# POST-TRANSCRIPTIONAL CONTROL OF IL-17 RECEPTOR-MEDIATED INFLAMMATION

by

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Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2018

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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Interleukin-17A (commonly known as IL-17) is a pro-inflammatory cytokine critical in host defense against microbial pathogens. However, dysregulated IL-17 receptor signaling drives many autoimmune conditions including psoriasis, multiple sclerosis and autoimmune glomerulonephritis. Activation of inflammatory gene transcription through NF-κB is one of the most well-characterized IL-17 signaling pathways. However, IL-17 is consistently found to be a modest activator of transcription in experimental settings. Thus, the profound biological functions of IL-17 in vivo are often attributed to post-transcriptional control of IL-17 target gene expression, but the fundamental mechanisms remain largely underexplored. In this dissertation, I have focused on understanding the basic principles underlying IL-17-induced post-transcriptional regulation of inflammatory genes. Here, I describe two novel RNA-binding proteins (RBP) that promote IL-17-induced inflammation. In chapter 3, I have focused on understanding how IL-17 promotes inflammation by regulating mRNA stability and mRNA translation through an RNA-binding protein called AT-rich interactive domain-containing protein 5A (Arid5a). Arid5a stabilizes mRNAs encoding IL-17-driven genes such as Il6, Cxcl1 and Cxcl5 by directly binding to their 3'UTR. Arid5a also enhances mRNA translation of key

transcription factors such as C/EBPβ and IκBξ, which in turn facilitate transcription of IL-17 target genes. In chapter 4, I identified another

RBP, which acts as an activator of IL-17 signaling. This RBP upregulates expression of IL-17-dependent genes at least in part by triggering NF-κB. Mice deficient in this RBP are less susceptible to IL-17-dependent autoimmune models such as experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune glomerulonephritis (EAGN). Interestingly, this RBP does not affect Th17 differentiation in vivo or in vitro. Using radiation chimeras, I found that this RBP functions dominantly in the non-hematopoietic compartment to induce kidney damage during EAGN. These results are consistent with a model in which this novel RBP positively regulates IL-17 mediated signaling rather than affecting Th17 cell differentiation. Therefore, my dissertation research has deepened our understanding of how RBPs play essential roles in controlling IL-17 signaling post-transcriptionally. Understanding these mechanisms would be beneficial for designing oligonucleotides or small molecule inhibitors that can restrain RBP-mRNA interactions to dampen inflammation during autoimmune conditions.

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| | |
|---|---|
| Act1 | NF-κB Activator 1 |
| ARE | AU-Rich Elements |
| AMP | Anti-Microbial Peptide |
| ANAP | Anaphase Promoting Complex |
| Arid5a | AT-rich Interactive Domain Containing Protein A |
| C/EBP | CCAAT/Enhancer Binding Protein |
| CBAD | C/EBPβ Activation Domain |
| CIKS | Connection to IκB kinase and Stress-activated protein kinases |
| CMC | Chronic Mucocutaneous Candidiasis |
| DUB | Deubiquitinase |
| EAE | Experimental Autoimmune Encephalomyelitis |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ERK | Extracellular signal-Regulated Kinase; |
| FN | Fibronectin III-like |
| Hsp | Heat Shock Protein |
| HuR | Human antigen R |
| IKK | Inhibitor of kB Kinase; |
| IL-17 | Interleukin-17 |

| | |
|---|---|
| IL-17RA | IL-17 Receptor subunit A |
| ILC | Innate Lymphoid Cell |
| IκBζ | Inhibitor of NF-κB ζ |
| JNK | c-Jun N-Terminal Kinase |
| MCPIP1 | MCP-1-induced Protein 1 |
| MMP | Metalloproteinase |
| RBP | RNA-binding Protein |
| SEFEX | SEFIR-Extension |
| SEFIR | Similar Expression of Fibroblast Growth Factor and IL-17R |
| SF2 | Splicing Factor 2 |
| Syk | Spleen Tyrosine Kinase |
| TAK1 | Transforming growth factor β-Activated kinase |
| TAK1 | TGF-b Activated Kinase 1; |
| TF | Transcription Factor |
| TLR | Toll-like Receptor |
| TRAF | TNF Receptor-Associated Factor; |
| UTR, | Untranslated Region. |
| WCL | Whole Cell Lysate |
| WT | Wild Type |

## ACKNOWLEDGEMENTS

This PhD dissertation is based on research work conducted in the laboratory of Dr. Sarah Gaffen. The journey of graduate school has been a great life experience. First of all, I would like to thank my mentor Dr. Gaffen. Words are not enough to describe how grateful I am to you. I appreciate all the time and effort you have spent on my training over the years. You have taught me not only to be a good scientist but also to be a good person. I am immensely grateful to the kindness and support that you have bestowed upon me. I hope to apply the training that I received from you for the benefit of science and, most importantly, human kind.

The work presented in this thesis would not have been possible without the help and support of all the past and present members of Gaffen lab. I would like to thank Abhishek, Agustin, Bianca, Felix, Akash, Giraldina, Chunsheng and Leticia for all the scientific advice and friendship throughout the years. I would also like to thank Partha Biswas, Mandy McGeachy and Saikat Majumder for being such great collaborators. The technical assistance and the scientific input that you have provided have been very helpful for my thesis research. I greatly also appreciate the incredible guidance provided by all my thesis committee members.

Finally, I would like to thank my family and friends for being so supportive throughout my life and in graduate school. The everlasting support of my family and friends is what drives me to achieve my goals in life.

# 1.0 INTRODUCTION



***Trends in Immunology*, May 2017, Volume 38, Issue 5**

**'IL-17 Signaling: The Yin and the Yang'**

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## 1.1 CYTOKINES IN THE IMMUNE RESPONSE

Cells within the immune system communicate either by direct contact or by exchanging signals in the form of small protein molecules such as cytokines, which are messenger proteins produced by cells in response to external stimuli. Cytokines were first discovered in the early 1950s (1). The first cytokines described were identified as endogenous pyrogens, which are now known as interleukin 1 (IL-1), nerve growth factor and interferon (IFN) (2). Today, the cytokine superfamily encompasses interleukins, chemokines, hematopoietins, interferons, transforming growth factors (TNF) and tumor necrosis factors (TGF). Cytokines act in autocrine, paracrine or endocrine fashions, and can be either pleiotropic or redundant in function (3). Different subclasses of cytokines have unique sets of cell surface receptors that share common structural features. Cytokine receptor families include Type I/hematopoietin, TypeII/Interferon, TNF receptor family, Immunoglobulin-type receptor family and G-protein coupled receptor family (3). Upon binding to their receptor, cytokines initiate intracellular signaling pathways resulting in activation of multiple transcriptional, post-transcriptional and post-translational events. These events can directly control a variety of cellular functions such as hematopoiesis, differentiation, migration, proliferation, survival, apoptosis, etc. Cytokine signaling often results in production of new effector molecules such as other cytokines, chemokines, anti-microbial peptides etc., which further amplify their biological functions. Thus, cytokines are a diverse set of multi-functional proteins that coordinate immunity.

Cytokines are produced by both hematopoietic and non-hematopoietic cells in response to external antigens such as microbes, environmental allergens, stimulus associated with cell injury/damage or to self-antigens. As a result of downstream signaling in target cells, different effector molecules are produced which promote the clearance of foreign invaders. However,

excessive or dysregulated production of cytokines can result in autoimmune pathologies, tissue damage and chronic inflammatory diseases. Another key but under-appreciated functional characteristic of cytokines is that they can synergize, resulting in profound biological outcomes. Synergistic responses of cytokines are especially important *in vivo* because the inflammatory milieu contains a complex mixture of cytokines. Hence, any inflammatory response is not due to actions of an individual cytokine, but rather a result of combined reactions to multiple cytokines. Therefore, cytokines are essential components of the immune system and potent mediators of inflammation.

Breakthroughs in cytokine signaling research have resulted in the development of biologics and inhibitors for the treatment of many autoimmune diseases. Use of monoclonal antibodies such as anti-TNF-α for the treatment of Crohn's disease and rheumatoid arthritis (RA), anti-IL6R for RA, and anti-IL-17A for psoriasis are a few examples of such advances (3, 4). While IL-17 blockade is clinically beneficial in some settings, the downside of this and all immune-modulating drugs is susceptibility to opportunistic infection. Therefore, better treatment strategies are needed that alleviate inflammation during autoimmune disease without making patients vulnerable to microbial infections or vice-versa.

In this dissertation, I have focused on studying the post-transcriptional control of cytokine interleukin-17A (IL-17) signal transduction. IL-17 is a proinflammatory cytokine critical in host defense and one of the major drivers of inflammation in many autoimmune pathologies. Downstream IL-17 receptor signaling initiates both transcriptional and post-transcriptional events. Transcriptional regulation of IL-17 signaling is the most-well studied. However, relatively little is known about post-transcriptional regulation of IL-17 signaling. Here,

I have identified two novel RNA-binding proteins that promote IL-17 signal transduction post-transcriptionally, Arid5a and IMP2.

## 1.2 INTERLEUKIN(IL)-17 AND ITS PRODUCERS

IL-17 (IL-17A, originally called CTLA-8) was cloned in 1993, but its functions remained obscure for close to a decade (5). In 2005, IL-17 came into prominence with the discovery of a new population of $CD4^+$ T helper (Th) cells characterized by expression of IL-17. This subset became known as "Th17 cells," and a large body of literature has since been devoted to understanding the mechanisms that direct development, differentiation and regulation of this lineage (6-9). Although Th17 cells are typically considered the principal source of IL-17, a subset of $CD8^+$ cells have also been shown to make this cytokine, and are termed "Tc17." In addition, a number of innate immune subsets make this cytokine, including innate-acting lymphocytes such as γδ-T cells, some natural killer T (NKT) cells and TCRβ+ 'natural' Th17 cells. Furthermore, IL-17-expressing Type 3 "innate lymphoid cells" (ILC3) have been described, which serve as innate counterparts of Th17 cells that lack an antigen receptor (10). Myeloid cells have also been reported to make IL-17, although not in large amounts, and in many cases the validity of this has been called into question (11). Collectively, IL-17-producing cells, whether adaptive or innate, are often termed 'Type 17' cells.

## 1.3 IL-17 FAMILY CYTOKINES AND RECEPTORS

The IL-17 family consists of six structurally related cytokines IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. IL-17A and IL-17F are the most closely related and are co-expressed on linked genes and are usually co-produced by Type 17 cells (12). Similarly, the IL-17R family comprises five receptor subunits, IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. IL-17RA is the founding member of the IL-17R family and is a co-receptor used by several other IL-17 family ligands. The IL-17 family of cytokines and their respective receptor subunits are summarized in **Figure 1.1**. The expression and functions of the extended IL-17/IL17R family are reviewed in detail elsewhere (13). IL-17 and IL-17F exist either as homodimers or as heterodimer, and all forms of the cytokine induce signals through an obligate dimeric IL-17RA and IL-17RC receptor complex.

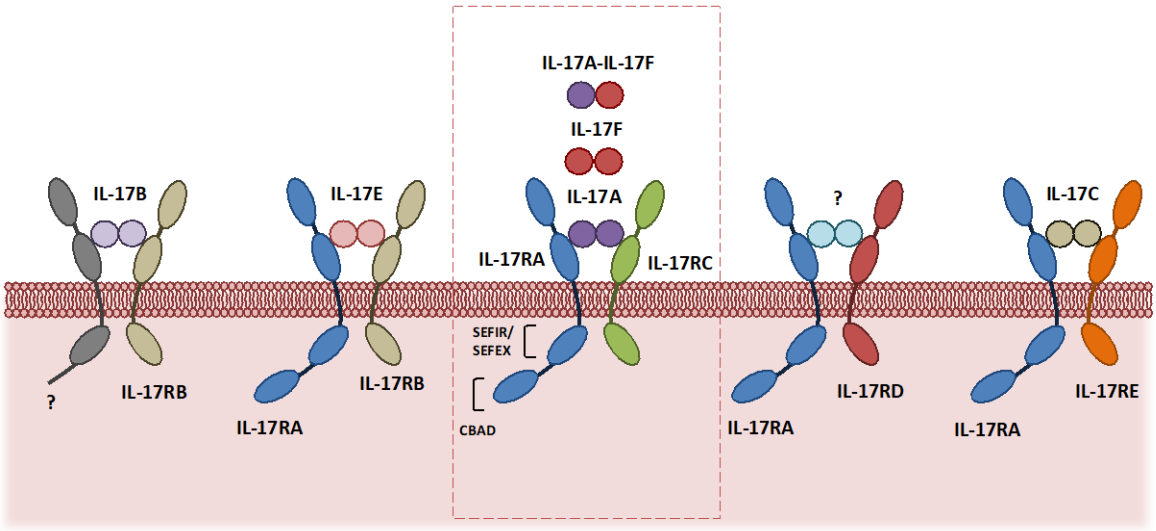


**Figure 1.1: IL-17 Cytokine and Receptor Family**

IL-17A is the prototypical cytokine of IL-17 family that includes five other cytokines. IL-17 receptor family consists of five different receptors, which share a common cytoplasmic motif known as the SEFIR domain. IL-17RA, the common subunit for all the other receptors, also consists of an inhibitory CBAD domain. Abbreviations: CBAD, C/EBPβ activation domain; SEFEX, SEFIR extension; SEFIR, similar expression of fibroblast growth factor and IL-17Rs.

## 1.4 STRUCTURAL FEATURES OF IL-17 RECEPTOR FAMILY: SEFIR AND BEYOND

Members of the IL-17R family are defined by conservation of a cytoplasmic motif known as the Similar Expression of Fibroblast growth factor and IL-17R (SEFIR) domain, a motif that is distantly related to the "Toll/IL-1R" (TIR) domain found in IL-1 and Toll-like receptor family members (14). Act1, also known as Connection to IκB kinase and Stress-activated protein kinases (CIKS), is a unique cytosolic adaptor required for activation of all known IL-17-dependent signaling pathways (15, 16). Act1 contains a SEFIR domain and interacts with IL-17RA and IL-17RC through homotypic SEFIR interactions (14, 17). Moreover, soluble decoy peptides that mimic the predicted Act1:IL-17RA interface have been shown to block both IL-17A and IL-25 (IL-17E) signaling activities (18).

In addition to the SEFIR domain, the first mutagenesis studies of IL-17RA identified a nonconserved region required for IL-17RA signaling function that extends ~100 residues beyond the SEFIR, termed a "SEFIR-Extension" (SEFEX) (19, 20). Subsequent X-ray crystallographic studies of the human IL-17RA cytoplasmic domain confirmed that the SEFIR and SEFEX regions together comprise a single composite structural motif (21). Deletion studies suggested that IL-17RC may also require at least a short sequence extending past the SEFIR region for receptor functionality (22). There are SEFIR domains in the other IL-17R family members, though their functions are not well defined. Interestingly the IL-17RB SEFIR region appears to adopt a very different 3-dimensional topology from its counterpart in IL-17RA (21, 23), suggesting there may be unique twists to how other IL-17R family members operate.

The cytoplasmic tail of IL-17RA additionally contains a distal domain, distinct from the SEFIR/SEFEX, whose function is associated with negative regulation of signaling. The first

studies of this motif were based on correlations with activation of the transcription factor CCAAT/Enhancer Binding Protein (C/EBP)β (see below) (19, 24). Hence, this region was named a "C/EBPβ activation domain" (CBAD). Later studies showed association of the CBAD with two signaling inhibitors, TRAF3 and A20 (25-27). Therefore, IL-17RA has at least two structurally and functionally discrete signaling domains within the cytoplasmic tail that control downstream signaling events.

The extracellular regions of IL-17R family members contain two fibronectin III-like (FN) domains, homologous to those in the extracellular regions of Class I and Class II cytokine receptors (14, 28, 29). These domains mediate protein-protein interactions as well as ligand binding. Yeast-two hybrid studies and FRET analyses revealed that the membrane proximal FN domain in IL-17RA (FN2) is capable of self-dimerization and accordingly mediates pre-assembly of IL-17R complex. This motif thus functions similarly to the ‘‘pre-ligand assembly domain’’ found in the TNF receptor family and is another potential way that assembly or blockade of IL-17R receptors might be manipulated (30-32).

## 1.5 THE YIN AND THE YANG OF IL-17 FUNCTION

IL-17 is strongly associated with pathology in many autoimmune conditions such as psoriasis, multiple sclerosis, ankylosing spondylitis, and autoimmune glomerulonephritis (33). Antibodies targeting IL-17A (secukinumab and ixekizumab) were approved in 2016 for the treatment of moderate-severe plaque psoriasis (34, 35). In many cases these drugs cause almost complete clearance of psoriatic plaques (36, 37). However, the efficacy of IL-17 blockade for other conditions has been less dramatic (11), though there are promising data from trials of ankylosing

spondylitis and psoriatic arthritis (11, 38). Disappointingly and rather surprisingly, trials of secukinumab and brodalumab (anti-IL-17RA) in Crohn's disease were terminated early due to worsening of disease in the treatment group (39, 40). This observation contrasted with the efficacy of anti-IL12/23 therapies (ustekinumab, briakinumab) and anti-IL-6 receptor antibody (tocilizumab), which target cytokines that control Th17 differentiation and hence IL-17 secretion (41-43). An explanation for this paradox came from studies in mice showing a dominant protective role for IL-17 in maintaining intestinal barrier integrity that apparently outweighs its tissue-damaging potential in inflammatory bowel disease (44-47). Thus, targeting IL-17 is an effective therapy for certain conditions, but its clinical use has revealed unexpected new insights into how Th17 cells function in humans.

Numerous studies in mice starting in the early 2000's indicated that IL-17R signaling is critical for protection against a variety of fungal and bacterial infections, particularly the commensal fungus *Candida albicans* and the pulmonary bacterium *Klebsiella pneumoniae* (15). Humans with IL-17 defects are especially prone to chronic mucocutaneous candidiasis (CMC). For example, rare mutations in IL-17 signaling genes (e.g., *IL17RA, IL17RC, ACT1*) are associated with CMC (48-50). Individuals with *AIRE* gene deficiency generate neutralizing autoantibodies against Th17 cytokines including IL-17A, and typically present with CMC as well. (51, 52). However, only a small percentage of patients undergoing anti-IL-17 treatment (2-4%) experienced mucosal candidiasis, suggesting that blockade of this cytokines may need to be profound in order to cause this side effect (37, 53).

