPIP1 targets *Il17ra* mRNA sequence between nucleotides 1 and 775. Further truncations will be required to determine the smallest possible *Il17ra* mRNA sequence targeted by MCPIP1.



IL-17RA Domain Map





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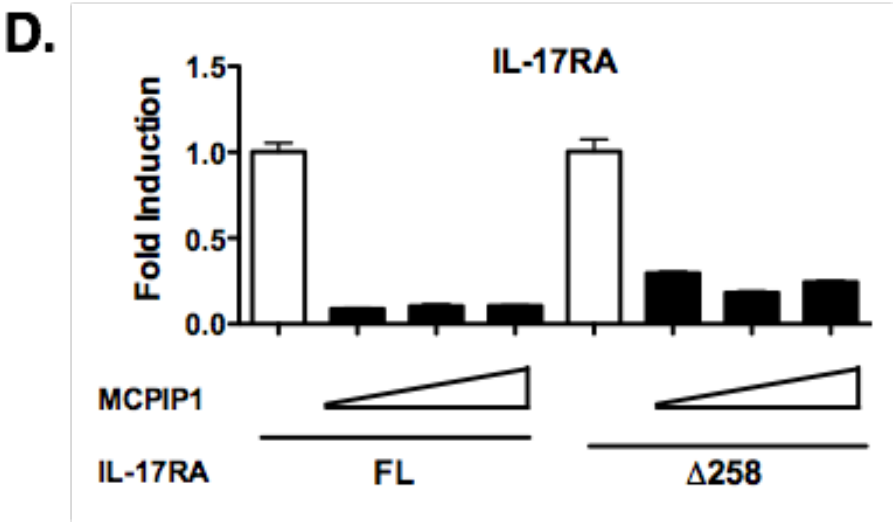


Figure 5.8: MCPIP1 targets the N-terminal region of IL-17RA.

(A) Schematic diagram of IL-17RA domains and C-terminal truncation mutants. ECD, extracellular domain; TM, Transmembrane; locations of SEFIR/SEFEX and CBAD domains are indicated. Also, the numbers depict the last expressed amino acid residue of that particular truncation construct. **(B)** MCPIP1 degrades IL-17RA in a SEFIR/SEFEX-independent manner. HEK293T cells were transfected with murine IL-17RA and indicated mutants (tagged at the C-terminus with HA) with or without murine MCPIP1. Anti-HA Abs was used to detect IL-17RA expression in WCL. Expression of MCPIP1 in WCL is indicated (bottom). **(C)** MCPIP1 does not target sequences that encode for C-terminal IL-17RA region. HEK293T cells were transfected with indicated murine IL-17RA mutants (C-terminal Myc-tagged) along with murine MCPIP1 and blotted for IL-17RA with anti-Myc Abs. **(D)** MCPIP1 degrades IL-17RA though its N-terminal coding sequence. HEK293T cells were transfected with murine IL-17RA (full length) or IL-17RAΔ258 along wit increasing concentration of murine MCPIP1 (0 to 0.5ug). *Il17ra* mRNA expression was determined by qPCR and normalized to the cells transfected with IL-17RA in the absence of MCPIP1.

5.2.9 IL-17RA deletion in MCPIP1 knockout mice does not result in enhanced survival.

MCPIP1 knockout mice (MCPIP1$^{-/-}$) have been generated and described previously [101, 183]. Deletion of MCPIP1 in mice results in growth retardation after weaning, severe splenomegaly and lymphadenopathy. Moreover, MCPIP1$^{-/-}$ mice have elevated serum levels of proinflammatory proteins such as TNFα and MCP-1 [101]. As a result, these mice die prematurely, with a median survival of approximately 7-8 weeks. Inflammation in MCPIP1$^{-/-}$ mice is due in part to a lack of regulatory function of MCPIP1 in T cells as well as LPS and IL-1β signaling [189, 190].

Although MCPIP1$^{-/-}$ mice have an elevated number of T_H17 cells, it is not known whether enhanced IL-17 production or signaling drives baseline inflammation in these mice. As we observed that MCPIP1 inhibits IL-17 signaling, we hypothesized that IL-17 is involved in driving inflammation in MCPIP1$^{-/-}$ mice. Therefore, we crossed MCPIP1$^{-/-}$ mice to IL-17RA$^{-/-}$ mice and obtained MCPIP1-IL-17RA double knockout mice (MCPIP1$^{-/-}$ IL-17RA$^{-/-}$). Surprisingly, similar to MCPIP1$^{-/-}$ mice, MCPIP1$^{-/-}$ IL-17RA$^{-/-}$ mice also display growth retardation and spontaneous inflammation in spleen and lymph nodes (data not shown). We also determined the survival kinetics in both groups of mice and found no difference **(Figure 5.9)**. Thus, we conclude that IL-17 signaling is not required for the persistent inflammation in MCPIP1$^{-/-}$ mice. In fact, a recent report suggests that inflammation in MCPIP1$^{-/-}$ mice is driven by endogenous microbiota of mucosal surfaces, particularly the intestinal microflora, since antibiotic treatment in drinking water alleviated the inflammation in MCPIP1$^{-/-}$ mice.

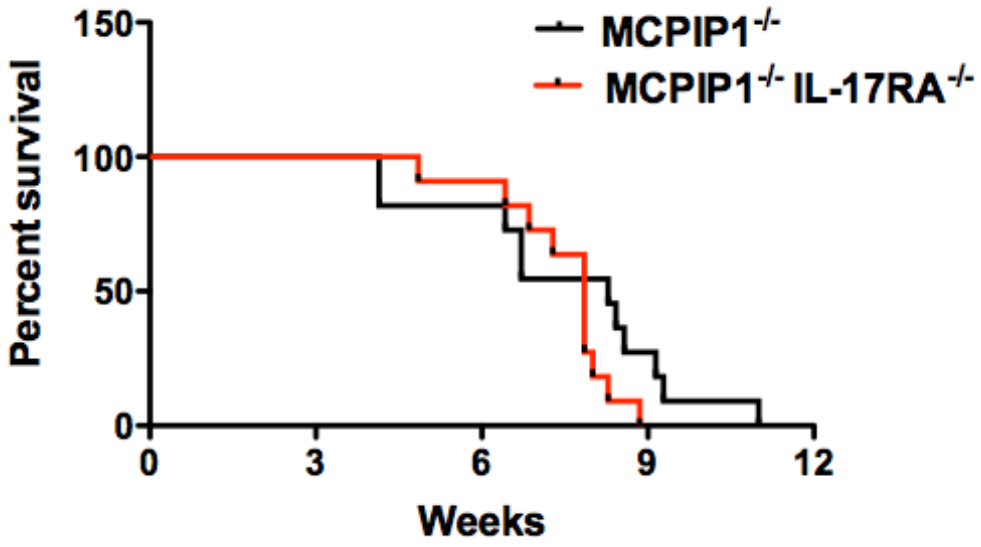


Figure 5.9: IL-17RA deletion does not enhance survival of MCPIP1 knockout mice.

Crossbreeding of MCPIP1$^{-/-}$ and IL-17RA$^{-/-}$ mice. To generate MCPIP1$^{-/-}$ IL-17RA$^{-/-}$ mice, we bred MCPIP1$^{+/-}$ to IL-17RA$^{-/-}$ mice. After several rounds of breeding, we obtained MCPIP1$^{-/-}$ IL-17RA$^{-/-}$ mice and compared the survival kinetics of MCPIP1$^{-/-}$ and MCPIP1$^{-/-}$ IL-17RA$^{-/-}$.

5.2.10 MCPIP1 deficiency in mice leads to enhanced protection from disseminated *Candida albicans* infection that is dependent on IL-17RA.