## 1.6 RNA-BINDING PROTEINS AND POST-TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION

Cytokine signaling induces expression of a plethora of inflammatory genes, which collectively promote cytokine-mediated immunity. Although cytokines evolved to protect host from pathogens and help in tissue repair (54), uncontrolled cytokine-induced inflammation can drive various pathologies and tissue damage (2, 55). Thus, cytokine-induced inflammatory gene expression is tightly controlled at multiple levels. Transcriptional gene regulation is the most well-studied mechanism downstream of most cytokine signaling pathways. Recruitment of specific transcription factors, epigenetic regulation such as histone modifications, and control of chromatin are a few mechanisms by which cytokines control gene expression (56). Although transcription is an essential component of cytokine signaling, post-transcriptional mechanisms are also important (57). Control of mRNA stability, mRNA splicing, mRNA export and protein translation are few important post-transcriptional gene regulatory mechanisms (57, 58). In contrast to transcription, these post-transcriptional mechanisms act directly on pre-formed mRNA transcripts allowing for rapid control of gene expression, and regulation of the strength and duration of immune responses (58). Thus, post-transcriptional regulation of gene expression is an important but not well understood facet of cytokine signaling.

Control of mRNA stability is one of the most important and best-characterized post-transcriptional mechanisms of gene regulation. According to several microarray analyses, control of mRNA stability accounts for almost 50% of changes in gene expression in response to external stimuli (59-61). Eukaryotic mRNAs are characterized by 7-methylguanosine cap at the 5’ end and a poly(A) tail at the 3’ end. Cytoplasmic RBPs such as eIF4E (eukaryotic translation initiation factor 4E) and the poly(A)-binding protein (PABP) associate with the 5’ cap and the

poly(A) tail respectively, and shield them from RNA decay factors such as exonucleases **(Figure 1.2)**. When these structures are compromised, multiple enzymes target either the 5'cap or the poly(A) tail, resulting in mRNA degradation. Decapping proteins such as Dcp1/Dcp2 can remove the 5' cap, and deadenylases such as PAN2-PAN3 (polyA nuclease) can remove the poly(A) tail from an mRNA. Additionally, endoribonucleases can also cleave an mRNA internally. All of these events result in generation of unprotected mRNA fragments making them prone to exonucleolytic attack (61).

One of the important determinants of mRNA stability is the presence of adenine and uridine-rich elements (AREs) in the 3' untranslated region (UTR) (61). AREs are classical features of unstable mRNA, and many transcripts encoding inflammatory genes such as cytokines have multiple AREs in the 3'UTR (62). In fact, AREs were first identified in the 3'UTR of mRNAs encoding TNF and GM-CSF (63, 64). AREs recruit ARE-binding proteins (ARE-BPs) that promote or inhibit mRNA stability and/or mRNA translation. Tristetraprolin (TTP), AU-rich binding factor-1 (AUF1), KH splicing regulatory protein (KSRP), T cell restricted intracellular antigen-1 (TIA-1) are few examples of such ARE-binding proteins. Once bound to the AREs on the target mRNA, these RBPs recruit various mRNA-decay factors (57). TTP (*Zfp36*) is one of the best-characterized ARE-binding proteins, and is known to destabilize transcripts encoding TNF, IL-1β, IL-2, IL-3, IL-6, IL-10, GM-CSF, etc (57). The function of TTP is tightly controlled by multiple signaling pathways. LPS promotes TTP expression and induces binding of TTP to its target mRNAs, which then recruits mRNA decay machinery. However, mitogen activated protein kinases (MAPK) such as p38 can phosphorylate TTP, which then gets sequestered by the chaperone protein 14-3-3, resulting in mRNA stability (65). Conversely, protein phosphatase 2A (PP2A) can dephosphorylate TTP and release it to its active

form (66). $TTP^{-/-}$ mice are more susceptible to LPS-induced shock, and develop severe inflammatory diseases such as severe cachexia, dermatitis, arthritis and myeloid hyperplasia (67, 68).

In contrast, ARE-BPs such as the embryonic lethal and abnormal vision (ELAV) family members Hu antigen R (HuR, ELAVL1) and Hu antigen D (HuD, ELAVL4) promote mRNA stability by competing with destabilizing ARE-BPs on their target mRNAs. HuR has been shown to bind to TTP target sequences on mRNA and inhibit TTP-mediated mRNA decay (58). *Tnf, Il1b, Il4, Ifng,* and *Il17a* are few examples of HuR target transcripts (57, 69). Accordingly, mice deficient in HuR are less susceptible to experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis, and show reduced IL-17A production by CD4+ T cells (69). Although HuR is primarily an activator of inflammatory processes, mice lacking HuR in the myeloid compartment are more susceptible to endotoxemia and chemical-induced colitis (70). Therefore, in some contexts HuR acts as an inhibitor of inflammation. In myeloid cells, HuR is known to cooperate with mRNA decay factor TIA-1 and inhibit translation of IL-1β and TNF (71). Moreover, a few studies suggest that HuR stabilizes transcripts of anti-inflammatory cytokines (58). Therefore, RBPs are highly specialized in their functions, and the interplay between various RBPs determines the fate of their target transcripts.

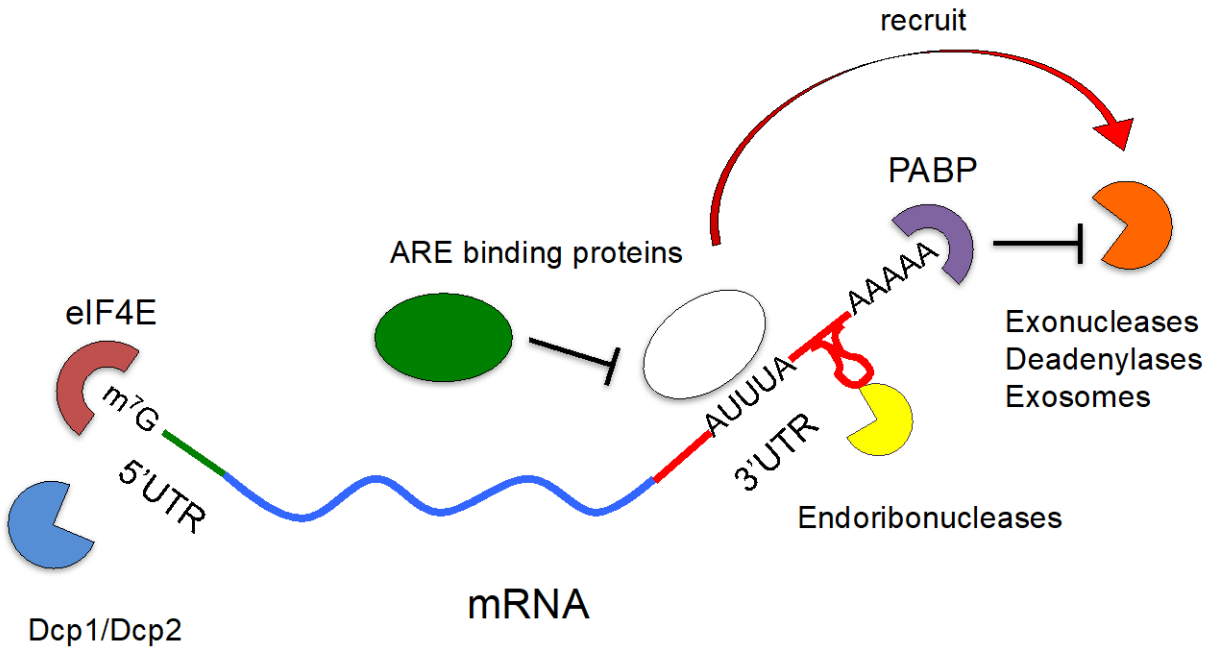


**Figure 1.2: Regulation of mRNA stability**

The 5' 7-methylguanosine cap and the 3' poly(A) tail are essential features of eukaryotic mRNAs. Various RBPs such as eIF4E and PABP associate with the 5' cap and the poly(A) tail, and protect mRNA from various RNA decay enzymes. Many transcripts of cytokines possess multiple AREs on their 3'UTR, a prototypical feature of an unstable mRNA. AREs are important cis-acting elements that recruit RBPs that either stabilize or degrade mRNAs. eIF4E: eukaryotic translation initiation factor 4E, PABP: poly(A)-binding protein, ARE: AU-rich elements, UTR

**Table 1.1: RBPs that control mRNA processing.**

| RBPs (Abbreviation) | Full name | Function |
|---|---|---|
| eIF4E | Eukaryotic Initiation Factor 4E | Initiates translation |
| PABP | Poly-A Binding Protein | Binds to poly(A) tail |
| Dcp1/Dcp2 | Decapping Protein | Trims poly(A) tail of nascent mRNA |
| PAN2-PAN3 | PABP-dependent poly(A) nuclease | Deadenylate mRNA |
| TTP (Zfp36) | Tristetraprolin | Degrades mRNA |
| AUF-1 | AU-rich binding factor-1 | Most forms degrade mRNA, some stabilizes mRNA |
| KSRP | KH splicing regulatory protein | Degrades mRNA |
| TIA-1 | T-cell-restricted intracellular antigen-1 | Inhibits translation |
| ELAV proteins (HuR and HuD) | Embryonic Lethal Abnormal Vision | Stabilizes mRNA |

## 1.7 MECHANISMS OF IL-17 SIGNAL TRANSDUCTION

IL-17 upregulates inflammatory gene expression either by inducing *de novo* gene transcription or by stabilizing target mRNA transcripts, and both of the pathways controlling these events are discussed in detail in the following sections. Although the IL-17R is expressed ubiquitously, non-hematopoietic cells are generally the primary responders of IL-17 **(Figure 1.2)** (11). The pro-inflammatory role of IL-17 was first demonstrated in fibroblasts, where IL-17 was shown to activate NF-κB and induce NF-κB-dependent cytokines (72-74). Subsequent studies delineated a characteristic IL-17 core gene signature that includes pro-inflammatory cytokines, chemokines, anti-microbial peptides (AMPs), matrix metalloproteinases (MMPs) and inflammatory effectors (**Figure 2A**). (75, 76). Additionally, there is a distinct, tissue-specific pattern of IL-17-dependent genes that underlies the diverse physiologic functions of this cytokine (**Figure 1.2**) (77). For instance, IL-17 regulates several genes largely restricted to gut epithelia, such as occludin (*Ocln*), regenereting islet-derived protein 3 gamma (*Reg3g*) and mucin 1 (*Muc1*), which all contribute to maintenance of intestinal barrier integrity. In NK cells, IL-17 was reported to induce expression of GM-CSF (*Csf2*), one of the few documented examples of IL-17-dependent signaling in hematopoietic cells (78). A recent report showed that IL-17 drives kallikrien 1 (*Klk1*) expression in renal epithelial cells to confer protection against disseminated candidiasis (79). IL-17-induced RANKL/OPGL expression in osteoblasts promotes osteoclastogenesis, perhaps accounting for some of the bone-destructive effects of IL-17 observed in models of arthritis or periodontal disease (80, 81), and IL-17 regulates expression of histatins in the salivary compartment that control infection with *C. albicans* (82). Another vitally important facet of IL-17 function is its capacity to synergize (or at least cooperate) with numerous other inflammatory stimuli, further compounding its biological activities (83, 84).

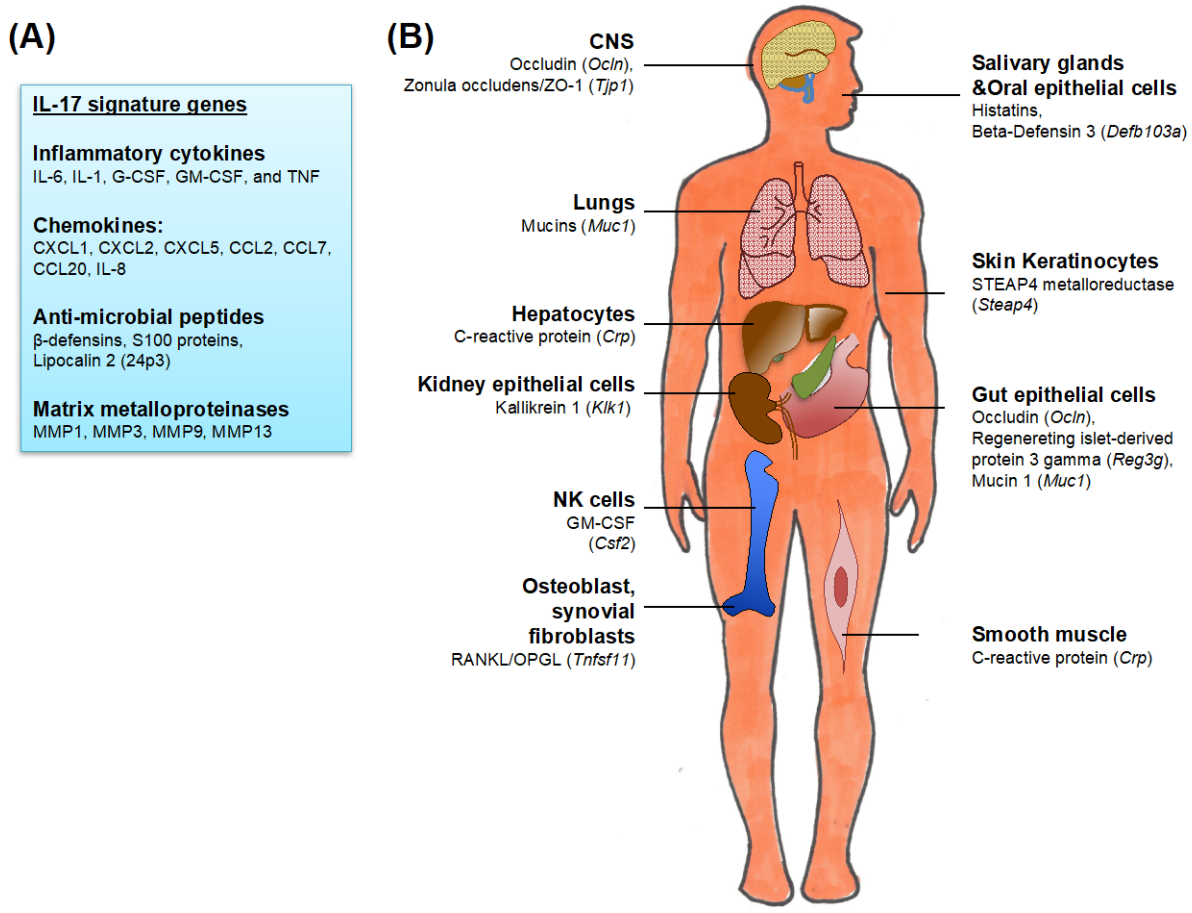


**Figure 1.3: Major Inflammatory Genes Regulated by IL-17**

IL-17 signaling controls inflammation by regulating expression of inflammatory genes in the cells of mostly non-hematopoietic compartment. The IL-17 signature genes (top left) are common inflammatory genes regulated by IL-17. Genes listed elsewhere are specific to respective tissue compartments and play a crucial role in mediating tissue-specific IL-17-dependent inflammation. Abbreviation: NK, natural killer.

### 1.7.1 Transcriptional regulation of IL-17 signaling

***NF-κB and MAPKs***. The IL-17R signaling pathway shares many features in common with IL-1R/TLR pathways, but there are notable differences, particularly in receptor-proximal signaling events. Most significantly, the earliest event in signaling following IL-17 receptor engagement is the induced association of the IL-17R with Act1 (CIKS), an adaptor not used by the IL-1R/TLR family. (17, 85). In addition to its role as an adaptor, Act1 is a Lysine-63 (K63) E3 ubiquitin ligase, which recruits and ubiquitinates TRAF6, also a K63 E3 ligase. Ubiquitination of TRAF6 provides a scaffold for the recruitment and activation of the transforming growth factor β-activated kinase (TAK)1 and the inhibitor of NF-κB kinase (IKK) complex composed of IKKα, IKKβ and IKKγ (NEMO) (85-89). IKK then phosphorylates the IκB subunit of the NF-κB:IκB complex, marking IκB for proteasomal degradation. Degradation of IκB exposes a nuclear localization signal on NF-κB, freeing it for rapid nuclear translocation and consequent inflammatory gene transcription **(Figure 1.3)** (90). IL-17 signaling does not activate the non-canonical NF-κB pathway (13). Consistently, most IL-17 target genes have essential NF-κB-binding elements (91).

While NF-κB is a undisputedly a key event in the IL-17 signaling cascade, IL-17 is consistently found to be only a modest activator of NF-κB (84). Despite increased understanding of factors controlling NF-κB activation, the underlying basis of this weak activation is not yet understood. Nonetheless, IL-17 is a powerful inducer of inflammatory cytokines due to its capacity to signal synergistically with other stimuli. Although best-studied for TNFα, IL-17 also cooperates with lymphotoxin (LT), IFNγ, LPS and others (76). Interestingly, the synergistic up-regulation of IL-17 and TNFα target gene expression is not mediated through NF-κB activation

(83, 84). Rather, co-stimulation with TNF-α and IL-17 results in stabilization of TNF-α-induced mRNA transcripts that are inherently unstable, which is characteristic of most chemokine and cytokine genes (92-95). IL-17 was shown to synergize with the fibroblast growth factor FGF2 in colitis driven by dysregulated intestinal microbiota. In contrast to its positive role in IL-17R signaling, Act1 is a negative regulator of FGF2; FGF2 and IL-17 co-stimulation resulted in preferential binding of Act1 to IL-17RA, thus dampening its suppressive effect on FGF2 activity (47). These combinatorial effects of IL-17 create a setting in which the physiological impact of IL-17 *in vivo* is profound, even though its individual effects in isolated experimental conditions do not always appear to be particularly potent. Nevertheless, some studies suggest that the activity of IL-17 is dependent on the cellular context. For example, in a reconstructed human epidermis (RHE) model, IL-17 was shown to regulate significantly more genes in differentiated, versus undifferentiated, keratinocytes. The differential IL-17 response was attributed to higher expression of the transcription factor C/EBPβ in differentiated keratinocytes (96).

Many IL-17-dependent genes are regulated by inhibitor of NF-κB (IκB)-ζ, a non-canonical member of the NF-κB transcription factor family. Despite its name, IκBζ acts primarily as a driver of transcription rather than an inhibitor. IL-17 induces both mRNA and protein expression of IκBζ, which in turn positively activates numerous IL-17 target genes in cooperation with NF-κB (93, 94, 97-99). Additionally, IκBζ facilitates IL-17-induced gene expression by suppressing expression of miR-23b, an inhibitor of IL-17 signaling (100). The transcript encoding IκBζ (*Nfkbiz*) is intrinsically unstable and IL-17 serves to enhance its stability thereby indirectly upregulating expression of its target genes. One case in point is Lipocalin 2 (Lcn2, also known as 24p3 or NGAL), a prototypical IL-17 target gene (**Figure 1.2**) whose expression is exquisitely IκBζ-dependent (91, 93, 98). Thus, IκBζ is a central regulator of

IL-17 signaling, and identifying novel IκBζ target genes important for IL-17-mediated inflammation may be an informative area for future studies.

IL-17 also activates MAPK pathways, which include extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK), although the dominance of these pathways in response to IL-17 appears to vary somewhat by cell type background **(Figure 1.3)** (84). Support for a role for MAPKs *in vivo* came from studies in which mice with impaired p38-MAPK signaling showed reduced pathology in IL-17-driven experimental autoimmune encephalomyelitis (EAE). Moreover, mice lacking the p38-MAPK inhibitor MKP1 (Dusp1) showed enhanced IL-17-dependent signaling in this setting (101). The TPL2 kinase is also upstream of IL-17-mediated MAPK activation. Upon IL-17 stimulation, IKK mediates p105 phosphorylation, releases TPL2 from p105 and activates p38 and JNK. Interestingly, the TPL2-TAK1 axis activates IKK, creating a positive-feedback loop that reinforces gene induction. TPL2 deficiency is associated with compromised IL-17-induced expression of chemokines and pro-inflammatory cytokines in CNS, and *Tpl2*$^{-/-}$ mice are resistant to EAE (102). In the TLR and TNFR pathways, TPL2 mediates ERK1/2 activation; however, TPL2 is dispensable for ERK activation in IL-17 signaling (102, 103). Hence, the mechanism that activates ERK in the IL-17 pathway is still unclear. Another recent study showed that IL-17 promotes formation of a multi-protein signaling complex that comprises of IL-17R-ACT1-TRAF4-MEKK3-MEK5. This complex activates ERK5 but not NF-κB, p38, JNK, or ERK1/2, which induces IL-17 target genes resulting in keratinocyte proliferation and tumor formation (104).

Although the IL-17 signaling pathway is primarily activated by serine/threonine kinases that characterize NF-κB and MAPK pathway activation, IL-17 was recently reported to activate the spleen tyrosine kinase (Syk) in keratinocytes. Upon IL-17 stimulation, Syk associated with

IL-17RA, Act1 and TRAF6, leading to activation of NF-κB (105). Recently, CARMA2 (encoded by *Card14*), a scaffold protein, was shown to associate with Act1 and TRAF6, and activate NF-κB, p38 and JNK but not ERK. Mice with E138A gain of mutation in *Card14* develop spontaneous psoriasis-like disease, and *Card14*$^{-/-}$ mice are more susceptible to imiquimod (IMQ)-induced dermatitis (106). Thus, there may be multiple pathways by which IL-17 activates NF-κB.

***C/EBPs***. CCAAT/enhancer-binding protein (C/EBP) transcription factors are additional transcriptional regulators that are modulated by IL-17. Both NF-κB and C/EBP binding sites are over-represented in promoters of IL-17 target genes (91), and expression of C/EBPβ and C/EBPδ is increased by IL-17 in a variety of settings (95). Several gene promoters absolutely require C/EBPβ and/or C/EBPδ for IL-17-dependent induction (e.g., *Il6* and *Lcn2*), even when the NF-κB sites are intact (19, 107, 108). Some data suggest that activation of C/EBP family members is part of the cooperative/synergistic activation of IL-17 with TNF-α (95). C/EBPδ is primarily induced transcriptionally, whereas C/EBPβ is regulated by IL-17 post-transcriptionally (alternative translation) and post-translationally (phosphorylation). For example, there are three methionine start sites within the *Cebpb* gene, and IL-17 triggers a shift to use of the less dominant sites (95). The biological significance of IL-17-induced changes in alternative translation is unclear, but one of the alternatively generated C/EBPβ isoforms contains only the DNA binding domain and is thus potentially a transcriptional repressor (95, 109). C/EBPβ is also inducibly phosphorylated at multiple sites following IL-17 stimulation, an event that is associated with reduced signaling. Specifically, ERK and glycogen synthase kinase 3 β(GSK-3β) phosphorylate C/EBPβ on two threonine residues within its regulatory domain (24). Intriguingly,

both these alternative translation and phosphorylation of C/EBPβ are mediated by the IL-17RA CBAD domain (19, 24), linking C/EBPβ to negative regulation of IL-17.

**Ubiquitin ligases-mediated transcriptional regulation.** Ubiquitin-related post-translational modifications play a major role IL-17 signaling. Ubiquitin ligases recognize and link ubiquitin chains to their respective substrate. Though all seven lysines can be used for ubiquitination, only $K^{48}$ and $K^{63}$ linked chains have been described thus far in IL-17 signaling. K48 ubiquitinated substrates typically undergo proteasomal degradation, whereas K63 chains promote signal transduction by enhancing protein-protein interactions (110). As described above, E3 ubiquitin ligases such as Act1 and TRAF6 are central activators of the IL-17 signaling pathway through K63-linked ubiquination events. The β-transducin repeat–containing protein (β-TrCP) is an F-box E3 ubiquitin ligase that ubiquitinates phosphorylated Act1 on K48 residues under prolonged stimulation, thus triggering Act1 degradation and restricting IL-17 activity (**Figure 1.4**) (111). Furthermore, heat shock protein 90 (Hsp90), a chaperone that helps in protein folding and assembly, maintains the integrity of Act1 at the protein level. Inhibition of Hsp90 leads to proteasomal degradation of Act1 and thus downregulates IL-17 activity (112) (**Figure 1.3**). As Act1 is the focal point of IL-17 signal transduction, it is not surprising that there are multiple pathways involved in fine-tuning Act1 expression and activation.

Ubiquitin ligases also influence signal transduction by competing for common substrates/complexes. Although the impact of their ubiquitin ligase activity is unclear, TRAF3 and TRAF4 both act as inhibitors of IL-17 signaling (**Figure 1.4**) As noted, TRAF3 binds to the CBAD domain in IL-17RA and interferes with IL-17RA-Act1 interaction. In contrast, TRAF4 competes with TRAF6 for occupancy of TRAF binding sites on Act1. Consequently, deficiency of TRAF3 or TRAF4 results in enhanced IL-17-activated gene expression and therefore

enhanced autoimmune disease severity (25, 113).

Removal of ubiquitin chains (mainly K63-linked) also serves to dampen IL-17-induced inflammatory response. This process is carried out by deubiquitinase enzymes (DUBs), and reverses the positive signaling induced by Act1-TRAF6. In the IL-17 pathway, the A20 (encoded by *Tnfaip3*, a common autoimmune gene locus (110)) and Usp25 have both been shown to deubiquitinate TRAFs (**Figure 1.4**) (26, 114). A20 deubiquitinates TRAF6 and this suppresses NF-kB and MAPK activation, while Usp25 deubiquitinates both TRAF5 and TRAF6 to restrict downstream gene expression. A20 expression is also upregulated by IL-17, making this another feedback regulatory response. Moreover, A20 is recruited to the CBAD domain of IL-17RA, explaining why deletion of this part of the receptor leads to exacerbated IL-17-dependent gene induction (19, 20). A20 interacts with the non-canonical adaptor Anaphase Promoting Complex Subunit 5 (ANAPC5, also called APC5), and knockdown of ANAPC5 enhances IL-17 signaling (115). Notably, since these DUBs act on other inflammatory pathways (TNFα, IL-1R, etc), their activation by IL-17 can have ripple effects that serve to restrict inflammation more globally. Cumulatively, ubiquitin-related protein modifications are rheostats that help control the magnitude of IL-17-induced inflammatory gene expression.

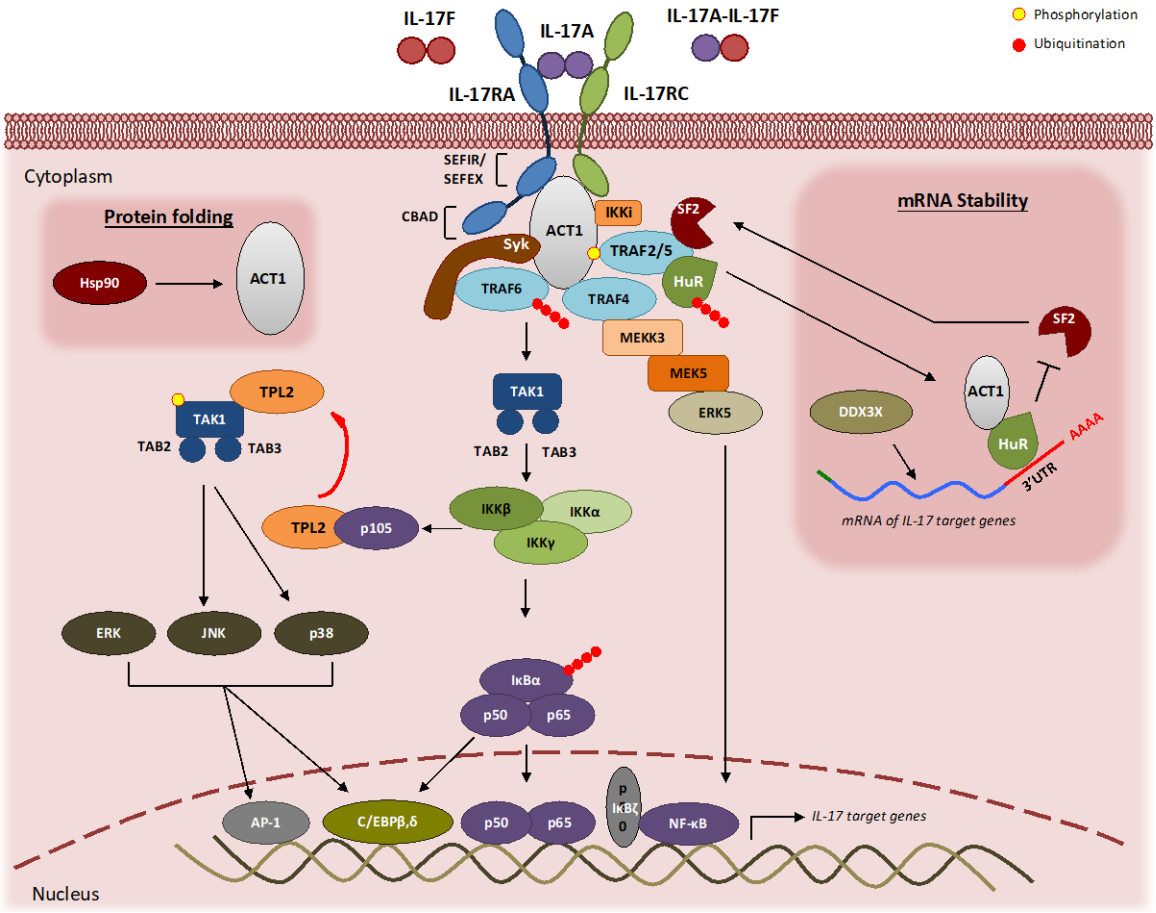


**Figure 1.4: Activation of IL-17 Signal Transduction**

IL-17 signaling starts with the binding of IL-17A/A, IL-17A/F, or IL- 17F/F cytokine to their receptors IL-17RA and IL-17RC. Upon ligand binding, Act1 activates multiple independent signaling pathways operating through different TRAF proteins. Activation of TRAF6 results in the triggering of NF-kB, C/EBPb, C/EBPd, and MAPK pathways. The IL-17R–Act1 complex also associates with MEKK3 and MEK5 via TRAF4, resulting in the activation of ERK5. While TRAF6- and TRAF4-mediated IL-17 signaling results in transcription of inflammatory genes, IL-17 signaling through the ACT1–TRAF2–TRAF5 complex results in the control of mRNA stability of IL-17 target genes. Abbreviations: ERK, extracellular signal-regulated kinase; Hsp, heat-shock protein; HuR, human antigen R, also known as ELVAL1; IKK, inhibitor of kB

kinase; JNK, Janus kinase; SF2: splicing factor 2; TAK1, TGF-b activated kinase 1; TRAF, TNF receptor-associated factor; UTR, untranslated region.

### 1.7.2 Post-transcriptional regulation of IL-17 signaling

In addition to turning on inflammatory genes by *de novo* transcription, IL-17 promotes expression of a large number of genes by modulating mRNA stability. Stabilized transcripts are stored in cytoplasmic granules, and can either be translated or degraded rapidly as needed (57). Indeed, mRNA stabilization appears to be one of the mechanisms by which IL-17 synergizes with other cytokines such as TNFα (92, 93). Moreover, many inflammatory cytokines and chemokines induced by IL-17 are intrinsically unstable. Since IL-17 is a poor stimulus for NF-κB and only induces modest transcription of inflammatory genes, its capacity to indirectly control inflammatory gene expression through control of factors like IκBζ is a fundamental feature of IL-17-mediated inflammation.

***RNA binding proteins*:** IL-17-induced regulation of mRNA stability is controlled in several non-canonical ways. For example, tristetraprolin (TTP), one of the best-characterized ARE-binding factors known to be activated by IL-1β and TNFα, does not stabilize mRNA in response to IL-17 (116). Like all known IL-17-dependent signals, the IL-17-mediated mRNA stability pathway is dependent on Act1, but surprisingly independent of TRAF6 (117). Instead, Act1 is inducibly phosphorylated (22), which triggers a shift in TRAF usage and subsequently determines which downstream pathway is preferentially activated (118). Using the *Cxcl1* mRNA transcript as a representative system, IL-17 was found to recruit the inducible IκB kinase (IKKi,

also known as IKKε) to the IL-17R-Act1 complex, leading to phosphorylation of Act1 on Ser 311.

Phosphorylated Act1 favors recruitment of TRAF2 and TRAF5, which sequester an RNA decay factor (the mRNA splicing regulatory factor 2, SF2) away from the 3' UTR of *Cxcl1* mRNA. This sequestration thus prevents degradation of *Cxcl1* (119). At the same time, IL-17 recruits the RNA binding protein human antigen R (HuR, also known as ELAVL1) to the Act1/TRAF2/TRAF5 complex and activates HuR through Act1-dependent ubiquitination. HuR directly binds to the 3'UTR of the *Cxcl1* transcript. Since HuR and SF2 recognize similar sequences within the 3'UTR, HuR sterically competes with SF2 and prevents SF2-mediated RNA decay (120) (**Figure 1.3**). While phosphorylation of Act1 by IKKi on Ser 311 mediates IL-17-induced mRNA stability, phosphorylation of Act1 on three other Ser sites by IKKi and IKK-related kinase, TANK Binding Kinase 1 (TBK1), suppresses IL-17 signaling **(Figure 1.4)**. IL-17 activates TBK1, which associates with and phosphorylates Act1. Additionally, Act1 phosphorylation interferes with binding of TRAF6 to Act1 and inhibits NF-κB activation (121). Thus, a complex series of events centered around the Act1 and TRAF proteins controls the activation of gene expression through both transcription and mRNA stability.

A recent study showed that Act1 can directly bind to a stem-loop structure on *Cxcl1* mRNA through the SEFIR domain, revealing that Act1 itself is a RBP. In this study, Act1 was shown to form three independent RNA-protein complexes in different cellular compartments, in an IL-17 dependent manner. In the nucleus, Act1 stabilizes *Cxcl1* mRNAs by counteracting SF2. In P-bodies, Act1 mediates TBK1-induced phosphorylation of decapping enzymes Dcp1 and Dcp2 on *Cxcl1* mRNAs, and prevents mRNA degradation. In polysomes, Act1 facilitates HuR binding to *Cxcl1* mRNAs and facilitates *Cxcl1* translation (122). Thus, Act1 as a central

orchestrator of IL-17 signaling pathways that ultimately induces both transcription and mRNA stability.

Although enhanced inflammation due to IL-17-mediated mRNA stabilization is beneficial during clearance of pathogens, constraint of this pathway is critical for prevention of severe inflammatory conditions. Recently we showed that the endoribonuclease MCPIP1 (MCP-1-induced protein 1, also known as Regnase-1) negatively regulates IL-17 signaling by degrading mRNAs encoding IL-17-dependent genes (98). IL-17 induces MCPIP1 mRNA (*Zc3h12a*) and protein in non-hematopoietic cells (98, 123, 124). Specifically, IL-17 induces MCPIP1 via NF-κB, but also stabilizes *Zc3h12a* transcripts through activation of the DEAD box protein DDX3X (98, 125). DDX3X forms a complex with Act1 upon IL-17 stimulation and promotes stability of *Zc3h12a* mRNA though, interestingly, not of *Il6, Cxcl1* or *Cxcl5* mRNA (125) (**Figure 1.3**). In turn, MCPIP1 acts as a negative feedback inhibitor by degrading IL-17-induced mRNA transcripts such as *Il6* and *Nfkbiz* (encoding IκBζ, see above) through their respective 3'UTRs (**Figure 1.4**). Moreover, MCPIP1 indirectly regulates expression of other IκBζ-dependent IL-17 target genes by controlling IκBζ mRNA stability and ultimately protein expression. Consequently, MCPIP1-deficient mice exhibit enhanced IL-17 signaling which makes them less susceptible to infection (e.g. by *Candida albicans*) while exacerbating IL-17-mediated pathology in mouse models of EAE, pulmonary inflammation and psoriasis (98, 126).

Intriguingly, MCPIP1 also degrades the transcripts encoding IL-17 receptor subunits *Il17ra* and *Il17rc.* Strikingly, this regulation occurs in a manner that is independent of the 3'UTR motifs, but rather through binding to sequences located within the coding region (98). Recently, the RNA binding proteins Roquin-1 and Roquin-2 were shown to cooperate with MCPIP1 to repress mRNAs of $T_H17$ differentiating factors such as IL-6, thus regulating IL-17 production

(127). Moreover, Roquins also regulate IL-6 production downstream of IL-17 signaling. However, the role of Roquins and MCPIP1 are not fully redundant in IL-17 pathway, as knockdown of both enhances signaling more than knockdown of either alone (98). Recently, MCPIP1 and Roquin-1 were shown to recognize stem-loop structural motifs in the 3'UTR of their target transcripts, rather than binding to specific sequences (128). A similar mechanism likely exists in IL-17 signaling.

***MicroRNAs*:** MicroRNAs regulate gene expression through mRNA decay or suppress translation. In IL-17 signaling, miR-23b was found to target signaling intermediates such as TAB2, TAB3 and IKK-α, which led to inhibition of NF-κB activation. IL-17 downregulates miR-23b transcription, resulting in feedback activation of IL-17 signaling (100). In addition, miR-30a degrades *Traf3ip2* mRNA (encoding Act1) and consequently inhibits IL-17-induced NF-κB and MAPK activation (**Figure 1.4**) (129). Doubtless more layers of miRNA-directed regulation of IL-17 signaling will come to light.