IL-17 is vital in controlling infections from fungi such as *Candida albicans*. Based on our observation that MCPIP1 inhibits IL-17 signaling and that *Zc3h12a* expression is increased during oral *C. albicans* infection, we hypothesized that MCPIP1 knockout have persistent IL-17 signaling *in vivo* and as a result, would be protected from *C. albicans* infection. However, MCPIP1$^{-/-}$ mice die prematurely and have multi-organ inflammation, precluding their use for experimentation. Therefore, we tested MCPIP1 heterozygous mice (MCPIP1$^{+/-}$) and conducted inflammatory gene expression analysis in multiple organs including kidneys, lungs and gut. Although the levels of inflammatory cytokine mRNA transcripts in most organs in MCPIP1$^{+/-}$ were comparable to WT mice, we detected elevated mRNA expression of inflammatory cytokines such as IL-6, IL-17 and IFN-γ in the gut of MCPIP1$^{+/-}$ mice (data not shown). However, these conclusions are preliminary, as these experiments were conducted only once with one animal per group, and so require further verification to draw any firm conclusions about inflammation in MCPIP1$^{+/-}$ mice. Nevertheless, compared to MCPIP1$^{-/-}$ animals, MCPIP1$^{+/-}$ mice do not have overt baseline inflammation and are suitable for use in experimental models.

We infected MCPIP1$^{+/-}$ mice with 2x10^5 *C. albicans* via their tail veins and compared them to age- and sex-matched MCPIP1 wild-type (WT or MCPIP1$^{+/+}$) mice. In this infection model, *C. albicans* disseminates to multiple organs, particularly kidneys [194, 195]. Two days post-infection, these mice were euthanized and fungal load in the kidneys was assessed. As expected, based on previous publications, *C. albicans* infection in WT mice resulted in a very high fungal burden in kidneys (approximately 10^6 cfu per gram of tissue). Although MCPIP1$^{+/-}$

mice do not completely clear the infection, these mice displayed a significantly lower fungal load, compared to WT mice **(Figure 5.10-A)**, supporting the idea that MCPIP1$^{+/-}$ mice have enhanced inflammatory signaling.

Previous studies have shown that IL-17RA$^{-/-}$ mice are more susceptible to disseminated *C. albicans* infection, indicating an important role of IL-17 in host defense against *C. albicans* [196]. Therefore, we aimed to verify that the reduced fungal load in MCPIP1$^{+/-}$ mice is due to enhanced IL-17 signaling. To ascertain the direct role of IL-17R signaling, we used MCPIP1$^{+/-}$ mice that were crossed to IL-17RA$^{-/-}$ mice. The resultant mice were heterozygous for MCPIP1 gene and were completely knocked out for IL-17RA expression (MCPIP1$^{+/-}$IL-17RA$^{-/-}$). These mice were also infected with 2x10^5 *Candida albicans* via the tail vein and compared to WT, MCPIP1$^{+/-}$ and IL-17RA$^{-/-}$mice. Consistent with earlier reports, IL-17RA$^{-/-}$ mice had higher fungal burdens than WT mice. Furthermore, we confirmed our earlier finding that MCPIP1$^{+/-}$ mice had reduced fungal load in kidneys. However, MCPIP1$^{+/-}$ IL-17RA$^{-/-}$ mice were unable to control the infection and had a fungal load similar to WT mice **(Figure 5.10-B)**. These data clearly demonstrate that the control of fungal infection in MCPIP1$^{+/-}$ mice is due to the role of MCPIP1 in regulating IL-17R signaling.