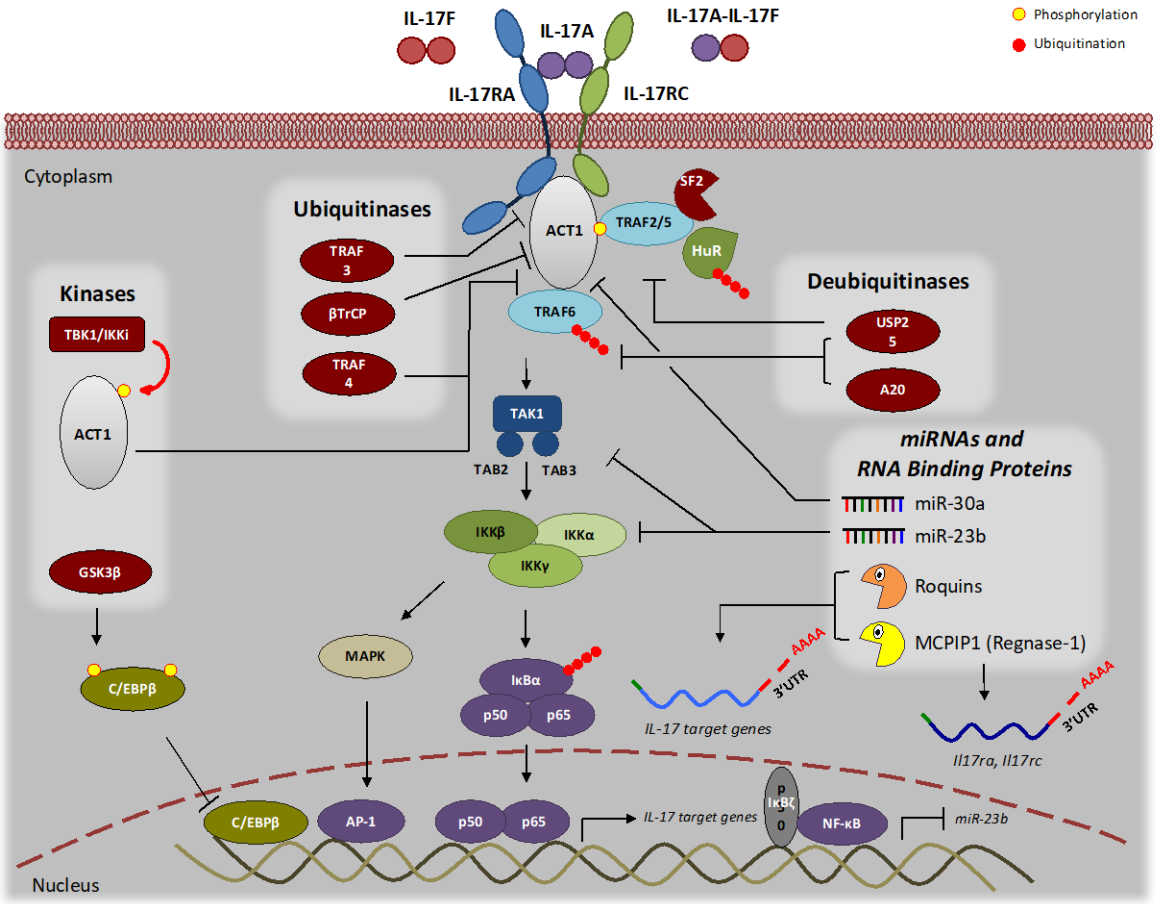


**Figure 1.5: Negative Regulation of IL-17 Signal Transduction**

Different classes of inhibitors such as ubiquitinases (TRAF3, TRAF4, and bTrCP), deubiquitinases (A20 and USP25), kinases (TBK1, GSK3b), endoribonuclease (MCPIP1/ regnase-1) and micro RNAs (miR-23b and miR-30a) negatively regulate IL-17 signaling through various independent mechanisms (Table 1). Abbreviations: GSK, glycogen synthase kinase; HuR, human antigen R, also known as ELVAL1; IKK, inhibitor of kB kinase; MAPK, mitogen-activated protein kinase; SF2, splicing factor 2; TAK1, TGF-b activated kinase 1; TBK1, TANK-binding kinase 1; TRAF, TNF receptor-associated factor; UTR, untranslated region.

**Table 1.2: Proximal Activators and Inhibitors of the IL-17 Signaling Pathway [reviewed in (88, 89, 130)]**

| Classes | Molecules | Function |
|---|---|---|
| **Ubiquitinases** | Act1 | Recruits and ubiquitinates TRAF6 |
| | TRAF2 | Mediates mRNA stability of IL-17 target genes |
| | TRAF5 | Mediates mRNA stability of IL-17 target genes |
| | TRAF6 | NF-κB and MAPK activation |
| | TRAF3 | Interferes with the formation of IL-17R-Act1-TRAF6 complex |
| | TRAF4 | Competes with TRAF6 for associating with Act1 |
| | βTrCP | Degradation of Act1 |
| **Deubiquitinases** | A20 | Deubiquitinates TRAF6 |
| | USP25 | Deubiquitinates TRAF5 and TRAF6 |
| **RNA Binding Proteins** | HuR | Stabilizes mRNA of *Cxcl1* and *Cxcl5* |
| | DDX3X | Stabilizes mRNA of *Zc3h12a* (MCPIP1) |
| | SF2/ASF | Degrades mRNA of *Cxcl1* |

Table 1.2 (continued)

| | | |
|---|---|---|
| | MCPIP1/ Regnase-1 | Degrades mRNA of IL-17 target genes and IL-17 receptors |
| | Roquin-1/2 | Degrades mRNA of IL-17 target genes |
| **micro RNAs** | miR-23b | Targets TAB2, TAB3 and IKK-α |
| | miR-30a | Targets Act1 |
| **Chaperones** | Hsp90 | Mediates folding of Act1 |
| **Kinases** | MAPKs | Activates AP-1 |
| | IKKα/β/γ | Activates NF-κB |
| | TAK1, TAB2, TAB3 | Activates NF-κB |
| | Syk | Mediates Act1-TRAF6 complex formation |
| | TPL2 | Phosphorylates TAK1 |
| | IKKi | Phosphorylates Act1 and mediates mRNA stability |
| | GSK3β | Phosphorylates C/EBPβ and inhibits IL-17 signaling |

Table 1.2 (continued)

| | | |
|---|---|---|
| | TBK1/IKKi | Phosphorylates Act1 and inhibits Act1 and TRAF6 binding |

## 1.8 SUMMARY

Although IL-17 is a critical mediator of inflammation, it is a modest activator of known signaling pathways, compared to other inflammatory stimuli. Nonetheless, its capacity to synergize with other cytokines makes this a vital inflammatory effector. This is highly biologically relevant in the context of an inflammatory environment, where conditions are not driven independently by a single cytokine, but rather as a result of concerted actions of multiple inflammatory mediators (83). This chapter highlights the pathogenic and protective aspects of IL-17 and discusses multiple regulatory mechanisms that keep IL-17 signaling in check. Since the positively-acting proteins in the IL-17 pathway can drive pathogenicity if not held in check, negative inhibitors are vital to keep the inflammation under control.

It is not well understood why so many non-redundant mechanisms exist to turn on or off IL-17 signal transduction (summarized in **Table 1**). One potential explanation is that these regulators individually are not sufficient to enhance or halt a response, but that a collective effort at multiple levels is necessary, a phenomenon described in many other immune settings (58). Similarly, the kinetics of induction or activity of each regulator may contribute uniquely at different stages of signaling. Moreover, once induced, these inhibitors can also regulate other signaling pathways, thus serving to fine-tune inflammation. Biologically, it is imperative that the

precarious balance between pathogenic and protective sides of pro-inflammatory IL-17 signaling be maintained. Either uncontrolled acceleration of the system or failure of the “brakes” can lead to persistent inflammation, resulting in tissue damage and/or autoimmune diseases. Gaining an in-depth understanding of these regulators may therefore help in the rational design of drug targets for maintaining balance during dysregulation or for treatment of diseases where IL-17 activity is beneficial. Furthermore, as more activators and inhibitors are identified, it will be critical to understand their specificity and the interplay between all these factors influencing IL-17-induced inflammation.

# 2.0 MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Cells and Reagents

ST2 cells (murine bone marrow stromal cell line) and HEK293T cells were cultured in α-MEM (mimimum essential medium, Sigma Aldrich, St. Louis MO) supplemented with 1% L-glutamine, 1% Pen-Strep and 10% FBS. Primary mouse embryonic fibroblasts (MEFs) were cultured in α-MEM supplemented with 1% L-glutamine, 20% FBS and no antibiotics. N/TERT2G cells were a gift from Johann E. Gudjonsson (Department of Dermatology, Taubman Center, University of Michigan, Ann Arbor, 48109, USA). N/TERT2G cells were cultured in Keratinocyte-serum free media (Gibco) supplemented with 30 μg/ml bovine pituitary extract-BPE, 0.2 ng/ml recombinant human epidermal growth Factor- EGF, $CaCl_2$ and antibiotics.

### 2.1.2 Cytokines

Recombinant murine IL-17, murine TNF-α and human IL-17A were purchased from Peprotech and were resuspended in 0.27% bovine serum albumin (BSA) containing sterile PBS. Murine cells were treated with mIL-17A at 200 ng/mL final concentration or with mTNF-α at 10ng/mL

final concentration. Human cells were treated with recombinant human IL-17 at 100 ng/mL final concentration.

### 2.1.3 Inhibitors

Actinomycin D (Cat # A9415) was purchased from Sigma and used at 5μg/mL final concentration. IKK inhibitor VII (Cat# 401486) was purchased from EMD Millipore was used at 10 μM final concentration.

### 2.1.4 Small Interfering RNA (siRNA)

ON-TARGETplus SMARTpool siRNAs against gene of interest and Non-targeting control siRNAs were purchased from Dharmacon. siRNAs were reconstituted in 1X siRNA buffer (Catalog # B-002000) at 50μM stock concentration and used at 50nM final concentration. List of siRNAs used are listed in Table 2.1.

**Table 2.1: SMARTpool ON-TARGETplus siRNAs**

| Gene | Catalog # | Organism | siRNA target sequences |
|---|---|---|---|
| Arid1a | L-040694-01-0005 | Mouse | AGAUGUGGGUGGACCGGUA<br>AAGCAUUGCCCAAGAUCGA<br>GGACAGGGGAUCAAUAGUA<br>CCUUGGGGAUGUUAAGUUA |
| Arid1b | L-053908-01-0005 | Mouse | CGAUGGACCCAAUGGUGAU<br>CAAGAAGGCUAUGGAACUA<br>CAGUUGUAUGGGAUGGGUA<br>CCAUGAAGACUUGAAUUUA |
| Arid2 | L-055278-01-0005 | Mouse | GGUCUUAGCAUGAGCGGAG<br>ACUAAACACAUCCGACUAA<br>AAACAUAACUCCAUCGUCA<br>CAGAAAUGCUGUAGUGAAU |
| Arid3a | L-054716-00-0005 | Mouse | GAGAUGAAUGGUAUUGUAU<br>CAAACAACUCCUUGCCUUA<br>CGCCCAAGAUCAAGAAAGA<br>GGACGUGGGCUCUGAUGAU |
| Arid3b | L-054720-01-0005 | Mouse | CCAGAGUAGUAUAUCGACA<br>CCAUUCAGGUUCUGCUGUA<br>UGAAGUAUCUAUAUGCCUA |

Table 2.1 (continued)

| | | | |
|---|---|---|---|
| | | | CGGGAGACCUUCUGGUAGU |
| Arid3c | L-068890-01-0005 | Mouse | CCUCUUUGCUCGACGCCAA<br>UGUAUGAACUCGAUGCGGA<br>UGGAAGUCAUCAACCGAAA<br>ACGUGGCACUAGAGAUCAA |
| Arid4a | L-043734-01-0005 | Mouse | GGACGCAGGUGAUGACGAU<br>GCGAUCAAAUUGUGCAUAU<br>GAUUUAGAUGAGAAGGAUA<br>ACCCAAAGAUUGUACAUAU |
| Arid4b | L-054961-01-0005 | Mouse | GUGCUUGGGUAUCGAAAUU<br>CCAUACAGAUUUGGUAAUA<br>GCACCAAAUCAAACCGUAA<br>UAACAUUGGCAAAGACGAA |
| Arid5a | L-054696-01-0005 | Mouse | CGUCACAACCUAUGCGGCA<br>GCUCAAAGGUGGCGUAUGA<br>GUGACCAAUGUGCGAGCAA<br>CCUCUAAACUUCACCGGUA |

Table 2.1 (continued)

| | | | |
|---|---|---|---|
| Arid5a | L-022856-02-0005 | Human | CCAGGUUGCCCACGGAAAA<br>CCACCGAGAGGCCGAAGAA<br>GCAGAGGACCCAGUUGUUA<br>UCUACAAAGCAGUGGAGAA |
| Arid5b | L-054678-01-0005 | Mouse | GGUCCAUGCUUAAACGGAU<br>CGGAGAAGAUCCACGUCAA<br>GUGAUGAGUUCGCGCCAAA<br>ACAAUAACUGUGACGGUAA |
| HuR<br>(*Elavl1*) | L-053812-00-0005 | Mouse | CAGUUUCAAUGGUCAUAAA<br>GAUCACAUGAGUGGGAUUU<br>GACCACAGGUUUGUCCAGA<br>GGUUGAAUCUGCAAAGCUU |
| IMP2<br>(*Igf2bp2*) | L-041793-01-0005 | Mouse | ACGACAUGCUGGCCGUUAA<br>CGUCAGAAUUAUCGGGCAU<br>GGCAUCAGUUUGAGGACUA<br>GGAUGGACCUAGACACCUA |
| MCPIP1<br>(*Zc3h12a*) | L-052076-01-0020 | Mouse | AGUACUGGUCUGAGCCGUA<br>CGAGAAGGCUCACCGCAGA<br>UGGACAACUUCCUUCGUAA<br>GGAAACGCUUCAUCGAGGA |
| TRAF2 | L-042814-01-0005 | Mouse | GAGUCUACUUGAAUGGCGA<br>CUGUCAUCGUGGCGGCAAA<br>CGGAGUGUCCUGCAUGUAA<br>AGUUCGGCCUUUCCAGAUA |

Table 2.1 (continued)

| | | | |
|---|---|---|---|
| TRAF5 | L-041340-01-0005 | Mouse | UAACGAGAGCUGCCGGGAA<br>CCUUGGACUUUGAGCCCGA<br>GCUCCGGAAAGACGUGAAA<br>GGGUGCAAUGCCAGGAUUA |

### 2.1.5 Plasmids

All plasmids used in this study are listed in Table 2.2.

**Table 2.2: List of plasmids.**

| Plasmid | Organism | Source | Catalog # | Gaffen # |
|---|---|---|---|---|
| Flag/Myc-Arid5a | Mouse | Origene | MR219216 | M77 |
| *Nfkbiz*+3UTR | Mouse | Dr. Ulrich Siebenlist (NIH, Bethesda MD) | N/A | H86 |
| pGL3-Luciferase-*Il6* 3UTR | Mouse | Dr. Keith L. Kirkwood (Medical University of South Caroline) | N/A | L12 |
| pcDNA3.1(+)-*Cxcl1* 3'UTR | Mouse | GeneArt/Thermo Fisher | Ref# 1917594 | N93 |
| pcDNA3.1(+)-*Csf2* 3'UTR | Mouse | GeneArt/Thermo Fisher | Ref# 1917598 | N94 |
| pCR2.1_Il6 3UTR | Mouse | Gaffen Lab (Nilesh Amatya) | N/A | N92 |
| Mouse MCPIP1 | Mouse | Gaffen Lab (Abhishek Garg) | N/A | M41 |
| Act1-Myc | Mouse | Gaffen Lab (Amarnath Maitra) | N/A | M15 |
| TRAF2-Myc | Human | Gaffen Lab (Amarnath Maitra) | N/A | M13 |
| pGL3-Basic_Imp2 proximal promoter | Mouse | Gene ART/Gaffen Lab (Nilesh Amatya) | N/A | L15 |

### 2.1.6 Antibodies

Antibodies used for western blot are listed in Table 2.3.

**Table 2.3: List of Western Blot Antibodies**

| Antibody | Vendor | Catalog # | Dilution |
|---|---|---|---|
| Act1 | Santa Cruz | sc-11444 | 1:1000 |
| Anti-mouse-HRP | Thermo Fisher | 31430 | 1:10000 |
| Anti-rabbit-HRP | Invitrogen | 31460 | 1:10000 |
| Arid5a | Sigma Aldrich | SAB2100147 | 1:500 |
| C/EBPβ | Biolegend | 606202 | 1:500 |
| eIF4G | Cell Signaling | 2469 | 1:1000 |
| FLAG-tag M2 | Sigma Aldrich | F3165 | 1:1000 |
| IMP2 | Cell Signaling | 14672S | 1:1000 |
| IMP2 | Harvard University | n/a | 1:1000 |
| IκBζ | Cell Signaling | 9244S | 1:500 |
| MCPIP1 | Santa Cruz | sc-136750 | 1:1000 |
| Myc-tag | Cell Signaling | 2276S | 1:1000 |
| TRAF2 | Santa Cruz | sc-876 | 1:500 |
| TRAF5 | Santa Cruz | sc-6195 | 1:500 |
| YY1 | Santa Cruz | sc-1703 | 1:1000 |
| α-tubulin | Abcam | ab40742 | 1:5000 |
| β-actin-HRP | Abcam | ab49900 | 1:25000 |

### 2.1.7 Cloning Primers

**Table 2.4: List of primers used for cloning**

| Gene | Description | Primer Sequence (5' to 3') |
|---|---|---|
| *Il6* 3'UTR | Fwd with 5' EcoRI<br>Rev with 3' HindIII | CCGAATTCTAGTGCGTT ATGCCTAAGC<br>CCAAGCTTGTTTGAAGACAGTCTAAAC |

### 2.1.8 qRT-PCR primers

All primers for qRT-PCR were purchased from Qiagen and are listed on Table 2.5.

**Table 2.5: List of commercial qRT-PCR primers.**

| Gene | Organism | Catalog # |
|---|---|---|
| *Arid5a* | Mouse | QT00119357 |
| *ARID5A* | Human | QT00000567 |
| *Ccl20* | Mouse | QT02326394 |
| *Ccl5* | Mouse | PPM02960F-200 |
| *Cebpb* | Mouse | QT00320313 |
| *Csf2* | Mouse | QT00251286 |
| *Csf3* | Mouse | PPM02989A-200 |
| *Cxcl1* | Mouse | QT00115647 |
| *CXCL1* | Human | QT00199752 |
| *Cxcl2* | Mouse | PPM02969F-200 |
| *Cxcl5* | Mouse | QT01658146 |
| *Elavl1 (HuR)* | Mouse | QT00135324 |
| *Gapdh* | Mouse | QT01658692 |
| *GAPDH* | Human | QT02504278 |
| *Igf2bp2/Imp2* | Mouse | QT00167951 |
| *Il17a* | Mouse | QT00103278 |
| *Il17ra* | Mouse | QT00112063 |
| *Il6* | Mouse | QT00098875 |
| *Lcn2* | Mouse | QT00113407 |
| *LCN2/NGAL* | Human | QT00028098 |
| *Nfkbiz* | Mouse | QT00143934 |

Table 2.5 (continued)

| | | |
|---|---|---|
| *Traf2* | Mouse | QT00103082 |
| *Zc3h12a* | Mouse | QT01166480 |

### 2.1.9 Mice

WT mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) in the C57BL/6 background or were bred in our facility. *Il17ra*$^{-/-}$ mice were from Amgen. *Act1*$^{-/-}$ mice were from Ulrich Siebenlist (National Institutes of Health (NIH)). *Imp2*$^{-/-}$ and *Imp2*$^{+/-}$ mice were gift from Joseph Avruch, Harvard University, Boston, MA. All mice 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.

### 2.1.10 RNA Immunoprecipitation Buffers

Polysomal lysis buffer was made using 100mM KCL, 5mM $MgCl_2$, 10mM Hepes pH 7.0, 0.5% NP-40, 1mM DTT) with 100 U/mL RNAse Out (Invitrogen). CHAPS lysis buffer was made using 0.3% CHAPS, 40mM HEPES pH7.5, 120mM NaCl, 1mM EDTA, 10mM Sodium Pyrophosphate, 10mM β-glycerophosphate, 50mM NaF, 1.5mM sodium orthovanadate, 1mM DTT), supplemented with Protease Inhibitor cocktail (Sigma). Nuclear extraction buffer was made with 20mM Hepes pH7.9, 0.4M NaCl and 1mM EDTA as previously described (131).

## 2.2 METHODS

### 2.2.1 *In vitro* cytokine treatment

Murine ST2 cells and primary MEFs were treated with recombinant murine IL-17 (Peprotech) at 200 ng/mL final concentration. Human N/TERT2G cells were treated with recombinant human IL-17 at 100 ng/mL final concentration (Peprotech). Murine TNF-α (Peprotech) was added at 10ng/mL final concentration.

### 2.2.2 Total mRNA extraction and qRT-PCR

RNeasy Mini Kit (Qiagen) was used to extract total RNA following the manufacturer's protocol. Note: ST2 cells and MEFs were homogenized in 350µl of RLT buffer using 20-gauge needle and 1mL insulin syringe. Cells were passed through the needle at least 10 times. Mouse spinal cords were homogenized in 500µl of Qiazol using M tubes (Miltenyi Biotech) and gentleMACS Dissociator (Miltenyi Biotech). Chloroform was added to the tissue homogenized in Qiazol and upper aqueous layer was collected. Manufacturer protocol supplied with RNeasy Mini Kit was followed to extract total RNA. Total RNA concentration was assessed using Nanodrop.

For the synthesis of complementary DNA (cDNA), equal concentration of total mRNA between samples was used. cDNA was prepared using Superscript III First-Strand (Invitrogen) by following manufacturer protocol. Expression of genes was determined by qPCR analysis using PerfeCTa SYBR Green FastMix ROX (Quanta BioSciences). Analysis was done on a 7300 Real-Time PCR System (Applied Biosystems). All primers used were commercially purchased and are listed in Table 2.5.

### 2.2.3 Western blot, immunoprecipitation and ELISA

*Western blot:* For whole cell lysates, cells were lysed with 1% NP40 comtaining lysis buffer complemented with protease inhibitors (Roche), sodium orthovanadate and PMSF (Phenylmethylsulfonyl fluoride). ST2 cells and primary MEFs were lysed in 1.5mL centrifuge tubes with intermittent mixing for 10 minutes. HEK 293T cells were lysed directly on the plate for 20 minutes on a rocker in the cold room. For cytoplasmic extracts, cells were lysed with 0.5% NP40 containing polysome lysis buffer. For nuclear extracts, nuclear pellet was lysed in extraction buffer containing 20mM HEPES pH7.9, 0.4M NaCl and 1mM EDTA supplemented with protease inhibitors (Roche), sodium orthovanadate, PMSF. Nuclear lysis was performed by vigorously vortexing nuclear pellet in 1.5mL centrifuge tubes in cold room for 15 minutes. Protein concentration was measured using Pierce BCA Protein assay kit (Thermo Fisher Scientific). Lysates were boiled with 4X SDS at 95°C for 5 minutes. 30μg – 50μg of total protein was loaded in the appropriate homemade polyacrylamide gels. Gels used for most protein analysis contained 10% polyacrylamide. For analysis of C/EBPβ 15% polyacrylamide gels were used.

*Immunoprecipitation:* Whole cell lysates from ST2 cells or HEK 293T cells were obtained as described above. Lysates were precleared with Protein A or Protein G agarose beads for 1 hour. Precleared lysates were then incubated with equal concentration of antibody of interest for 1.5 hours. Protein A or Protein G agarose beads were then added to the pre-cleared lysate + antibody mixture and incubated for further 2.5 hours. Lysates were then washed at least 5 times with ice cold 1% NP40 lysis buffer. Finally, immunoprecipitated fractions were

resuspended in appropriate volume of 1% NP40 lysis buffer with SDS, boiled at 95°C for 5 minutes.

*ELISA:* Mouse and human IL-6 and IL-17A ELISA kits were obtained from eBiosciences. Murine CXCL1 and CXCL5 ELISA kits were obtained from R&D systems. ELISA were performed following manufacturer's protocol. For each experiment, samples were analyzed in at least in duplicates.

### 2.2.4 siRNA and plasmid transfection

ON-TARGETplus SMARTpool siRNAs against genes of interest (listed in Table 2.1) and scrambled control siRNAs were reconstituted in 1X siRNA buffer at 50µM stock concentration. 10µl stocks were stored in -20°C. One day before transfection ST2 cells, primary MEFs or N/TERT2G were plated in 0.4mL of antibiotic free α-MEM supplemented with 1% L-glutamine and 10% FBS in 24 well tissue culture plates. Transfection was done only when the confluency of cells was approximately less than 50% the following day. On the day of transfection, 50µM stock siRNAs were diluted 1:10 with 1X siRNA buffer to get 5µM working stock solution. Cells were transfected with siRNAs at 5nM final concentration using DharmaFECT Reagent 1 (Dharmacon) according to manufacturer's protocol. After 24 hours, media was replaced with fresh antibiotic free α-MEM supplemented with 1% L-glutamine and 10% FBS. Cells were treated with IL-17 for indicated times 48 hours post transfection. Since siRNA transfection can be toxic, cell viability was visually assessed. Knockdown efficiency was tested by qRT-PCR or Western blot. For testing knockdown efficiency by western blot higher number of cells were required so knockdown was performed in 6 well plates or 100cm dishes. For transfection in

larger tissue culture plates, cell number, volume of media, volume of siRNAs and DharmaFECT reagent 1 were scaled up according to manufacturer's protocol. Final concentration of siRNAs was always maintained at 50nM. ST2 cells were transfected with plasmids listed in Table 2.2 using Fugene HD following manufacturer's protocol. 293 T cells were transfected using $CaPO_4$ transfection method.

### 2.2.5 Assessment of mRNA half-life using Actinomycin D

ST2 cells were transfected with control siRNAs or siRNAs against Arid5a as described in section 2.2.4. 48 hours later cells were primed with TNFα (final concentration 10ng/mL) for 3 h in order to induce gene expression. Cells were then washed once with warm 1X PBS, and treated with fresh media containing 5μg/mL actinomycin D (ActD) with or without 200ng/mL IL-17 for 30 minutes, 60 minutes and 90 minutes. Expression of mRNA of interest were assessed at indicated times by qRT-PCR. For each mRNA, remaining quantity (%) was calculated by normalizing ddCt for different time points to the ddCt of samples primed with TNFα.

### 2.2.6 RNA Immunoprecipitation (RIP) using Myc antibodies

*Transfection and IL-17 treatment:* Approximately 700,000 ST2 cells were plated in 150mm dishes (2 plates per IP condition) in 20mL of 10% FBS containing antibiotic free α-MEM. [Note: For each condition, lysates from two plates were used for immunoprecipitating with Myc or isotype control]. Next day, cells were transfected with 16μg/μl of either empty vector or pCMV6-Arid5a-Myc/FLAG per 150mm dish using Fugene HD (Note: DNA:Fugene HD ratio used was 1:4, and 1600μl of transfection mixture was added to 20mL of cells ). 24 hours later,

cells were treated with 20mL of media containing IL-17A at a final concentration of 200ng/mL for 3 hours. [Note: It is important that the cells are not over confluent. Confluency at 80% is desired].

*Pre-coating of protein G beads:* One day before RIP, 100ul of protein G beads per IP condition were washed once with ice cold NT2 buffer and resuspended to its original volume. 10ug of IgG2a (ABCAM ab91361 stock 0.1 ug/ul) or 10ug of anti-myc (Cell Signaling 9B11 stock 0.318 ug/ul) was mixed with 100 ul of 50% vol/vol slurry Protein G beads [Note: concentration of anti-myc may vary between lots]. 500 ul ice cold NT-2 buffer was added to each tube and incubated overnight in a rotator in 4°C cold room. Next day, antibody-coated beads were centrifuged at 13,000 rpm for 5 min at 4°C and supernatants were discarded. Beads were then washed 5 times with 1 ml of chilled NT-2 buffer.

*Cell harvest and lysis:* 3 hours after IL-17 treatment, cells were washed with RNAse-free ice-cold PBS and trypsinized. Cells were spun down @ 2000rpm for 5 mins at 4°C. Cell pellet was washed 3 times with ice-cold PBS, and lysed with 200µl – 300µl of polysome lysis buffer supplemented with RNase inhibitors and protease inhibitors. Lysis was performed for 10 mins in ice with intermittent pipetting. [Note: Pellet were not vortexed to prevent nuclear lysis]. After lysing, cells were centrifuged at 13000 rpm for 30 minutes at 4°C, and supernatants were transferred to fresh RNase-free tubes.

*Pre-clearing and RIP:* Cytoplasmic lysates were pre-cleared with 5µg of IgG2a isotype control for 30 mins rotating at 4°C, and subsequently with 50ul of protein G beads with for 30 mins rotating at 4°C. Pre-coated protein G beads were prepared for RIP by adding 700µl of NT-2 buffer with 10ul of 0.1M DTT, 10ul of RNase Out and 33ul of EDTA. (Note: DTT should not be directly added to the antibody]. Pre-cleared lysates were then added to the Pre-coated protein G

beads and extra NT2 buffer was added to make the total volume 1mL. Lysate + Ab + protein G beads were then incubated for 4 hours rotating at 4°C. After RIP, samples were spun down at 13000 rpm for 5 minutes, and IP fractions were washed for 7 times with 1mL ice-cold NT2 buffer. After final wash 100µl of NT2 buffer containing 5µl of DNase I (2U/ul) was added and incubated at 37°C for 10 minutes. IP fractions were then washed with 1mL of NT2 buffer and centrifuged at 8000 rpm for 5 minutes at 4°C and supernatant were discarded. Next, 100 µl of NT2-buffer with 2.5µl of proteinase K (stock 20mg/mL), 1µl of 10% SDS were added to each tube and incubated at 55°C for 30 minutes (tubes were flicked intermittently). Samples were then centrifuged at 8000 rpm for 5 minutes at 4°C and supernatants were saved. Beads in the pellet were washed once with 200µl of NT2 buffer and supernatants were added to the fractions collected earlier.

*RNA extraction:* 300µl of lower layer acid phenol was added to each sample and vortexed for 1 minute at maximum speed. Samples were then centrifuged for 1 minute at 13000 rpm at room temperature. 250µl of upper layer was collected and were mixed with 25µl of 3M sodium acetate (pH 5.2), 625µl of 100% EtOH and 5µl of glycoblue. Samples were mixed well and incubated overnight at -80°C. Next day, samples were thawed on ice and mixed by inverting 5 times. Samples were then centrifuged at 13000 rpm for 30 minutes at 4°C and supernatants were discarded. Blue pellets were then washed with 1mL 70% EtOH and mixed by inverting 5 times. Samples were then centrifuged at 13000 rpm for 2 minutes at 4°C and supernatants were discarded. Centrifugation at the same speed was repeated one more time, leaving the lid open to let the EtOH evaporate. Pellet were air dried for 5-10 minutes and were resuspended in 12µl of RNase free water. RNA was used to prepare cDNA. mRNA enrichment was assessed by qPCR.

### 2.2.7 RNA Immunoprecipitation (RIP) using eIF4G antibodies

*Cell preparation:* Approximately 190,000 ST2 cells were plated in 100mm dishes (1 plate per condition) in 9.6mL of 10% FBS containing antibiotic free α-MEM. [Note: Cells used were growing at early passage 4-6. Conditions included were siRNA control – Untreated (2 plates), siRNA Control – IL-17 treated (2 plates), siRNA Arid5a – Untreated (2 plates) and siRNA – IL-17 treated (2 plates). For each condition, lysates from one plate was used for immunoprecipitating with anti-eIF4G and other plate was used for immunoprecipiating with isotype control].

*Transfection and IL-17 treatment:* Next day, cells were transfected with either control siRNA or siRNA against Arid5a at a final concentration of 50nM using DharmaFECT Reagent 1. [Note: 2400µl of transfection mixture was added to 9.6mL of cells to make final volume 12mL. Refer to section 2.2.4 for transfection protocol]. 24 hours post transfection media was replaced with fresh 10% FBS containing antibiotic free α-MEM. 48 hours after transfection, cells were treated with 10mL of IL-17A at a final concentration of 200ng/mL for 3 hours. [Note: It is important that the cells at this stage of experiment are at 80% confluent state. Translation efficiency is best captured when cells are at a proliferating state. Therefore, over confluent cells may hinder your results (132) ].

*Cell harvest and lysis:* Cells were then washed once with 5mL of ice-cold RNase free 1X PBS. 1mL of CHAPS buffer supplemented with 50mM NaF, 1.5mM Sodium Orthovanadate, 1mM DTT and 1X complete protease inhibitors was added to each 100mm dishes for cell lysis. Dishes were placed in the cold room for 45 minutes on a rocker. Cell extracts were then collected by scraping, and centrifuged for 10 minutes at 20,000xg at 4°C. Lysate volume was divided

equally for eIF4G RIP and INPUT. I usually get 900ul of cytoplasmic lysate, and use 450ul of lysate for RIP and 450ul of lysate for INPUT. INPUT cell lysate can be saved for immunoblotting purposes. Extracts were either flash frozen with LN2 and stored in -80°C or RIP was performed on the same day (Note: It is better to perform RIP on the same day for maximum sensitivity).

*eIF4G RIP*: Protein G-conjugated Magnetic Dynabeads were prepared by washing once with CHAPS extraction buffer and CHAPS extraction buffer equal to original volume was added to the beads. Lysates were pre-cleared with 10ul of Protein G-conjugated Magnetic Dynabeads for 30 minutes rotating in 4°C. [Note: I switched to Protein G-conjugated Magnetic Dynabeads from Protein A agarose beads to reduce background]. After pre-clearing, 15ul i.e. 3.2 µg of eIF4G antibody or equal concentration of normal rabbit IgG (isotype control) were added to the respective lysates, and incubated for 1 hour rotating in 4°C (Note: concentration of eIF4G antibody varies with LOT#). Then 25ul of Protein G-conjugated Magnetic Dynabeads were added to the lysate + antibody mixture and incubated for 45 minutes rotating in 4°C. Protein G-conjugated magnetic dynabeads now bound to antibody + protein (eIF4G) were collected using a magnetic rack, and were washed 2 times with 1mL CHAPS extraction buffer in cold room.

*RNA extraction, cDNA preparation and qPCR:* After final washing 450µl of Trizol reagent was added to the beads followed by 5 seconds of vortex. Then, 450µl of CHAPS extraction buffer was added to the Trizol/Beads mixture. Similarly, 450µl of Trizol reagent was added to the 450µl of lysates saved for INPUT, followed by 5 seconds of vortex. Then, 180ul of chloroform was added to each sample and mixture was vortexed vigorously for 1 minute. Samples were centrifuged at 16,000g for 15 minutes at 4°C. The upper layer was collected and transferred to a new tube. 1mL isopropanol and 2µl glycoblue were added to the supernatants

and stored overnight in -80°C. Next day, samples were thawed in ice. Samples were then centrifuged at 16000g for 15 minutes at 4°C. Pellets were washed once with 70% ethanol and centrifuged at 16000g for 5 minutes at 4°C. Finally, ethanol was removed and pellet were air dried for 5 minutes. RNA pellet was dissolved in 11µl RNase-free water. RNA obtained from RIP are precious and low yield, therefore concentration obtained by nano drop on such low yield RNA can be relatively inaccurate (133). Therefore, 9µl of RNA (equal volume) from each sample was used to make cDNA. cDNA was diluted 1:2 with RNase-free water and qPCR was done to assess mRNA enrichment. Fold mRNA enrichment for each IP fraction was calculated by normalizing to untreated isotype control IP fraction. For INPUT analysis, RNA was diluted 1:10 and 5ul of total RNA was used to make cDNA. Fold change expression of respective genes for INPUT was calculated by normalizing to untreated samples.

### 2.2.8 *in vitro* biotinylated RNA pull-down

*in vitro transcription:* pCR2.1 or pcDNA3.1 (+) constructs (listed in Table 2.2) encoding 3'UTR of *Il6*, *Cxcl1* and *Csf2* were linearized using appropriate restriction enzymes. 3'UTR of *Il6*, *Cxcl1* and *Csf2* were transcribed *in vitro* using TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, catalog #K0441) and subjected to *in vitro* transcription assay following manufacturer's protocol. Bio-11-CTP nucleotide (Enzo Life Sciences, ENZ-42818) was added to the nucleotide mix to biotinylated *in vitro* transcribed RNA. Linearized plasmids were purified using QIAquick PCR purification kit Cat #28104. DNase I digestion was done to purify RNA contaminated with plasmid DNA. RNA transcripts were then purified using RNeasy Mini Kit (Qiagen). RNA concentration was measured using nano drop.

*For Protein-RNA reaction*: 1µg of biotinylated-RNA, desired amount of protein or whole cell lysate (from HEK 293T cells overexpressing protein of interest), 2.5µl of RNase Out and 2X TENT Buffer was added to make the final volume 50ul. Protein-RNA mixture was incubated for 30 minutes with gentle rotation at room temperature. *Biotinylated RNA pull down:* Dynabeads M-280 Streptavidin (*Invitrogen*, Catalog #11205D) were resuspended by vortexing for 30 seconds. Desired volume of Dynabeads (10µl ~ 10µg per sample) were transferred to a new tube. Dynabeads were washed with at least 1mL of polysome lysis buffer or buffer used to lyse cells in the respective experiment. Tubes containing dynabeads were placed on a magnet to separate the dynabeads. Dynabeads were washed twice with 200ul of solution A and once with solution B. Finally, beads were resuspended with 10ul of 1X TENT Buffer. 10µl of pre-washed Dynabeads M-280 Streptavidin were added tubes containing biotinylated-RNA + protein and incubated for another 30 minutes with gentle rotation at room temperature. Dynabeads M-280 Streptavidin bound to biotinylated-RNA was separated using magnet for 2-3 minutes. Beads were washed twice with ice cold RNAse free 1X PBS. After final wash, 10µl of 4X SDS was added to beads and boiled at 95°C for 5 minutes. Protein bound to RNA of interest was detected using western blot.

### 2.2.9 Luciferase assay

HEK293T cells were transfected with firefly luciferase reporter constructs as listed in Table 2.2. Renilla luciferase constructs were used as a transfection control. After 24 hours, cells were lysed with 1% NP40 containing lysis buffer. Dual-Luciferase Reporter Assay system (Promega) was used to assess luciferase activity on whole cell lysates following manufacturer's protocol.