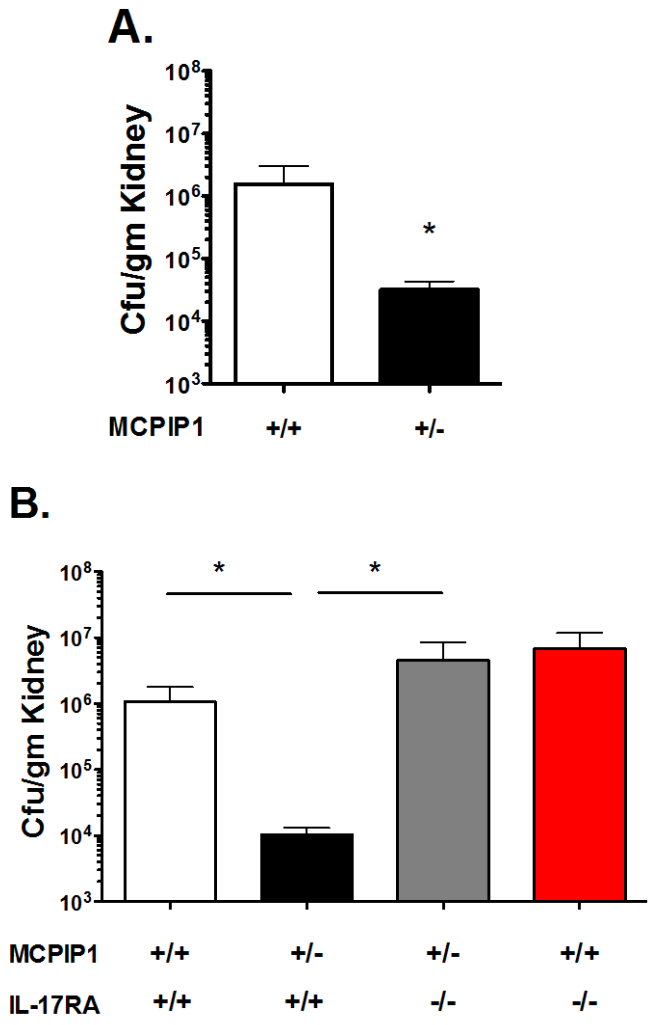


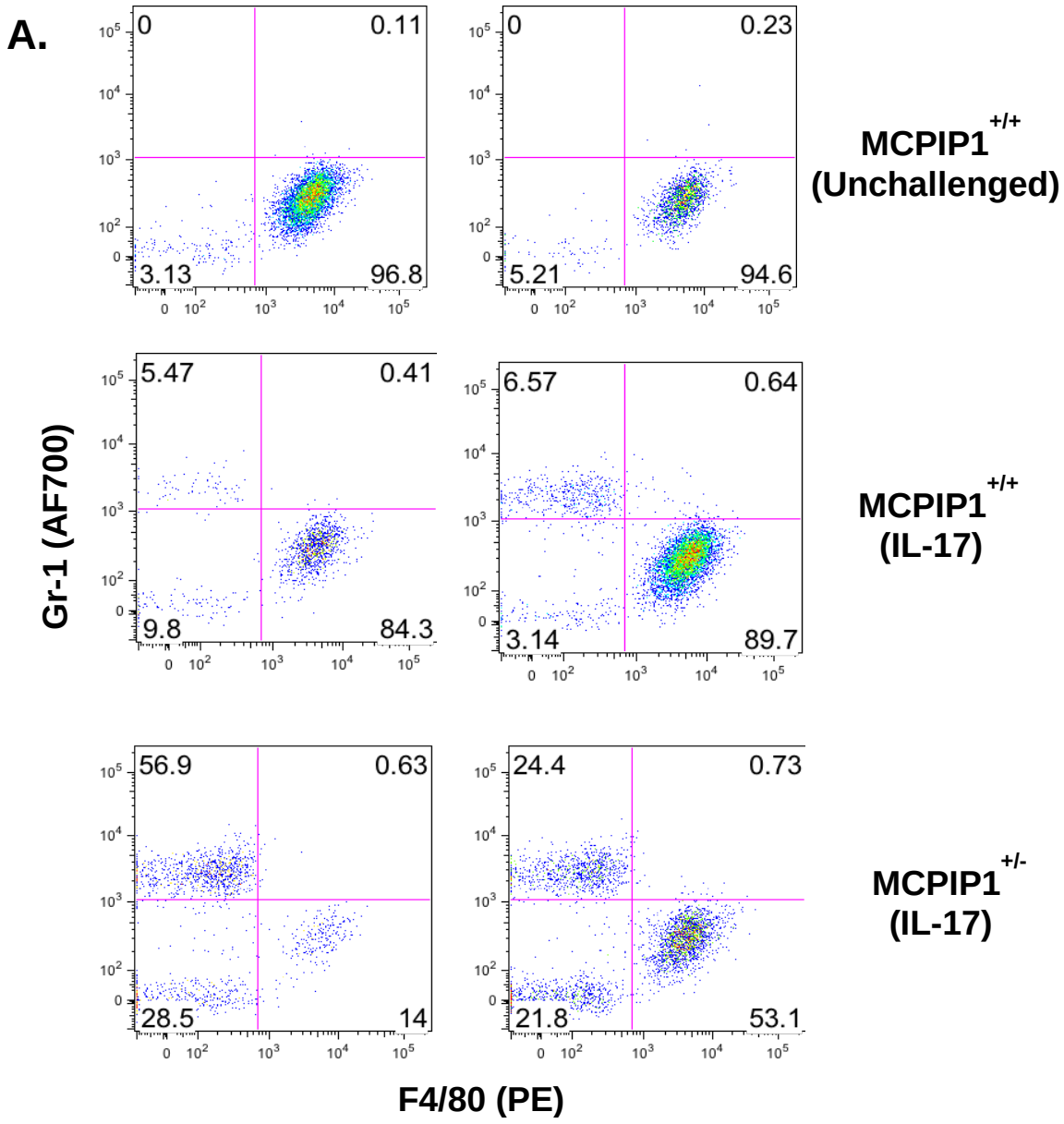
Figure 5.10: MCPIP1 deficiency in mice leads to enhanced protection from disseminated *Candida albicans* infection that is dependent on IL-17RA.

(A) Regulation of disseminated candidiasis infection by MCPIP1 heterozygous mice. MCPIP1$^{+/+}$ (WT) and MCPIP1$^{+/-}$ mice (*n*=5 per group) infection with *C. albicans* and kidney harvest have been explained in Section 2.2.6. *C. albicans* colony forming units (cfu) in kidneys were determined by counting *C. albicans* colonies on YPD agar plates. **(B)** Control of *C. albicans* infection in MCPIP1 heterozygous mice is dependent on IL-17RA. MCPIP1$^{+/+}$ (WT) and MCPIP1$^{+/-}$, MCPIP1$^{+/-}$ IL-17RA$^{-/-}$ and IL-17RA$^{-/-}$ mice (*n*=5 per group) were infected and assessed for kidney *C. albicans* cfu as described earlier. Data presented as Arithmetic mean ± SEM. These experiments were conducted in collaboration with Drs. Heather Conti, Natasha Whibley and Partha Biswas, University of Pittsburgh.

5.2.11 IL-17 induced pulmonary inflammation is enhanced in MCPIP1 heterozygous mice.

Administration of IL-17 through the airways induced the production of inflammatory cytokines and chemokines production that leads to the recruitment of neutrophils to the lungs [53, 92]. Therefore, to verify that MCPIP1 regulates IL-17 signaling *in vivo*, we treated mice with recombinant IL-17 through the intranasal route. WT and MCPIP1$^{+/-}$ mice were either left unchallenged or treated with IL-17 (300 ng/mouse). Twenty-four hours later, we analyzed immune cell infiltrate in BALF and well as inflammatory gene expression in the lungs.

As expected, we only detected a very low percentage of neutrophils (Gr-1^{+}, F4/80^{-}) in the BALF of unchallenged mice, which increased significantly upon IL-17 challenge. Furthermore, there was a significant increase in the percentage of neutrophils in MCPIP1$^{+/-}$ mice as compared to WT mice **(Figure 5.11-A)**. Consistent with the enhanced neutrophil infiltration, we detected elevated expression of IL-17 target genes such as *cxcl1* and *cxcl5* in the lungs (but not *Il6*), measured by qPCR **(Figure 5.11-B)**. Although the changes in gene expression were not statistically significant between WT and MCPIP1$^{+/-}$ mice, these data are consistent with our earlier observations and warrants further investigation with additional mice to confirm the results and statistical significance. To summarize, these preliminary data further support an inhibitory role for MCPIP1 in IL-17R signaling *in vivo*.


A.
0
0.11
3.13
96.8
0
0.23
5.21
94.6
MCPIP1+/+
(Unchallenged)
5.47
0.41
9.8
84.3
6.57
0.64
3.14
89.7
MCPIP1+/+
(IL-17)
56.9
0.63
28.5
14
24.4
0.73
21.8
53.1
MCPIP1+/-
(IL-17)
Gr-1 (AF700)
F4/80 (PE)

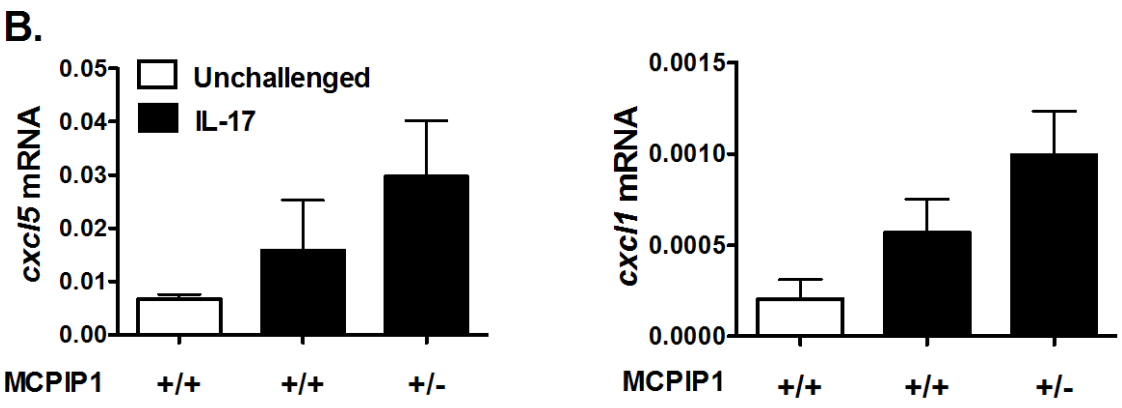

B.
Unchallenged
IL-17
cxcl5 mRNA
0.05
0.04
0.03
0.02
0.01
0.00
MCPIP1 +/+ +/+ +/-
cxcl1 mRNA
0.0015
0.0010
0.0005
0.0000
MCPIP1 +/+ +/+ +/-

Figure 5.11 Contd.

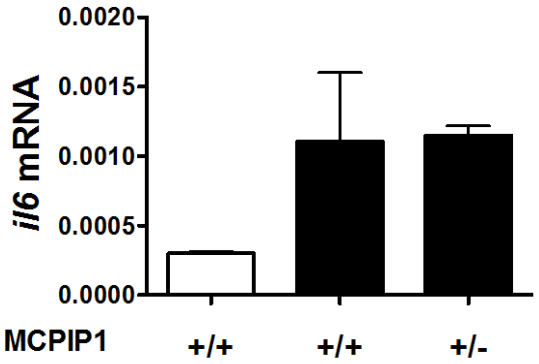


Figure 5.11: IL-17 induced pulmonary inflammation is enhanced in MCPIP1 heterozygous mice.