### 2.2.10 Mouse model of oral candidiasis

On day -1, *Candida albicans* (strain CAF2-1) was grown in YPD broth at 30°C for 16 hours with continuous shaking. On day 0, yeast suspension was prepared at the final cell density of $2x10^7$ cells/mL. Mice were anesthetized and were sub-lingually infected with cotton balls dipped in *Candida albicans* culture. Weight of mice was assessed daily from day -1 to day 5. On day 5, mice were euthanized and tongues were harvested. Tongues were then homogenized in MACS dissociator using C-tubes. (Miltenyi Biotech). Homogenates were plated in YPD agar plates at 1:1, 1:10 or 1:50 dilution. Plates were incubated at 30°C for 24 hours and yeast colonies were counted manually. For *Arid5a* gene expression analysis using qRT-PCR, tongues were harvested 48 hours post infection.

### 2.2.11 Experimental autoimmune encephalomyelitis (EAE) - mouse model of multiple sclerosis

Myelin oligodendrocyte glycoprotein (MOG) peptide (35-55) (Biosynthesis, Lewisville, Texas, USA) at stock concentration 1mg/mL was mixed at the ratio 1:1 with complete Freund's adjuvant. Note: Complete Freund's adjuvant was prepared by adding 100ug of M tuberculosis strain H37Ra (DIFCO Laboratories, Detroit, Michigan, USA) in 1mL of incomplete Freund's adjuvant. C57BL/6 *Imp2*$^{-/-}$ mice or littermate controls were immunized subcutaneously in two sites on the back with a 50ul of MOG + CFA mix. Mice were also injected intraperitoneally with 100ng of pertussis toxin on day 0 and day 2. Mice were assessed daily for symptoms of EAE and were given the clinical scores as follows: 1 – flaccid tail, 2 – impaired righting reflex and hind limb weakness, 3 – partial hind limb paralysis, 4 – complete hind limb paralysis, 5 – hindlimb

paralysis with partial fore limb paralysis and 6 – moribund. Mice were euthanized on day 21. For flow cytometry analysis, mice were euthanized on day 10 and spleen and lymph nodes were harvested. For gene expression analysis, mice were euthanized on day 21 and spinal cords were harvested.

### 2.2.12 Experimental autoimmune glomerulonephritis

On day -3, Rabbit IgG (0.1mg/mL) was emulsified at 1:1 ratio with complete Freund's adjuvant. C57BL/6 *Imp2*$^{-/-}$ mice or littermate controls were immunized with rabbit IgG + CFA intraperitoneally. On day 0, mice were i.v. injected with serum containing anti-glomerular basement membrane antibodies. On day 14, mice were euthanized and blood and kidneys were harvested. Blood was incubated at 4°C overnight in a tilted position, and serum was collected the next day. Kidneys were stored in formalin. Blood urea nitrogen was assessed using Blood Urea Nitrogen Enzymatic Kit (Bioo Scientific, cat# 5602-01) following manufacturer's protocol. Serum creatinine was assessed using Quantichrom Creatinine Assay Kit (BioAssay Systems, Cat#DICT-500) following manufacturer's protocol. Kidneys were sent to Department of Pathology, Development Laboratory at University of Pittsburgh for H&E staining. Representative pictures of H&E stained kidney sections were taken using EVOS FL Auto Imaging System.

### 2.2.13 Bone marrow chimera experiment

WT C57BL/6 mice on CD45.1 background were obtained from The Jackson Laboratory. On day -1, bone marrow recipient mice WT (CD45.1) or *Imp2*$^{-/-}$ (CD45.2) were given antibiotics

(Sulfamethoxazole and Trimethoprim) containing water. On day 0, bone marrow recipient mice were irradiated. On day 1, bone marrow was extracted from donor mice and 5 million bone marrow cells were i.v. injected to the recipient mice. Mice were given antibiotics containing water for at least 10 days. After 6 weeks of bone marrow reconstitution, mice were subjected to experimental autoimmune glomerulonephritis. At the end of experiment, bone marrow reconstitution was validated by flow cytometry.

### 2.2.14 Imiquimod-driven dermititis

On day -1, *Imp2*$^{+/+}$, *Imp2*$^{-/-}$ and *Il17ra*$^{-/-}$ mice were put under mild anesthesia using isoflurane. Dorsal skin of these mice was shaved using an electric razor and Nair cream was applied to completely remove dorsal hair, and to facilitate cutaneous absorption of treatment. From day 0 to day 5, 6.25 mg of 5% imiquimod (IMQ; 3M; Aldara) was applied daily to the dorsal skin. On day 6, mice were euthanized and skin of the mice were harvested. Five random sections of skin were taken for flow cytometry analysis.

### 2.2.15 *in vitro* Th17 differentiation

Naïve CD4$^{+}$ T cells were isolated from either *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ mice using CD4+ T cell isolation kit (Miltenyi Biotech, Cat# 130-104-454). 200,000 CD4+ T cells were plated in 96 well flat-bottom plates pre-coated with anti-CD3 and anti-CD28 (final concentration 5µg/mL). Cells were either treated with full RPMI for Th0 conditions or with RPMI containing Th17 cocktail (TGF-β 10ng/mL, IL-6 100ng/mL, IL-1β 40ng/mL and IL-23 20ng/mL). After 72 hours, supernatants were taken for IL-17A ELISA. Cells were then treated with PMA/ionomycin (1:2000 dilution)

with golgi-plug and incubated for 4 hours. Percentages for Th17 cells were assessed using flow cytometry.

### 2.2.16 Flow Cytometry

To assess Th1 and Th17 percentages in spleen and lymph nodes during day 10 of EAE, cells were stimulated with PMA/ionomycin + Golgi Plug for 4 hours. Cells were then stained with CD4 (Qdot 605), IL-17A (PerCp Cy 5.5), GM-CSF (FITC) and IFNγ (PE). To assess neutrophil infiltration in skin during IMQ-induced dermatitis, cells were stained with CD45 (Alexa Fluor 700), CD68 (Pacific Blue), CD11B (FITC) and Ly6G (PE). To assess in vitro Th17 differentiation, in vitro differentiated CD4+ T cells were stained with Ghost Dye, CD4 (BV605), IL-17 (PerCp Cy 5.5), GM-CSF (PE), IFNγ (Pacific Blue). To validate reconstitution of bone marrow, spleenocytes were stained with CD45 (APC), CD45.1 (FITC) and CD45.2 (Pacific Blue). All antibodies were used at 1:1000 dilution. Samples were analyzed using BD LSR Fortessa in the Division of Rheumatology and Clinical Immunology at the University of Pittsburgh.

### 2.2.17 Statistics

To assess statistical significance, student's t test was used for pairwise comparison and ANOVA with post hoc Tukey's test or dunnett's were used for multiple comparison. Data presented are means ± SEM of experimental replicates or biological replicates. N for each experiment is listed on respective figure legends. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, n.s. = non-significant.

# 3.0 IL-17 INTEGRATES MULTIPLE SELF-REINFORCING, FEED-FORWARD MECHANISMS THROUGH THE RNA-BINDING PROTEIN ARID5A

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***Science Signaling*, Oct 2018, Volume 11, Issue 551**

**'IL-17 integrated multiple self-reinforcing feed-forward mechanisms through the RNA-binding protein Arid5a'**

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http://stke.sciencemag.org/content/11/551/eaat4617

## 3.1 BACKGROUND

Interleukin-17A (IL-17) is an inflammatory cytokine that is crucial for host defense against microbial pathogens, particularly fungi such as the opportunistic fungus *Candida albicans* (134). IL-17 also drives immunopathology in autoimmune and chronic inflammatory conditions (11). The successful clinical outcomes of drugs blocking IL-17 or its receptor for plaque psoriasis underscores the utility of targeting the IL-17 pathway for therapeutic benefit (11). Therefore, understanding the mechanisms by which IL-17 functions has important clinical implications.

IL-17 is produced by Th17 cells and innate lymphocytes (10) and signals through a dimeric receptor composed of IL-17RA and IL-17RC (135). The IL-17 receptor recruits NF-κB Activator 1 (Act1), an adaptor and E3 ubiquitin ligase that is upstream of nearly all known IL-17-dependent activities. Act1 binds to TNF receptor associated factor 6 (TRAF6), which in turn activates the canonical nuclear factor kappa B (NF-κB) pathway and mitogen activated protein kinases (MAPK). Together, these pathways drive *de novo* transcription of IL-17-induced signature genes encoding cytokines [IL-6, granulocyte-colony stimulation factor (G-CSF)], antimicrobial proteins [lipocalin 2 (Lcn2), β-defensins] and chemokines (15, 135). Although activation of NF-κB is often considered the major IL-17 signaling event, IL-17 also induces other transcription factors (TFs) including CCAAT/Enhancer binding proteins (C/EBP) and the Activator Protein 1 (AP1) complex (135). Confirming their importance, DNA binding sites for C/EBP, NF-κB and AP1 are enriched in the proximal promoter regions of IL-17 target genes (91).

IL-17 signaling in cell culture systems is typically modest compared to potent inflammatory stimuli such as tumor necrosis factor α (TNFα) (11, 95). Nonetheless, the

biological impact of IL-17 is profound, because a deficiency in IL-17 signaling or Th17 cells results in protection from autoimmunity (e.g., experimental autoimmune encephalomyelitis, EAE, a model of multiple sclerosis) and increased susceptibility to pathogens (e.g., the fungus *C. albicans*) (136). One explanation for this paradox is that IL-17 synergizes potently with other inflammatory stimuli commonly found in an inflammatory environment. The molecular basis for this synergy is not fully understood, but is mediated in part through cooperative activation of IL-17-induced TFs that activate downstream genes (93, 94). For example, IL-17 induces IκBζ (NF-κB Inhibitor ζ, encoded by *Nfkbiz*) (137), an atypical member of the NF-κB family that facilitates transcription of several IL-17-dependent genes in cooperation with TNFα (99, 138, 139). Similarly, C/EBP family members are implicated in functional cooperativity between IL-17 and TNFα (95, 124, 137).

In addition to inducing new transcription, IL-17 promotes stabilization of some mRNAs (140). Many IL-17-induced transcripts possess AU-rich elements (AREs) or other stability-determining sequences in their 3' untranslated region (UTR) (57). IL-17 promotes mRNA stabilization of numerous genes, including *Il6*, C-X-C motif chemokine ligand 1 (*Cxcl1*) and *Cxcl5*, among others (140). RNA-binding proteins (RBPs) such as Hu-Antigen R (HuR) and DEAD-box helicase 3 X-Linked (DDX3X) are recruited to the IL-17R/Act1 complex through the adaptors TRAF2 and TRAF5. HuR binds to and stabilizes *Cxcl1* and *Cxcl5* by competing for 3'UTR occupancy with the RNA decay factor splicing factor 2 (SF2) (119, 120, 125). Another IL-17 target gene that influences mRNA stability is the endoribonuclease MCP-1-induced protein 1 (MCPIP1, also known as Regnase-1, encoded by the gene Zinc Finger CCCH-Type Containing 12A, *Zc3h12a*) (124, 127). IL-17 induces *Zc3h12a* expression and promotes its mRNA stability through DDX3X (98, 125). MCPIP1 mediates 3'UTR-mediated mRNA decay of

*Il6, Nfkbiz* and other transcripts and thus is a feedback inhibitor of IL-17 signaling (98). While these studies provided insights into the importance of mRNA control in the IL-17 pathway, the full extent to which IL-17 regulates mRNA remains incompletely understood.

In this chapter, I showed that the RBP AT-rich interactive domain-containing protein 5A (Arid5a) promotes IL-17 signaling. Arid5a was originally discovered as a DNA-binding transcriptional coactivator (141). Previous studies have demonstrated that Arid5a cooperates with SRY-box containing gene 9 (*Sox9*), and activates transcription of genes important for chondrocyte differentiation (142). Arid5a (also known as MRF-1) belongs to the family of ARID (AT-rich interaction domain)-containing proteins. Both human and mouse ARID family members are classified into seven subfamilies based on the degree of sequence identity. These seven subfamilies include ARID1, ARID2, ARID3, ARID4, ARID5, JARID1 and JARID2, which are further divided into 15 ARID members (Table 3.1). The sequence identity of ARID region between members of each subfamily ranges from 70-83%. The ARID domain is a helix-turn-helix DNA-binding module, which was initially demonstrated to interact with AT-rich sequences. However, the DNA-binding ability of most ARID family members has not been fully characterized (141, 143). ARID family proteins are mostly involved in transcriptional regulation, play important roles in cell differentiation and proliferation, and are implicated in many forms of human cancer (143).

The novel RNA-binding function of Arid5a was described very recently in 2013. The first study assessing the RNA-binding capacity of Arid5a demonstrated that Arid5a directly binds to the AREs on the 3'UTR of *Il6* transcripts, and promotes *Il6* mRNA stabilization in response to LPS in macrophages (144). This group found that *Arid5a* deficient mice are less susceptible to EAE, and have lower levels of IL-6 in serum resulting in lower frequency of Th17

cells (144). Later, Arid5a was shown to promote Th17 differentiation by directly stabilizing *Stat3* transcripts, which encodes a critical transcription factor necessary for Th17 generation, further supporting the role of Arid5a in promoting Th17-induced autoimmunity (145). Since then multiple studies have shown that Arid5a enhances mRNA stability of *OX40* and *Tbx21* (Tbet) (146). These studies further showed that Arid5a stabilizes mRNA by counteracting the binding of endoribonuclease MCPIP1/Regnase-1-on the AREs and stem-loop structures present in the 3'UTR of target mRNAs (143-146). However, the role of Arid5a downstream of IL-17 signaling in non-hematopoietic compartment has not been explored.

Here I show that IL-17 stimulation of target cells increased the abundance of Arid5a and triggered its recruitment to the adaptor TRAF2. Arid5a promoted the expression of several IL-17-dependent cytokine mRNA transcripts (*Il6, Cxcl1, Cxcl5)* by binding to their 3' UTR sequences, enhancing mRNA stability, and counteracting the negative effects of MCPIP1. However, Arid5a also promoted expression of certain IL-17-dependent genes (e.g., *Lcn2*) without impacting mRNA stability. This observation led us to interrogate the impact of Arid5a on the IL-17-induced TFs C/EBPβ and IκBζ. Arid5a stabilized mRNA encoding *Nfkbiz* but not *Cebpb*. However, Arid5a strongly enhanced IL-17-induced translation of both C/EBPβ and IκBζ proteins, revealing its potential to control translational circuitry.

**Table 3.1: List of ARID containing proteins.**

| | ARID subfamilies | ARID members |
|---|---|---|
| 1 | ARID1 | ARID1A |
| | | ARID1B |
| 2 | ARID2 | ARID2 |
| 3 | ARID3 | ARID3A |
| | | ARID3B |
| | | ARID3C |
| 4 | ARID4 | ARID4A |
| | | ARID4B |
| 5 | ARID5 | ARID5A |
| | | ARID5B |
| 6 | JARID1 | JARID1A |
| | | JARID1B |
| | | JARID1C |
| | | JARID1D |
| 7 | JARID2 | JARID2 |

## 3.2 RESULTS

### 3.2.1 IL-17 increases Arid5a expression in an NF-κB-dependent manner.

To identify RBPs that might participate in IL-17 signaling, I screened for IL-17-dependent genes encoding RBPs that were induced during a strongly IL-17-dependent immune response (123), oropharyngeal candidiasis (OPC). Expression of *Arid5a* was enhanced in the oral mucosa (tongue) of wild type (WT) mice after oral *C. albicans* infection, and was impaired in *Il17ra*$^{-/-}$ mice (**Figure 3.1-A**). This finding paralleled our observations with another RBP, *Zc3h12a* (encoding MCPIP1), which is induced by IL-17 in the tongue during OPC (98). Similarly, IL-17 stimulated the expression of *Arid5a* in the IL-17-responsive stromal cell line, ST2 **(Figure 3.1-B)** (98). IL-17-induced *Arid5a* expression was blocked by an NF-κB inhibitor, suggesting that its expression is NF-κB-dependent (**Figure 3.1-C).**

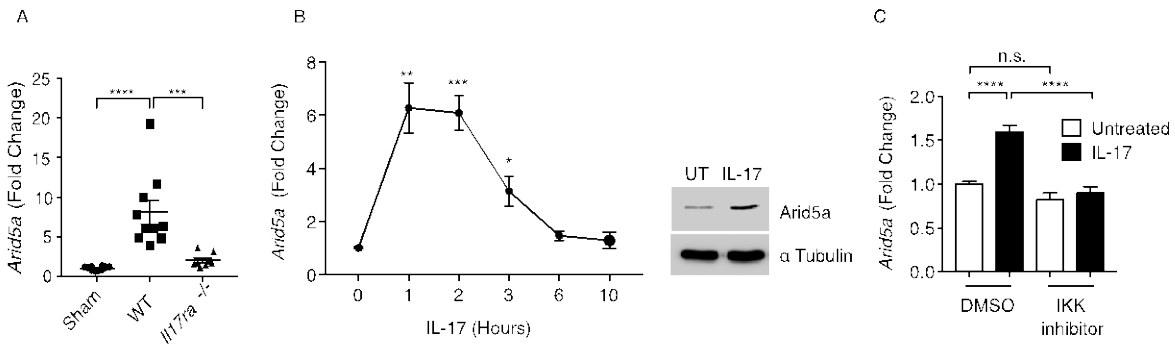


**Figure 3.1: IL-17 upregulates Arid5a expression in an NF-κB-dependent manner.**

**(A)** qRT-PCR analysis of *Arid5a* mRNA expression in the tongue tissue of WT or *Il17ra*$^{-/-}$ mice at 24hrs after oral exposure to PBS (sham) or *C. albicans* (CAF2-1). Fold-change data are means ± SEM of at least 8 mice/group from 2 independent experiments. (**B)** Left: qRT-PCR analysis of *Arid5a* mRNA expression in ST2 cells treated with IL-17 for the indicated times. Fold-change data are means ± SEM from 3 independent experiments. Right: Western blot analysis of Arid5a on lysates from ST2 cells treated with IL-17A for 4 h. Blots are representative of 3 independent experiments. **(C)** qRT-PCR analysis of *Arid5a* expression in ST2 cells pre-treated with 10uM IKK inhibitor VII for 30 minutes following IL-17 treatment for 3 h. Fold-change data are means ± SEM from 3 independent experiments. * P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with Dunnett's test (B) or post hoc Tukey's test (C).

### 3.2.2 IL-17 promotes association between Arid5a and TRAF2

Some IL-17-dependent pathways that lead to mRNA stabilization are initiated through Act1, TRAF2 and TRAF5 (119, 120, 125). To test the hypothesis that Arid5a uses similar proximal signaling intermediates, I transfected a Flag/Myc-Arid5a construct into HEK293T cells (commercial Abs against endogenous Arid5a are ineffective for IP) and co-immunoprecipitated

Arid5a and TRAF2. Arid5a associated with TRAF2 (**Figure 3.2-A**). To determine if the TRAF2-Arid5a association is IL-17-dependent, ST2 cells were transfected with Flag/Myc-Arid5a, stimulated with IL-17 for two hours and lysates were subjected to IP with Myc Abs. Endogenous TRAF2 co-immunoprecipitated with Arid5a, and the TRAF2-Arid5a interaction peaked at ~15-60 minutes after IL-17 treatment **(Figure 3.2-B)**. Together, these data show that IL-17 enhances Arid5a expression, which inducibly associates with TRAF2.

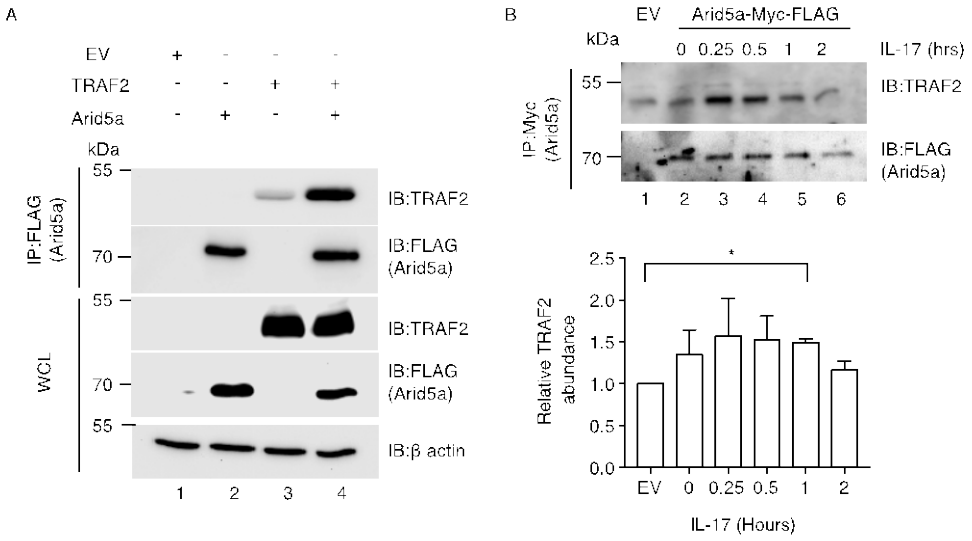


**Figure 3.2: Arid5a associated with TRAF2 in response to IL-17.**

**(A)** Co-immunoprecipitation analysis of Arid5a interaction with TRAF2 in lysates from HEK293T cells transfected with empty vector (EV), Flag/Myc-Arid5a or TRAF2 and immunoprecipitated for Flag. Blots are representative of 2 independent experiments. (**B**) (Top): Co-immunoprecipitation analysis of Arid5a interaction with TRAF2 in lysates from ST2 cells transfected with Flag/Myc-Arid5a, treated with IL-17 for the indicated times and immunoprecipitated with antibody against Myc. Blots are representative of 3 independent experiments. Quantified band intensity values are means ± SEM from all experiments. * $P<0.05$ by ANOVA with post hoc Tukey's test.

### 3.2.3 Arid5a augments the cellular response to IL-17

To ascertain whether Arid5a impacts the response to IL-17, ST2 cells were transfected with pooled siRNAs against Arid5a (knockdown efficiency was typically 40-60%, **Figure 3.3-C)**. Cells were treated with IL-17 for 3 h, and expression of IL-17 target genes was assessed. Knockdown of Arid5a suppressed IL-17-mediated induction of *Il6* mRNA and secreted IL-6 protein (**Figure 3.3-A and B**). Similar results were obtained for other canonical IL-17 target genes, including CXCL1 and CXCL5 (**Figure 3.3-A and B)**. [Note: In ST2 cells, significant induction of *Arid5a* and greater than 60% *Arid5a* knockdown efficiency were necessary to detect Arid5a-mediated regulation of IL-17-induced expression of *Cxcl1* and *Cxcl5* **(Appendix A)**]. However, not all IL-17-driven genes were detectably regulated by Arid5a, such as *Ccl20* and *Csf2* (**Figure 3.3-A**). Together, these data show that Arid5a is required for the expression of a subset of IL-17 target genes.

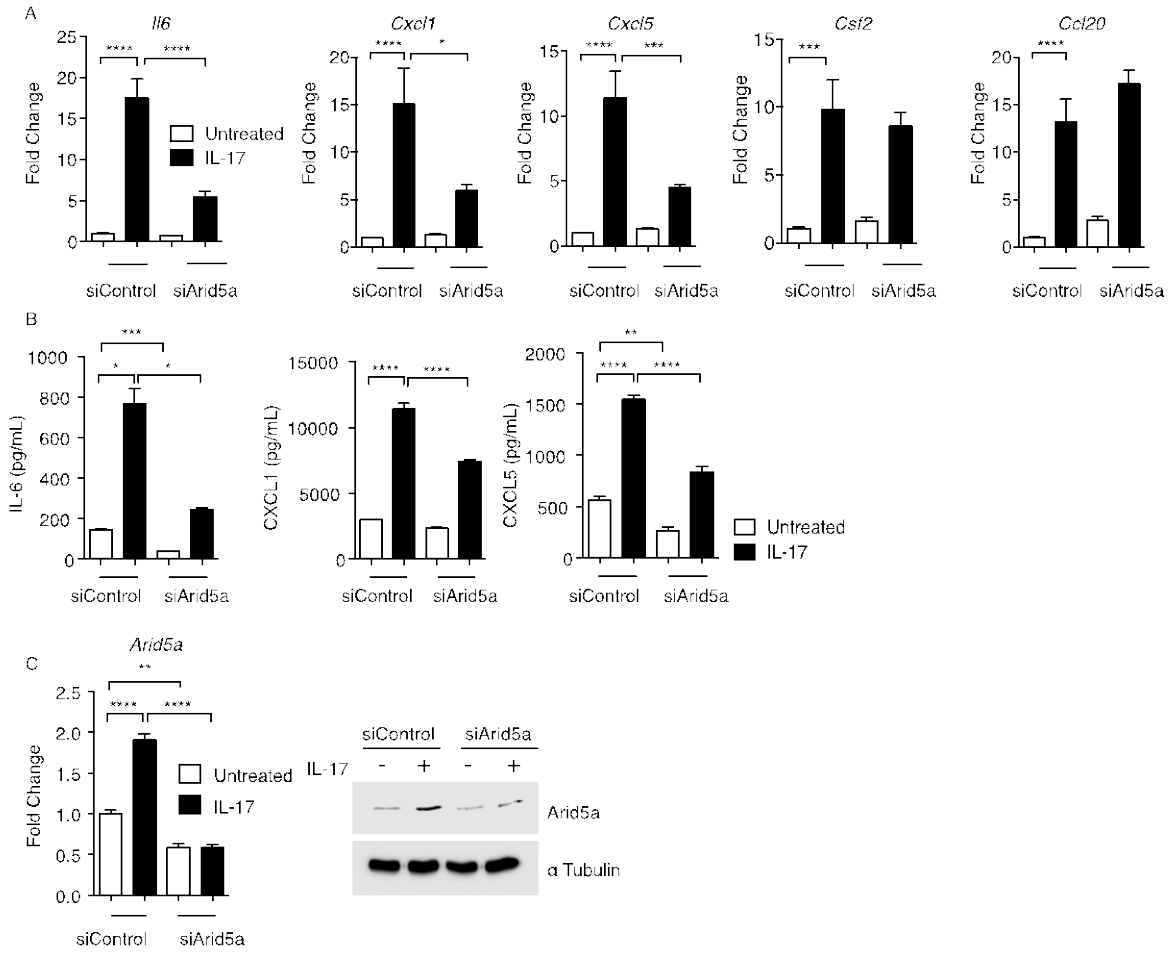


**Figure 3.3: Arid5a promotes cellular responses to IL-17.**

(**A and B**) qRT-PCR or ELISA analysis of the indicated genes in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 3 h. Fold-change data are means ± SEM from 3 independent experiments. (**C**) Left: qRT-PCR analysis of *Arid5a* expression in ST2 cells transfected with pooled siRNAs targeting Arid5a or control siRNA followed by IL-17 treatment for 3h. Fold-change data are means ± SEM from 3 independent experiments. Right: Western blot analysis of Arid5a performed on lysates from ST2 cells treated with IL-17A for 4 hours after transfection with siRNAs targeting Arid5a or control siRNA. Blots are representative of 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

### 3.2.4 Arid5a counteracts the inhibitory function of MCPIP1

In the IL-17 and other inflammatory signaling pathways, competition among RBPs helps determine the overall level of target mRNA transcripts (119, 120, 135). IL-17 induces expression of the endoribonuclease MCPIP1, which remains elevated during prolonged stimulation and binds to a similar RNA binding site as Arid5a (98, 144, 147). Because the net effect of IL-17 is to enhance expression of mRNAs that can be inhibited by MCPIP1, *e.g.*, *Il6* or *Lcn2* **(Figure 3.4-A)**, this paradox suggested that IL-17 augments the activity of RBPs that offset the negative effects of MCPIP1. To determine whether Arid5a might serve in this capacity, I knocked down Arid5a and MCPIP1 together (efficiency ~80%, **Figure 3.4-D**) and assessed *Il6* and *Lcn2* expression. As reported, MCPIP1 knockdown enhanced IL-17-induced expression of *Il6* and *Lcn2* mRNA (**Figure 3.4-B and C**) and IL-6 protein (**Figure 3.4-B**), but knockdown of Arid5a partially offset the inhibitory effect of MCPIP1 deficiency (**Figure 3.4-B and C**). Therefore, Arid5a counteracts the activity of MCPIP1 stimulated by IL-17.

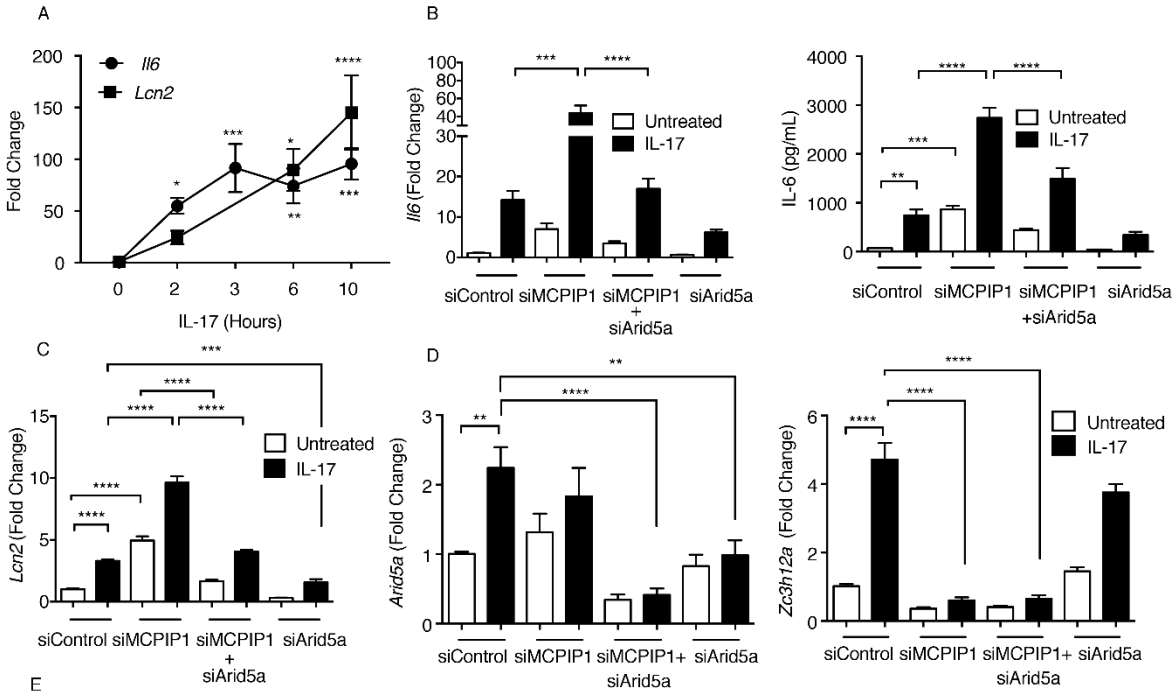


**Figure 3.4: Arid5a counteracts the inhibitory function of MCPIP1 on IL-17 signaling.**

**(A)** qRT-PCR analysis of *Il6* or *Lcn2* mRNA expression in ST2 cells treated with IL-17 for the indicated times. Fold-change data are means ± SEM from 3 independent experiments. (**B and C**) qRT-PCR analysis of *Il6* or *Lcn2* or ELISA analysis of IL-6 in ST2 cells transfected with pooled siRNAs targeting Arid5a ± MCPIP1 or scrambled control and treated with IL-17 for 3 h. Fold-change data are means ± SEM from 3 independent experiments. **(D)** qRT-PCR analysis of *Arid5a* or *Zc3h12a* (MCPIP1) in ST2 cells transfected with pooled siRNAs targeting Arid5a ± MCPIP1 or scrambled control and treated with IL-17 for 3 h. Fold-change data are means ± SEM from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with dunett's test (A) and post hoc Tukey's test (B-D).

### 3.2.5 Arid5a promotes mRNA stability of IL-17 target genes

IL-17 increases expression of *Il6* through effects on both its proximal promoter and also by stabilizing its mRNA transcript (95, 148, 149). However, I saw no impact of Arid5a on IL-6 promoter activation, as co-expression of Arid5a with a luciferase reporter driven by the mouse *Il6* promoter did not increase luciferase activity **(Figure 3.5-F).** To determine whether Arid5a promotes IL-17-dependent stability of *Il6* mRNA, ST2 cells were transfected with siRNAs against Arid5a and primed with TNFα for three hours in order to induce *Il6* mRNA without activating IL-17 signaling. After the cells were washed and treated with actinomycin D (ActD) to block further transcription, I assessed the half-life ($t_{½}$) of IL-17 target transcripts over 90 minutes (longer treatments led to cell toxicity) in the presence or absence of IL-17. As previously reported, IL-17 reduced the rate of decay of *Il6* transcripts (149), but *Il6* stabilization was impaired upon Arid5a knockdown **(Figure 3.5-A)**. Arid5a similarly stabilized *Cxcl1* and *Cxcl5* transcripts **(Figure 3.5-B and C)**. When I co-transfected HEK293T cells with Arid5a and a luciferase reporter fused to the *Il6* 3'UTR (150), expression of Arid5a increased luciferase activity (**Figure 3.5-F**). Control expression of either Act1 enhanced *Il6* 3'UTR reporter activity whereas MCPIP1 suppressed activity, as expected (122, 151). However, Arid5a knockdown did not affect mRNA stability of *Lcn2* **(Figure 3.5-D)**. Thus, these data suggest that Arid5a stabilizes some, but not all, IL-17 target mRNA transcripts encoding inflammatory cytokines and chemokines, through interaction with the 3' UTRs of these mRNAs.

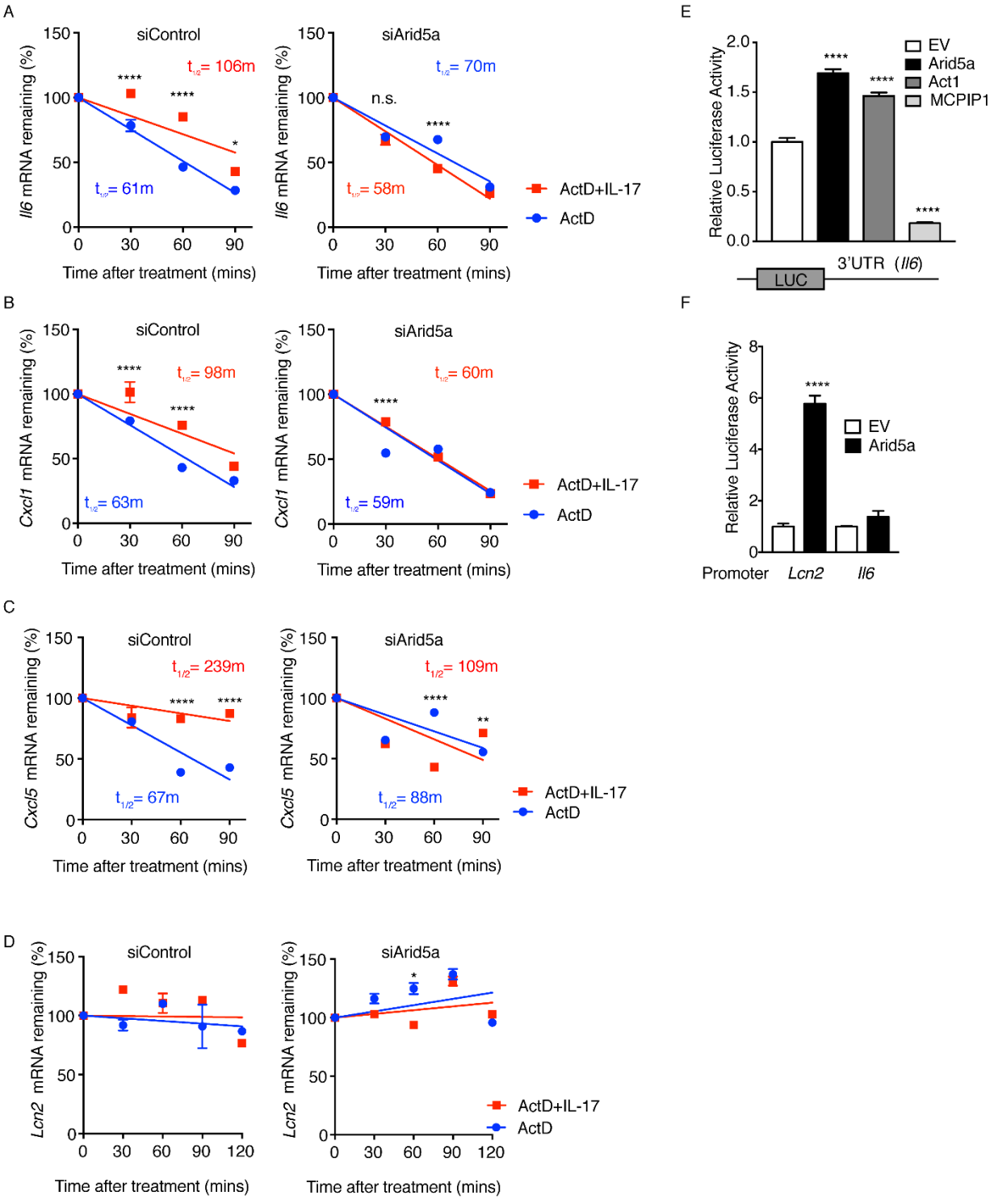


**Figure 3.5: Arid5a stabilizes mRNA of IL-17 target genes.**

(**A-D**) qRT-PCR analysis of *Il6*, *Cxcl1*, *Cxcl5* and *Lcn2* mRNA in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control, pretreated with TNFα for 3 h, then treated with Actinomycin D and IL-17 for the indicated times. Remaining mRNA compared to time=0 data are means of ± SEM representative of 3 independent experiments. (**E**) Luciferase assay of *Il6* 3'UTR activity in HEK293T cells at 24 hours after transfection with a luciferase reporter and either empty vector (EV), Flag/Myc-Arid5a, Act1-Myc, or MCPIP1 and analyzed after 24 h. **(F)** Luciferase activity analysis on lysates from HEK293T cells transfected with a luciferase reporter fused to the *Il6* proximal promoter together with EV or Flag/Myc-Arid5a for 24h. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test; half-lives ($t_{½}$) were determined using equations that defined decay kinetics as shown by colored lines in the graph, as described (92) (E).