(A) IL-17-induced pulmonary inflammation is enhanced in MCPIP1$^{+/-}$. MCPIP1$^{+/+}$ (WT) and MCPIP1$^{+/-}$ mice (*n* = 2 per group) were given intranasal injection of IL-17 (300 ng in 50 µl PBS) or left unchallenged. Infiltration of neutrophils (Gr-1^{+}F4/80^{-}) into the BALF was assessed 24 hours after injection (described in detail in Section 2.2.7). (**B**) CXCL1, CXCL5 and IL-6 mRNA expression in the lungs isolated from mice treated in (**A)** was determined by qPCR. These experiments were conducted in collaboration with Dr. Kong Chen and Dr. Jay Kolls, University of Pittsburgh.

5.2.12 MCPIP1 deficiency exacerbates EAE severity.

IL-17 signaling plays a critical role in the development of EAE, an autoimmune disease mouse model of human multiple sclerosis [66, 90, 92]. Enhanced IL-17 signaling has been associated with increased severity of disease in mice [90, 92]. To investigate the role of MCPIP1 in IL-17 signaling during autoimmune disease, we induced EAE in WT and MCPIP1$^{+/-}$ mice and monitored the mice every day for signs of clinical disease using a scoring scheme described in section 2.2.8.

We observed that the onset of disease as well as initial clinical scores were similar in WT and MCPIP1$^{+/-}$ mice. However, as the disease progressed, the severity of EAE pathology was significantly greater in MCPIP1$^{+/-}$ mice **(Figure 5.12)**, indicating that MCPIP1-mediated regulation of IL-17 signaling is relevant in the control autoimmune disease progression. To confirm these findings and to eliminate the possible contribution of additional inflammatory pathways in the development of EAE, we plan to repeat these experiments with more mice as well as with MCPIP1$^{+/-}$ IL-17RA$^{-/-}$ mice.

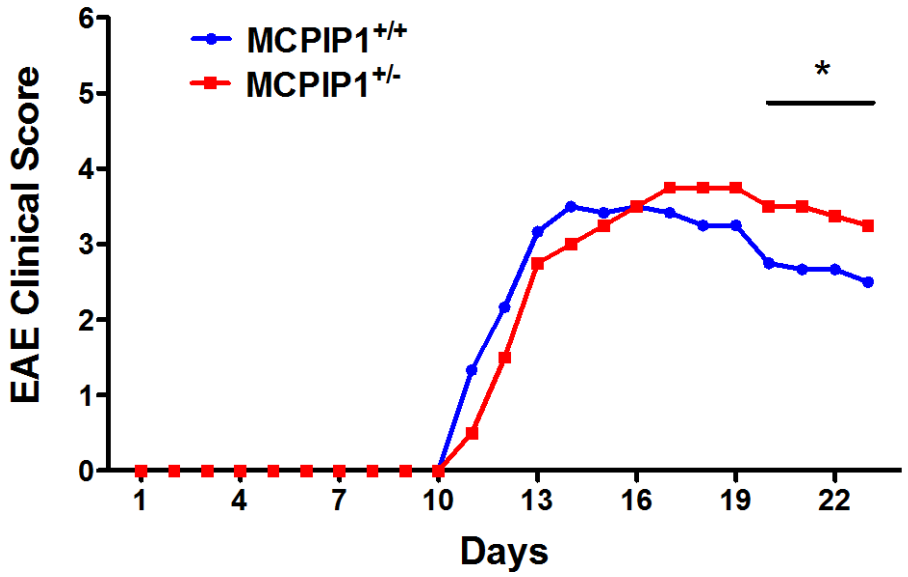


Figure 5.12: MCPIP1 deficiency exacerbates EAE severity.

EAE severity is enhanced in the absence of MCPIP1. (**a**) MCPIP1$^{+/+}$ (WT) and MCPIP1$^{+/-}$ mice (n = 4 per group) were challenged with MOG-CFA and pertussis toxin to induce EAE. The mice were scored everyday for signs of clinical disease on a scale of 1 to 6. (described in Section 2.2.8). This experiment was conducted in collaboration with Dr. Mandy McGeachy, University of Pittsburgh.

5.3 DISCUSSION

The proinflammatory activity of IL-17 is vital in regulating extracellular bacterial and fungal infections both in mice and humans. IL-17 exerts these functions by inducing the expression of several cytokines, chemokines and anti-microbial peptides. Similar to other inflammatory cytokines such as TNFα and IL-1β, IL-17 induces these genes by activating a complex cascade of signaling molecules. Conversely, inhibition of IL-17 signaling is important to avoid collateral damage from IL-17-induced inflammation. Therefore, multiple inhibitory molecules work together to 'put brakes' on IL-17 signaling. The work described earlier in this thesis includes two inhibitors of IL-17 signal transduction, A20 and AnapC5.

In an effort to identify additional novel feedback inhbitors, we mined previously-generated microarray data analyzing IL-17-dependent gene expression upon oral *C. albicans* infection [28]. Through this screen we identified *Zc3h12a*, which was initially discovered as a gene induced by MCP-1. Subsequently, other inflammatory stimuli such as IL-1β and LPS have also been shown to induce *Zc3h12a* expression. *Zc3h12a* encodes MCPIP1, which belongs to a family of CCCH-type zinc finger proteins that exhibit RNase activity. Indeed, MCPIP1 functions as an RNase and degrades *Il6* and *Il12* mRNA [183]. Furthermore, MCPIP1 also has DUB acitivity and deubiquitinates TRAFs [101]. Presumably as a cumulative result of these enzymatic actions, MCPIP1 inhibits TLR4, IL-1R and TNFR signaling pathways. IL-17 shares many activation mechanisms and target genes with IL-1β and TLRs. Therefore, we examined whether MCPIP1 also inhibits the IL-17 pathway. Accordingly, here we extend MCPIP1 function as an inhibitor of IL-17 signal transduction.

Using RNA silencing and MCPIP1$^{-/-}$ cells, we show that MCPIP1 inhibits IL-17-induced target gene expression **(Figure 5.1)**. In TLR and IL-1R pathways, the inhibitory function of

MCPIP1 is attributed mainly to the degradation of target gene mRNA and deubiquitination of adaptor proteins. In IL-17 signaling, IL-17RA and IL-17RC have been previously linked with direct recruitment of both activators and inhibitors of IL-17 signaling **(Chapter 3-4).** Therefore, we tested whether IL-17RA and IL-17RC physically associate with MCPIP1. Surprisingly, co-expression of MCPIP1 with IL-17RA or IL-17RC led to a substantial reduction in the expression of both receptor subunits **(Figure 5.2).** Upon further analysis, we found that MCPIP1 mediates *Il17ra* mRNA degradation, which ultimately results in reduced surface expression of IL-17R. Degradation of *Il17ra* and *Il17rc* appears to be specific, as MCPIP1 had no effect on Act1 and TRAF6 expression **(Figure 5.3-5.6)**.

MCPIP1-mediated degradation of *Il17ra* transcript occurs through the RNase and zinc finger domains of MCPIP1, as mutants lacking either of these domains lack the ability to degrade the receptor transcripts **(Figure 5.7)**. MCPIP1 degrades *Il6* mRNA by targeting a stem-loop structure in its 3'UTR [183]. Interestingly, the *Il17ra* sequence targeted by MCPIP1 in the *in vitro* experiments expresses the coding sequence but not the 3'UTR. Therefore, to assess whether the IL-17RA mRNA sequence also contains a putative stem-loop structure that is recognized by MCPIP1, we tested MCPIP1-mediated degradation of several IL-17RA constructs expressing truncated and mutant forms of IL-17RA. This analysis revealed that MCPIP1 targets a sequence in *Il17ra* mRNA between nucleotides 1-775 **(Figure 5.8)**. Additional experiments are required to identify the precise sequence and structure of IL-17RA sequence recognized by MCPIP1; nonetheless, degradation of IL-17RA coding sequence is an exciting finding for multiple reasons. First, MCPIP1 has never been reported to target the coding sequence of its target genes. Secondly, degradation of *Il17ra* and *Il17rc* mRNA is the first report of MCPIP1 degrading its inducing receptor. Based on our findings, we questioned

whether MCPIP1-mediated degradation of IL-17R is a broad mechanism of inflammatory receptor regulation. Indeed, co-expression of MCPIP1 and TLR4 resulted in reduced expression of TLR4 **(Figure 5.8)**. Therefore, we have not only uncovered MCPIP1 as an inhibitor of IL-17 signaling but also identified a novel mechanism by which MCPIP1 potentially regulates other inflammatory signaling pathways **(Figure 5.13)**.

MCPIP1, like A20 and TTP, is a key player in regulating tonic inflammatory signaling. As a result, MCPIP1$^{-/-}$ mice suffer from systemic multi-organ inflammation, growth retardation and premature death [101, 183]. Therefore, we crossed MCPIP1$^{-/-}$ mice to IL-17RA$^{-/-}$ mice to determine whether IL-17-induced inflammation is the root cause of abnormal inflammation in MCPIP1$^{-/-}$ mice. However, MCPIP1$^{-/-}$ IL-17RA$^{-/-}$ mice were not rescued from systemic inflammation and early death **(Figure 5.9)**, suggesting that IL-17 could not be involved in driving inflammation in MCPIP1$^{-/-}$ mice.

To verify further the physiological relevance of MCPIP1-mediated inhibition of IL-17 signaling, we examined MCPIP1$^{+/-}$ mice in a variety of mouse models. As expected, deficiency of MCPIP1 results in enhanced protection from disseminated *C. albicans* infection, which is dependent on IL-17RA. Furthermore, as compared to WT mice, MCPIP1$^{+/-}$ mice have more severe disease symptoms when subjected to EAE. Finally, direct treatment with recombinant IL-17 through an intranasal route results in enhanced neutrophil recruitment and inflammatory chemokine expression in MCPIP1$^{+/-}$ mice **(Figure 5.10-5.12)**. Cumulatively, these data suggest that MCPIP1 is involved in the regulation of IL-17 signaling *in vivo*.

In order to address the complete scope of MCPIP1-mediated inhibition of IL-17 signaling, more in depth exploration is clearly required. MCPIP1 functions as an RNase and degrades multiple other cytokine mRNAs in addition to that encoding IL-17R. However, it is

still unknown what determines the specificity of MCPIP1 target sequences. In some cases, enzymes recruit adaptor proteins to aid their activity, but it is not known whether MCPIP1 uses any adaptors. We discovered that MCPIP1 degrades inflammatory receptor transcripts such as those encoding IL-17R and TLR4, but it is not known whether MCPIP1-mediated degradation of receptor mRNA is a widespread function or is limited to MCPIP1-inducing receptors (IL-17R, TLR4). Also, MCPIP1 DUB activity is important in the regulation of TLR4 signaling, but its role in IL-17 signaling still needs to assessed.

MCPIP family members are closely related but not identical. Understanding the function of MCPIP1, the prototypical member function of the MCPIP family, could provide interesting insight into the function of other family members. For example, owing to their similarlities in sequence, MCPIP family proteins could either act as dominant negative factors or work as compensatory proteins in the absence of MCPIP1. Finally, as MCPIP1 inhibits multiple inflammatory signaling pathways, any interesting insight into the mechanism of MCPIP1-mediated regulation of IL-17 signaling could potentially be applicable to LPS, IL-1β and TNFα signaling.

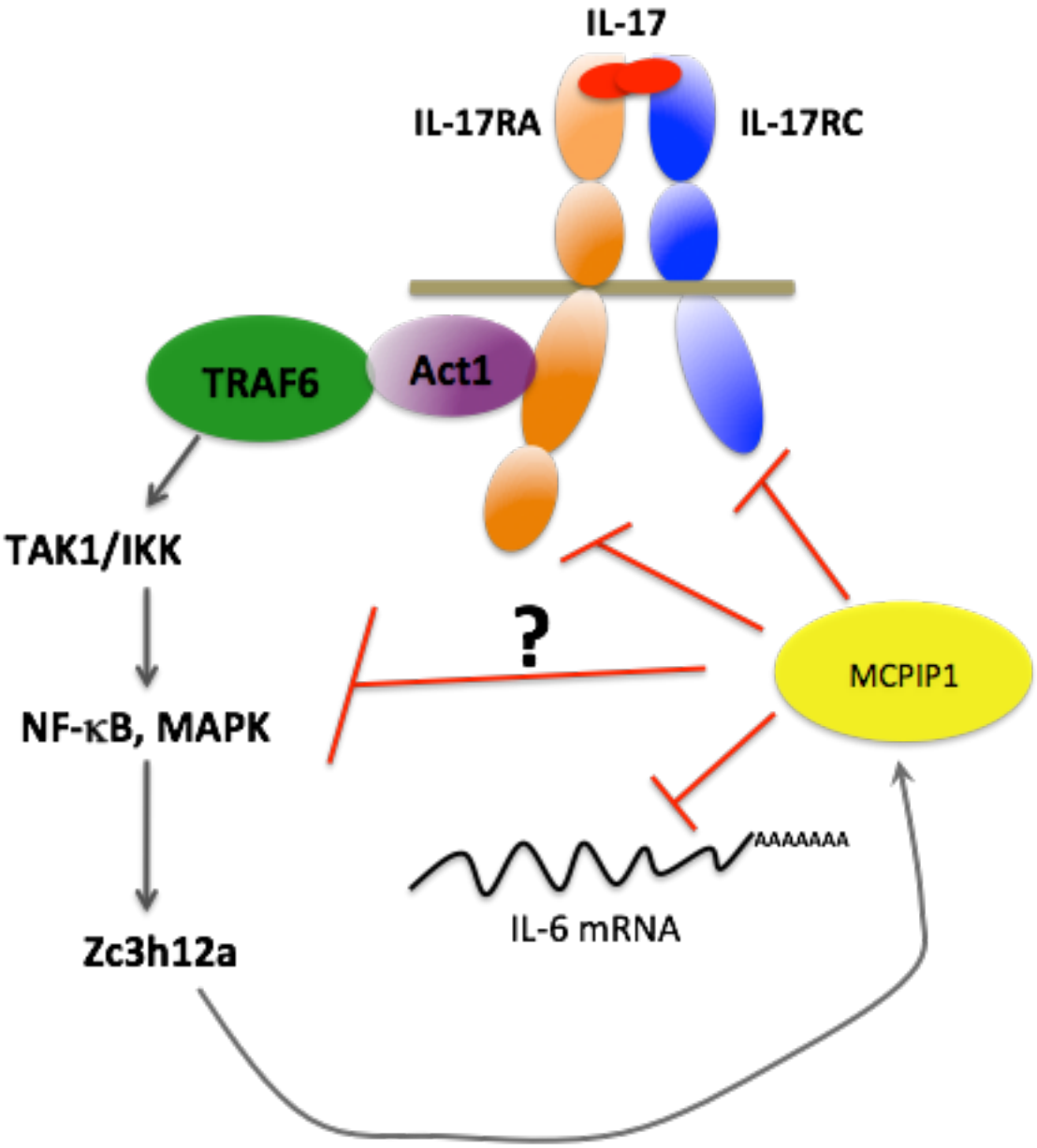


Figure 5.13: Schematic diagram of MCPIP1-mediated inhibition of IL-17 signaling.

MCPIP1 serves as a feedback inhibitor of IL-17 signaling by inhibiting IL-17 target gene expression. MCPIP1 also directly degrades IL-17RA and IL-17RC mRNA, suggesting a model where MCPIP1 inhibits IL-17 signal transduction through multiple mechanisms.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

6.1 SUMMARY

The IL-17 family is the newest addition to the inflammatory cytokine families, which include TNF and IL-1. IL-17 induces proinflammatory gene expression by activating a signal transduction pathway involving Act1 and TRAF6, ultimately resulting in the activation of NF-κB, C/EBPs and MAPKs. Additionally, several proteins are involved in the inhibition of IL-17R signaling. The data presented in this thesis have advanced our understanding of the mechanisms involved in the inhibition of IL-17 signal transduction. Here we identified three proteins that negatively regulate IL-17 signaling by different mechanisms.

Our studies demonstrated that A20 is a feedback inhibitor of IL-17R signaling **(Chapter 3)**. IL-17 induced *Tnfaip3* gene expression through NF-κB, which resulted in elevated A20 protein levels. A20 directly associated with TRAF6 in an IL-17-dependent manner and deubiquitinated TRAF6. These events resulted in reduced NF-κB and MAPK activity and subsequent reduction on IL-17-induced gene expression. A20 bound directly to IL-17RA; however, rather than interacting with the SEFIR domain, which is required for IL-17-dependent NF-κB activation, A20 bound to the IL-17RA distal domain (CBAD), which inhibits IL-17 signaling. Together, these data describe a previously unrecognized mechanism of restraining IL-17 signaling, revealing a dimension of A20 activity that may help to explain its widespread effect on autoimmunity in humans.

Furthermore, we analyzed the role of TAX1BP1 and Itch in facilitating A20-mediated inhibition of IL-17 signaling. Both TAX1BP1 and Itch are scaffolding proteins that mediate A20 inhibition in the TNFα and IL-1 signaling pathways. However, we did not detect any effects of TAX1BP1 or Itch knockdown on IL-17-mediated gene expression, suggesting either

that A20 functions alone in IL-17 signaling regulation or that novel signaling intermediates assist A20. Therefore, we tested the function of APC complex protein, AnapC7, identified as an IL-17RC-associating protein through a yeast-two hybrid screen. Although AnapC7 knockdown did not impact IL-17-induced gene expression, we found that a related APC family protein, AnapC5, inhibited IL-17R signaling. Strikingly, both AnapC7 and AnapC5 associated with IL-17RA through the CBAD domain of the receptor. A20 also binds to the IL-17RA CBAD; therefore we tested direct association of A20 and AnapC5. Indeed, we observed that AnapC5 also associated with A20, suggesting a model in which AnapC5, rather than TAX1BP1 or Itch, is a novel adaptor and negative regulator of IL-17 signaling pathways and possibly assists A20 in its role as an inhibitor of IL-17R signaling **(Chapter 4)**.

The studies described in this thesis also identify MCPIP1 as a novel inhibitor of IL-17 signaling pathway **(Chapter 5)**. *Zc3h12a*, the gene-encoding MCPIP1, is also an IL-17 target gene. MCPIP1 functions as an RNase in this pathway and targets IL-17 receptor expression by degrading *Il17ra* and *Il17rc* mRNA. The significance of MCPIP1-mediated inhibition of IL-17 signaling was demonstrated by enhanced IL-17-induced pulmonary inflammation and severe EAE disease severity in MCPIP1$^{+/-}$ mice. Furthermore, amplified inflammation in MCPIP1$^{+/-}$ mice resulted in a superior protection from disseminated *C. albicans* infection in an IL-17-dependent manner. Finally, we discovered that regulation of receptor expression by MCPIP1 is not limited to IL-17R proteins. MCPIP1 also regulates TLR4, indicating a common mechanism of inflammatory signaling regulation by MCPIP1.

Although the inhibitors of IL-17R signaling described in this study function by different means, these proteins lead to a common endpoint of restricting IL-17-induced inflammation and limiting any resulting collateral damage. In light of these findings, we update the current

paradigm of IL-17 signaling to include these feedback inhibitors of IL-17 signaling **(Figure 6.1)**.

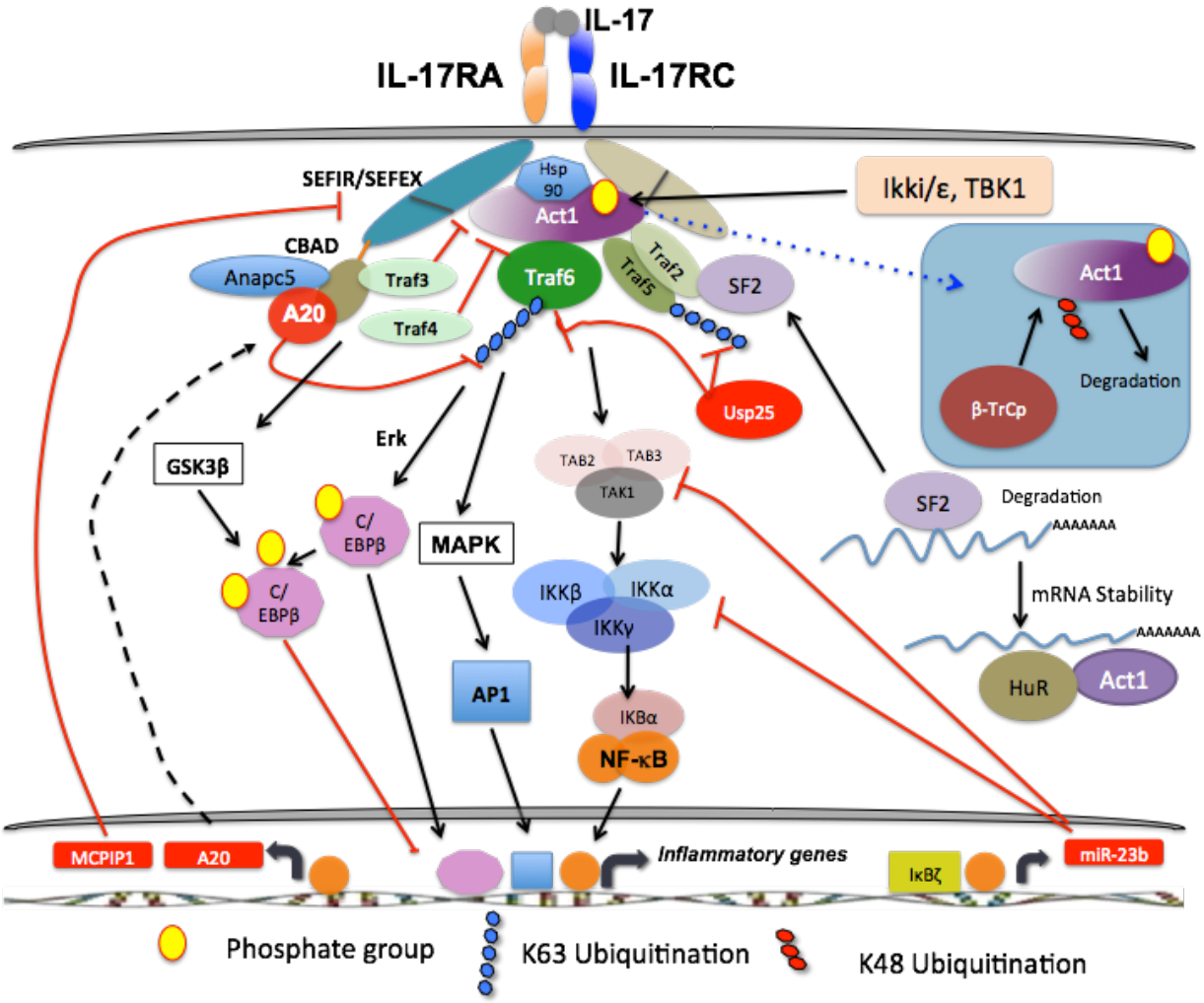


Figure 6.1: Schematic model of IL-17 signal transduction.

Activation of IL-17 signaling involves a complex array of proteins that regulate IL-17 target gene expression. Likewise, several inhibitors are involved in facilitating the inhibition of IL-17 signaling. This diagram is a representation of our current understanding of IL-17 signaling including the three inhibitory proteins described in this thesis.

6.2 OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

The studies presented here have focused on the inhibitory pathways involved in the regulation of IL-17 signal transduction. A20 is well-recognized inhibitor of inflammatory pathways such as TNFα and IL-1β, and now we extend its role to IL-17 signaling. However, further research is required to determine fully the contribution of A20-mediated inhibition of IL-17 pathway *in vivo*, this question could potentially be addressed by using $A20^{+/-}$ or $A20^{-/-}$ mice in IL-17-dependent disease models. Disseminated candidiasis (fungal infection model) or EAE and intranasal IL-17 challenge (models of autoimmunity) are examples of some settings where IL-17 plays a critical role in host protection or inducing autoimmune inflammation. Furthermore, like $MCPIP1^{-/-}$ mice, $A20^{-/-}$ mice also have systemic inflammation and die prematurely [124]. Earlier studies ascribed inflammation in $A20^{-/-}$ mice to enhanced TLR signaling initiated by gut resident microflora [122]. Whether IL-17 plays any role in driving inflammation in $A20^{-/-}$ mice can be addressed by crossbreeding $A20^{-/-}$ to either $IL\text{-}17A^{-/-}$ or $IL\text{-}17RA^{-/-}$ mice.

Another interesting observation in this study was the direct association of A20 and IL-17RA. This is the first report of A20 directly associating with its inducing receptor, suggesting a possible mechanism by which A20 quickly restricts IL-17 signaling. Since A20 also functions as a feedback inhibitor for TNFR, TLR and IL-1R, it would be interesting to test whether these signaling pathways also employ receptor-mediated recruitment of A20. Unlike TNFR and IL-1R pathways, the adaptor proteins TAX1BP1 and Itch do not seem to be relevant in A20-mediated regulation of IL-17 signaling. Instead, we discovered a novel role of AnapC5 as it directly associates with A20 and may aid A20 function in the IL-17 pathway. In addition to further exploring the importance of AnapC5 and related proteins in the recruitment of proteins

to IL-17 receptor proximal complex, it would be intriguing to test whether AnapC5 (or related proteins) is also involved in other pro-inflammatory signaling pathways.

MCPIP1 is another feedback inhibitor of IL-17 signaling identified in this study. MCPIP1 regulates IL-17 signaling *in vitro* and *in vivo*. However, deletion of IL-17RA in MCPIP1$^{-/-}$ mice did not alleviate systemic inflammation, suggesting that the baseline inflammation in MCPIP1$^{-/-}$ mice is independent of its role in IL-17 signaling. In fact, a recent study elegantly demonstrated that MCPIP1 regulates immune homeostasis, owing to its role in CD4^{+} T cells [190]. Another report suggested that the MCPIP1$^{-/-}$ mice treated with antibiotics have a significant reduction in inflammatory cytokine expression and display prolonged survival [189]. These observations demonstrate that intestinal microflora are at least partially responsible for inducing inflammatory phenotype in MCPIP1$^{-/-}$ mice, indicating the significance of MCPIP1 in inhibiting TLR and IL-1R signaling. To directly assess the relevance of MCPIP1 in controlling TLR/IL-1R pathways, we have bred MCPIP1$^{-/-}$ mice to MyD88$^{-/-}$ mice. We recently obtained a single MCPIP1$^{-/-}$ MyD88$^{-/-}$ mouse and observed a significant increase in survival (data not shown). However, loss of MyD88 and subsequent lack of TLR/IL-1R signaling did not result in complete abrogation of inflammation in MCPIP1$^{-/-}$ mice, suggesting that the inflammation in MCPIP1$^{-/-}$ mice is probably driven by multiple stimuli. Therefore, it would be interesting to further mine the scope of MCPIP1-mediated regulation of inflammatory pathways. A few stimulating questions include: Do IL-17 or TNFα drive inflammation in MCPIP1$^{-/-}$ MyD88$^{-/-}$ mice? Is MCPIP1-mediated inhibition of TLR and IL-17R signaling cell type specific? What are the relative contributions of immune and non-immune compartments? Does MCPIP1 also regulate signaling in other immune cells such as dendritic cells (DCs) or

macrophages? Tools such as MCPIP1$^{-/-}$ MyD88$^{-/-}$ and MCPIP1$^{-/-}$ IL-17RA$^{-/-}$ mice could prove to be useful in answering these questions.

Besides providing valuable insight into the mechanisms of IL-17 signaling inhibition, this study raises some interesting questions about the regulation of IL-17 signaling and inflammatory pathways in general. It is intriguing that several proteins are involved in inhibiting IL-17 signal transduction. Additionally, proteins such as A20 and MCPIP1 regulate multiple pathways including IL-17, TNFα, LPS and IL-1 signaling. There are a few possible explanations of this overlap in inhibitor function. First, the mechanisms employed in the regulation of IL-17 signaling are not identical to TNFα, LPS or IL-1 pathways. For example, A20 inhibits TNFα signaling by deubiquitinating RIP1, whereas in the IL-17, LPS and IL-1 pathways, TRAF6 is the target protein [197]. Secondly, in the setting of autoimmune conditions such as RA, proinflammatory cytokines such as IL-17, TNFα and IL-1 are upregulated [42]. Under such circumstances, these cytokines could act in synergy to further induce inflammatory cytokines and chemokines. Therefore, the presence of multiple inhibitors that could potentially regulate most of these pathways is possibly an effective strategy of counterbalancing inflammatory cytokine pathways.

Finally, it is possible that the kinetics of inhibition vary for different feedback inhibitors, allowing the inhibitors to act at different stages after the activation of the signaling pathway. For example, A20 and MCPIP1 expression are both induced by IL-17. However, the expression kinetics for these proteins are dissimilar. While A20 mRNA expression is induced very early and goes down rapidly, MCPIP1 mRNA expression is more gradual and remains elevated for a longer period of time. mRNA expression kinetics in addition to the stability of the encoded proteins, could potentially determine the timing and efficacy of inhibition. An approach to

perform in-depth analysis of such kinetics is to build bioinformatics models of the signaling pathways. This kind of modelling approach has been used in the past to determine the kinetics of NF-κB activation at steady state as well as in response to inflammatory stimuli such as TNFα (reviewed in [198]). We are collaborating with Dr. James Faeder (University of Pittsburgh) to develop a computational model of IL-17R signal transduction and A20-mediated inhibition of IL-17 signaling. To integrate the biological data and a representative mathematical model, we will use the existing data as an input for the construction and calibration of the model. These inputs include the kinetics of feedback inhibitor expression, target gene expression and the activation status of different proteins involved in the pathway. Furthermore, this model could be expanded to include other inhibitors such as MCPIP1 or could be overlayed with models of TNFα, LPS and IL-1 signaling to determine the basis of cross talk between the inflammaotry signaling pathways.

A goal of exploring signaling pathways is to identify drug targets that help improve the effectiveness of patient care. A standard treatment for many autoimmune conditions is inflammatory cytokine neutralization using biologics such as anti-cytokine antibodies. However, neutralizing antibodies also decrease the ability to fight infections, and as a consequence, patients on biologic therapies are more susceptible to bacterial and fungal infections. Also, not all patients respond equally to the neutralizing antibody treatments and are treated with different biologics until the effective therapy is identified. Therefore, defining molecular signaling intermediates, especially enzymes such as A20 and MCPIP1, has the potential to reveal strategies for developing small-molecule therapeutics that target IL-17 signaling as well as TNFα and IL-1β. Drugs that enhance endogenous inhibitor function could

help restrain the effects of IL-17 and other inflammatory cytokines on autoimmune inflammation without losing the effector function in host defense.

However, it is challenging to use chemical drugs to induce gain of function in proteins. We postulate that a way for this therapeutic strategy to be effective would be to regulate the expression of inhibitory molecules. There is precedent in literature for a similar strategy that has proven to be effective in downregulating inflammation. Specifically, an F box protein, Fbx12, regulates K48-linked ubiquitination and proteasomal degradation of TRAFs 1-6. F box proteins belong to the SCF superfamily of E3 ubiquitin ligases and are important for substrate recognition by these ubiquitin ligase complexes. Another E3 ubiquitin ligase F box component, Fbxo3, mediates the degradation of Fbx12, thereby resulting in enhanced TRAF protein expression as well as increased cytokine production. Targeting Fbxo3 with highly specific small-molecule inhibitors resulted in increased Fbx12 expression and a subsequent reduction in TRAF protein levels and inflammatory cytokine production. Fbxo3 inhibitors also effectively lowered systemic cytokine-driven inflammation as well as the severity of colitis *in vivo* [199] **(Figure 6.2)**.

Feedback inhibitors such as A20 and MCIPIP1 are regulated transcriptionally by IL-17 and other inflammatory stimuli. Therefore, identifying transcriptional repressors of the corresponding genes could potentially be an effective strategy to enhance A20 and MCPIP1 expression, and subsequently, their inhibitory function. A recent report identified two transcription factors, DREAM and USF1, that repress A20 gene expression by binding to regulatory elements in the *tnfaip3* promoter [200]. Therefore, drugs that inhibit DREAM or USF1 activity could be helpful in enhancing anti-inflammatory activity of A20. Finally, targeting A20 or MCPIP1 is particularly attractive as these proteins restrict multiple

inflammatory pathways. Based on our observations and previous reports, a therapy involving enhanced A20 or MCPIP1 function would simultaneously restrict IL-17, TNFα, IL-1β and LPS induced inflammation, thus possibly be more effective in the treatment of autoimmune conditions.

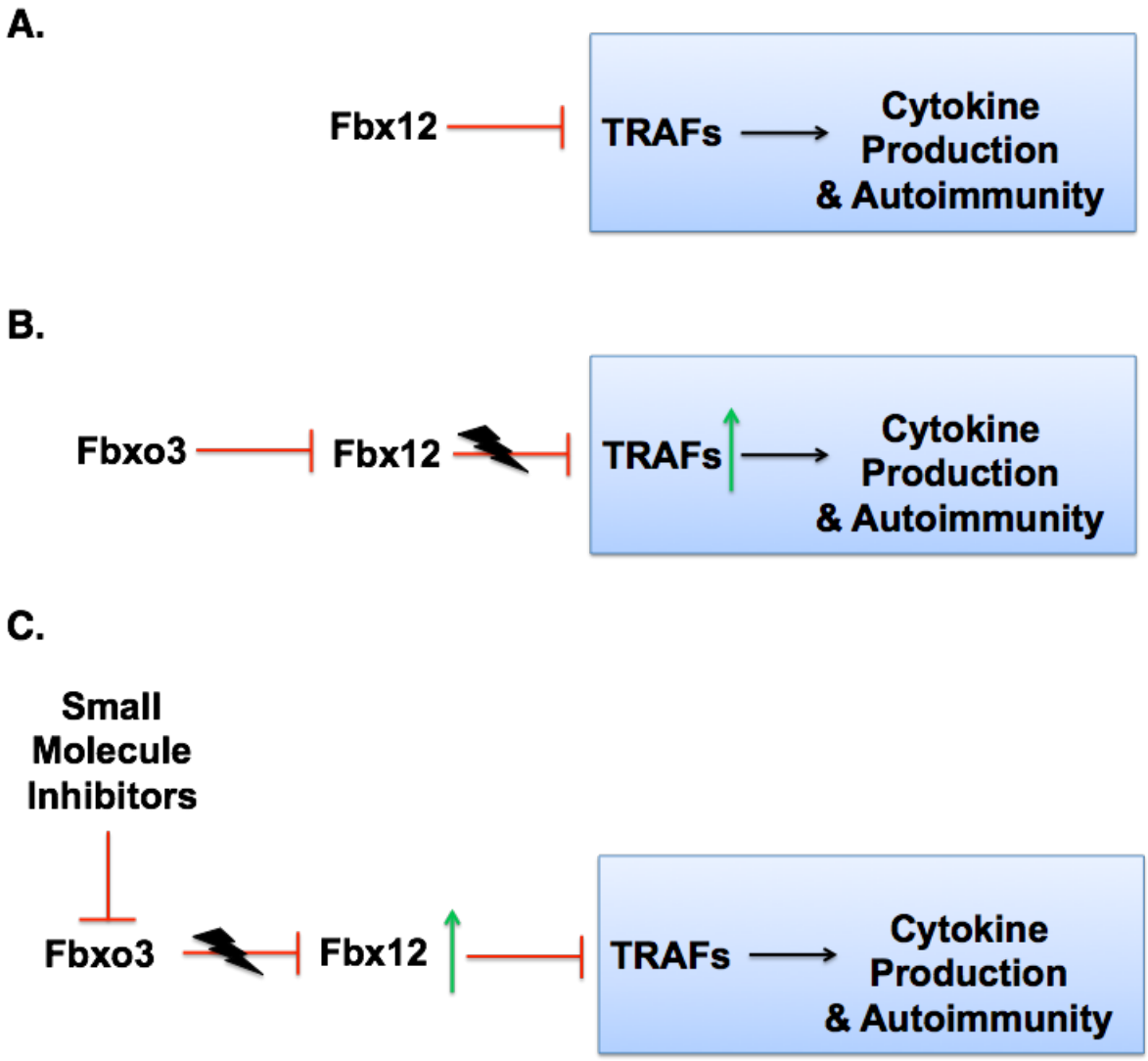


Figure 6.2: Schematic representation of targeting TRAF-mediated inflammation by using small molecule inhibitors.

(A-C) This diagram represents an indirect drug targeting strategy to enhance the expression of TRAF inhibitory protein; Fbx12. Fbx12 targets TRAFs 1-6 to proteasome-mediated degradation, thus inhibiting TRAF-mediated inflammatory cytokine production.

6.3 CONCLUDING REMARKS

The work presented in this thesis has improved our knowledge of the mechanisms involved in the inhibition of IL-17R signaling. We demonstrated that deubiquitination is an important mechanism involved in IL-17 signaling inhibition. In addition to A20, other DUBs such as CYLD and ABIN1 are known to act in TNFα and IL-1β signaling. Furthermore, MCPIP1 also functions as a DUB by deubiquitinating TRAFs. Therefore, it would be interesting to test whether additional DUBs are involved in inhibiting the IL-17 pathway. Also, additional analysis is required to assess the contribution of these inhibitory pathways *in vivo*. Given what we know about the involvement of IL-17 in inducing autoimmune inflammation, it is not surprising that there are multiple layers of inhibition in the IL-17 pathway. However, further investigation is required to fully grasp the interplay between these inhibitory proteins. In conclusion, feedback inhibition of IL-17 signaling seems to be a relevant approach in regulating this pathway and presents a promising opportunity to treat IL-17-mediated autoimmune conditions.

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