### 3.2.6 Arid5a promotes translation of C/EBPβ

Unlike *Il6* transcripts, IL-17 does not alter the mRNA stability of *Lcn2* (91). Moreover, Arid5a knockdown did not increase the half-life of *Lcn2* mRNA **(Figure 3.5-D).** Even so, when Flag/Myc-Arid5a was co-expressed with a luciferase reporter driven by the mouse *Lcn2* promoter (91), luciferase activity was significantly increased **(Figure 3.6-A)**. Because there are no apparent Arid5a recognition sites within the *Lcn2* promoter, these data raised the possibility that Arid5a may transactivate the *Lcn2* promoter indirectly through an IL-17-dependent TF. Indeed, the *Lcn2* promoter contains a C/EBP binding element required for IL-17-dependent induction (91, 93), and Arid5a failed to activate a *Lcn2* promoter with a C/EBP binding site mutation **(Figure 3.6-A)** (91). In response to IL-17, *Cebpb* expression increased 2-4-fold

**(Figure 3.6-B),** consistent with prior observations (19, 95, 107, 152) and raising the possibility that Arid5a increases *Lcn2* by enhancing C/EBPβ expression. However, Arid5a knockdown did not impair expression of IL-17-induced *Cebpb* mRNA **(Figure 3.6-C**). By western blot, I confirmed that IL-17 increases the abundance of C/EBPβ protein isoforms known as LAP (liver activated protein) and LIP (liver inhibitory protein), which are generated by alternative translation (19). I found that silencing of Arid5a strongly inhibited IL-17-induced expression of all C/EBPβ protein isoforms **(Figure 3.6-D)**. Furthermore, neither IL-17 stimulation nor Arid5a knockdown affected the mRNA stability of *Cebpb* transcripts **(Figure 3.7-A).** Thus, Arid5a appears to promote C/EBPβ protein abundance but not its mRNA expression.

These data suggested that Arid5a may increase C/EBPβ translation. Therefore, I employed RNA-immunoprecipitation (RIP) assays to determine whether Arid5a affects the occupancy of *Cebpb* within the eukaryotic eIF4F translation initiation complex (**Figure 3.7-B**) (153). Accordingly, ST2 cells were transfected with Arid5a siRNA, treated with IL-17, and lysates were immunoprecipitated with Abs against eIF4G, a scaffolding subunit of eIF4F associated with mRNA undergoing active translation (154). I found that *Cebpb* transcripts were enriched in the eIF4G IP fraction after IL-17 stimulation, consistent with increased translation (**Figure 3.7-C**). Moreover, Arid5a knockdown impaired enrichment of *Cebpb* transcripts in the eIF4F complex, supporting a role for Arid5a in promoting translation of C/EBPβ. Total *Cebpb* mRNAs in the cytoplasmic lysate used as the input for RIP were not affected by Arid5a knockdown **(Figure 3.7-D)**. To determine whether Arid5a itself interacts with the translation initiation complex, I transfected ST2 cells with Flag/Myc-Arid5a and stimulated these cells with IL-17 for two hours. When lysates were immunoprecipitated with Myc Abs, I found that endogenous eIF4G co-immunoprecipitated with Arid5a in an IL-17-dependent manner **(Figure**

**3.7-E)**. These data indicate that Arid5a promotes IL-17-induced translation of C/EBPβ by interacting with eIF4F translation initiation complex.

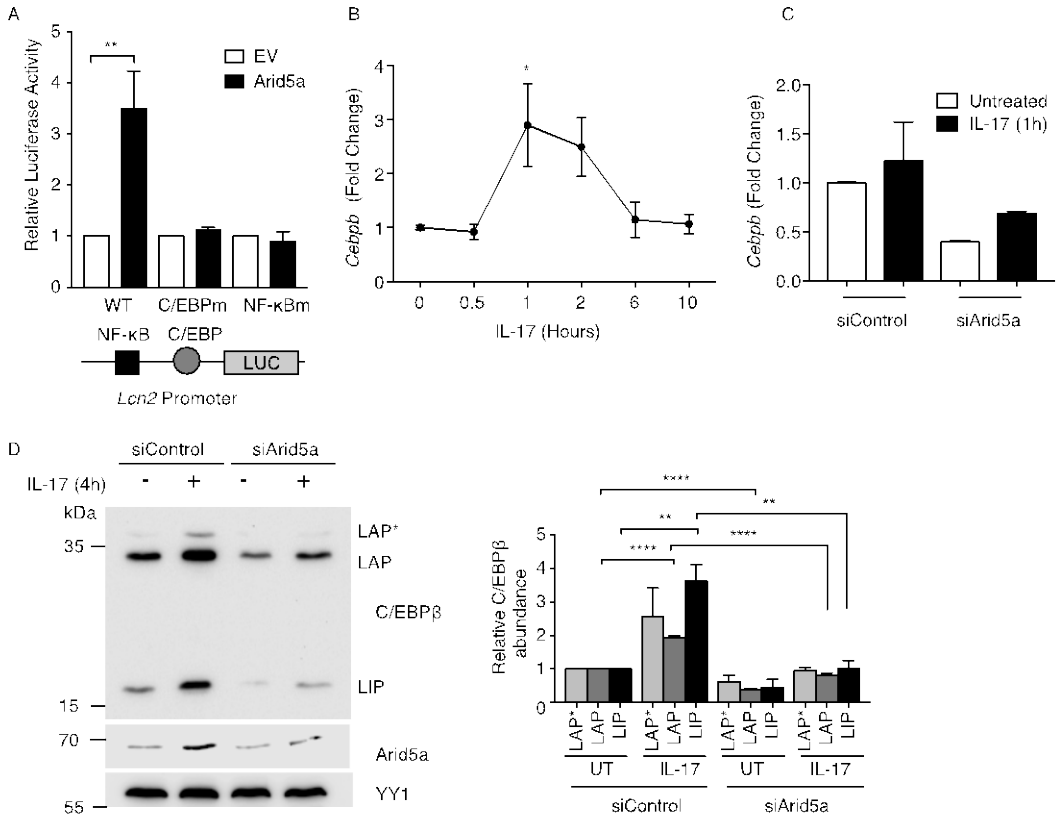


**Figure 3.6: Arid5a promotes C/EBPβ expression.**

(**A**) Luciferase assay of *Lcn2* proximal promoter activity in HEK293T cells transfected with a luciferase reporter together with EV or Flag/Myc-Arid5a and analyzed after 24 h. Fold-change data are means ± SEM from 3-5 independent experiments. (**B**) qRT-PCR analysis of *Cebpb* mRNA expression in ST2 cells treated with IL-17 for the indicated times. Fold-change data are means ± SEM from 3 independent experiments. (**C**) qRT-PCR analysis of *Cebpb* mRNA expression in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control

and treated with IL-17 for 1 h. Fold change data are means ± SEM from 3 independent experiments. (**D**) Western blot analysis of C/EBPβ isoforms (LAP*, LAP and LIP) in nuclear extracts from ST2 cells transfected with siRNAs targeting Arid5a or control siRNA and treated with IL-17A for four hours. Blots are representative of 3 independent experiments. Quantified band intensity values are means ± SEM from all experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test (A, C and D) or Dunnett's test (B).

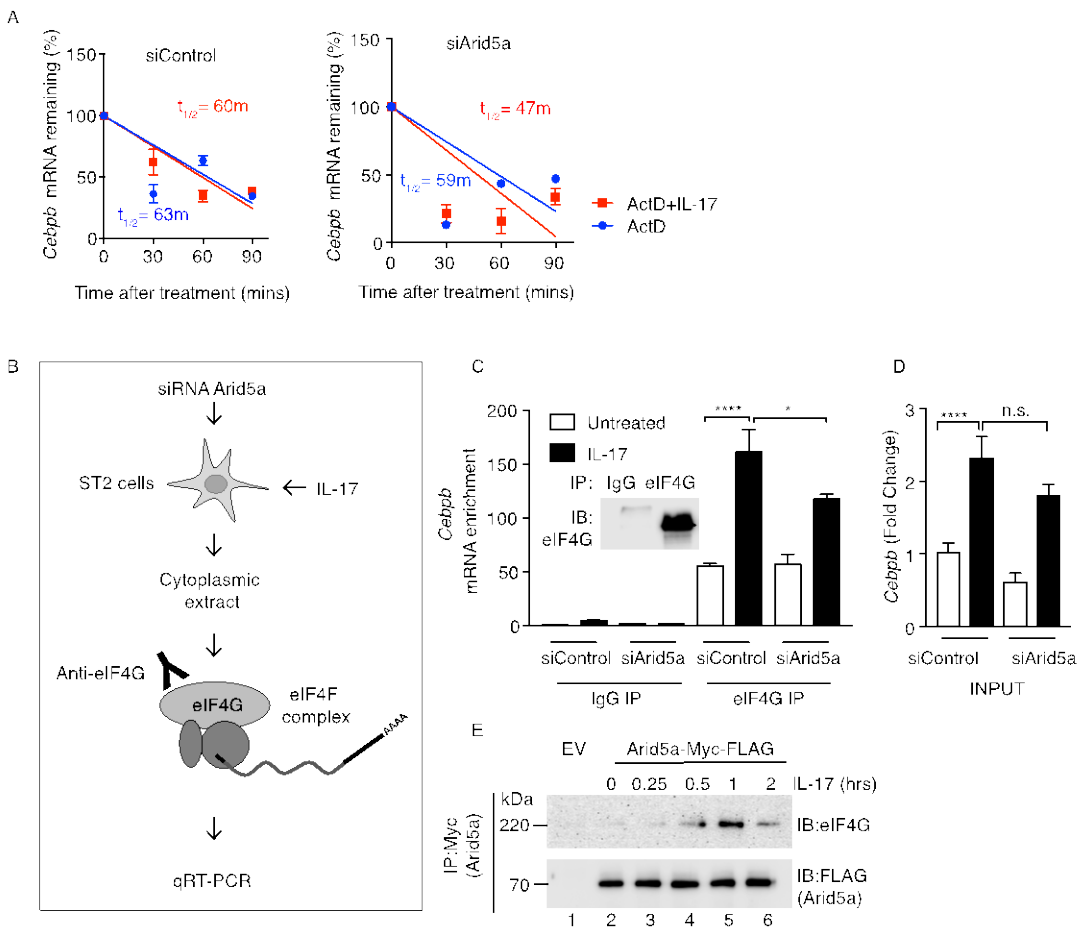


**Figure 3.7: Arid5a mediates translation of C/EBPβ.**

(**A**) qRT-PCR analysis of *Cebpb* mRNA in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control, pretreated with TNFα for 3 h, then treated with Actinomycin D and IL-17 for the indicated times. Remaining mRNA compared to time=0 data are means of ± SEM representative of 3 independent experiments. **(B)** Schematic diagram of eIF4G RIP assay. **(C)** RIP assay of *Cebpb* mRNA amount by qRT-PCR analysis on IgG or eIF4G immunoprecipitates from cytoplasmic extracts of from ST2 cells after transfection with siRNAs targeting Arid5a or control siRNA and treatment with IL-17 for 3 h. Inset: Western blot analysis of eIF4G in cytoplasmic fractions immunoprecipitated with IgG or eIF4G. Data are fold-change means ±

SEM representative of 3 independent experiments. **(D)** qRT-PCR analysis of *Cebpb* in 'input' used for eIF4G RIP. Fold-change data are means ± SEM from 3 independent experiments. (**E**) Co-immunoprecipitation analysis of Arid5a interaction with eIF4G in lysates from ST2 cells transfected with Flag/Myc-Arid5a, treated with IL-17 for the indicated times and immunoprecipitated with antibody against eIF4G. Blots are representative of 3 independent experiments. Quantified band intensity values are means ±SEM from all experiments *P<0.05, **P<0.01, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

### 3.2.7 Arid5a promotes mRNA stability and translation of IκBζ.

The NF-κB family also mediates responses to IL-17, and the *Lcn2* promoter contains an NF-κB site required for IL-17 activation. This site interacts with IκBζ (encoded by *Nfkbiz*), a non-canonical member of the NF-κB family that is induced by IL-17 and that cooperates with NF-κB p50 (91, 93, 124). Arid5a did not activate a luciferase construct driven by the *Lcn2* promoter mutated at its NF-κB binding site **(Figure 3.6-A)**, which suggested that Arid5a may enhance expression of IκBζ. I found that IL-17 enhanced expression of IκBζ both at mRNA and protein levels **(Figure 3.8-A and B)**, consistent with prior reports (98, 124, 155, 156). Arid5a silencing only modestly inhibited IL-17-induced mRNA levels of *Nfkbiz* in ST2 cells and had no effect in primary MEFs **(Figure 3.8-C and D)**. In contrast to *Cebpb* transcripts, Arid5a knockdown impaired IL-17-induced stabilization of *Nfkbiz* mRNA **(Figure 3.8-F).** However, Arid5a knockdown reduced IL-17-induced IκBζ protein abundance in both ST2 cells and MEFs **(Figure 3.8-C and D)**. Consistently, I found that co-expression of Arid5a with IκBζ in HEK293T cells increased IκBζ abundance **(Figure 3.8-E)**. As with *Cebpb* transcripts, *Nfkbiz* mRNA was less

abundant in eIF4G RIP fractions following Arid5a knockdown, which suggested that Arid5a promotes *Nfkbiz* translation **(Figure 3.8-G)**. Thus, our results showed Arid5a enhanced both mRNA stability and translation of IκBζ. Collectively, these data suggest that Arid5a indirectly stimulates target genes that are activated by the IL-17-inducible TFs C/EBPβ and IκBζ.

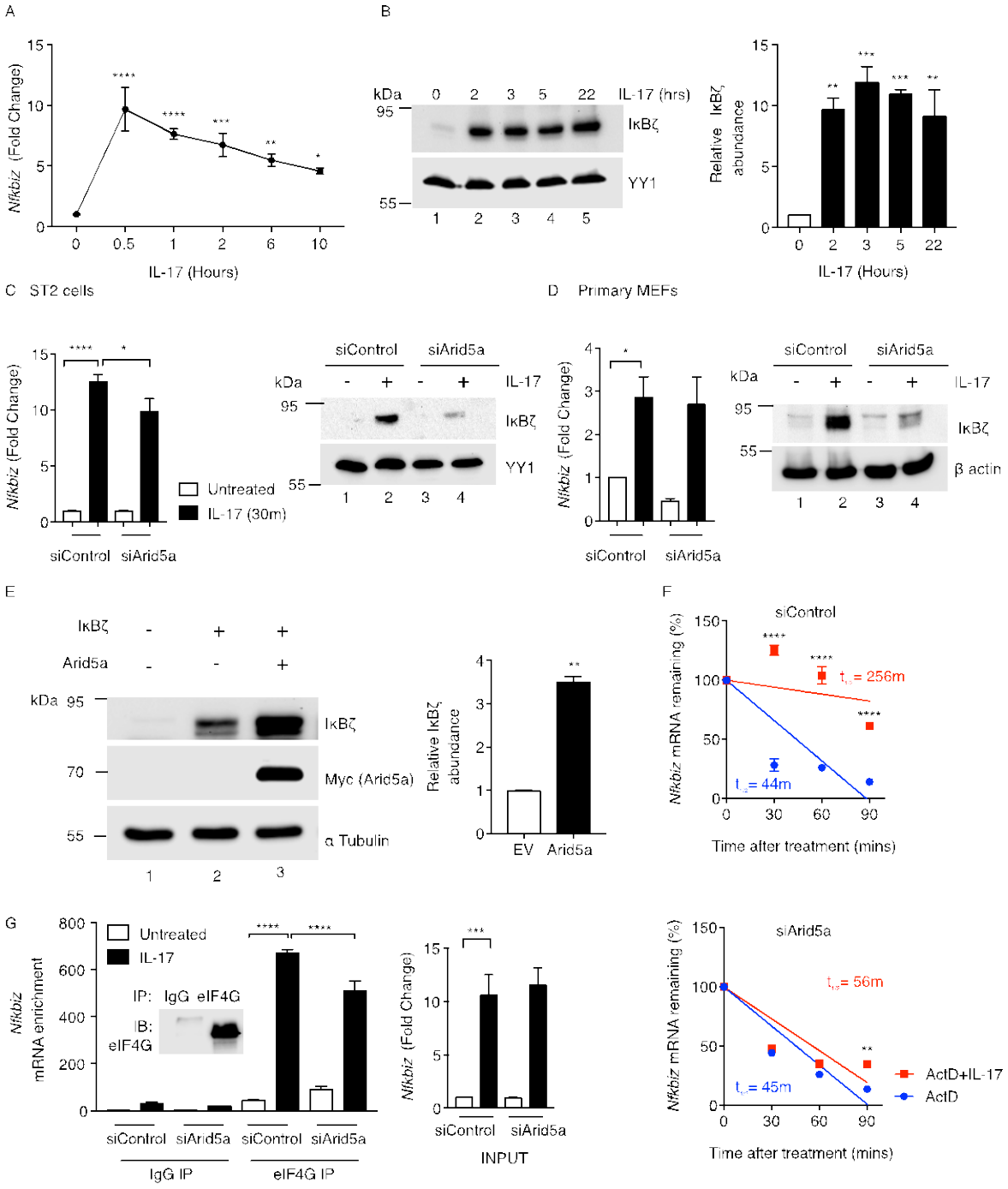


**Figure 3.8: Arid5a mediates mRNA stability and translation of IκBζ.**

(**A**) qRT-PCR analysis of *Nfkbiz* mRNA expression in ST2 cells treated with IL-17 for the indicated times. Fold-change data are means ± SEM from 3 independent experiments. (**B**) Western blot analysis of IκBζ in nuclear extracts from ST2 cells treated with IL-17A for

indicated times. Blots are representative of 3 independent experiments. Quantified band intensity values are means ± SEM from all experiments. (**C**) Left: qRT-PCR analysis of *Nfkbiz* mRNA expression in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 30 min. Fold-change data are means ± SEM from 2 independent experiments. Right: Western blot analysis of IκBζ in nuclear extracts from ST2 cells transfected with siRNAs targeting Arid5a or control siRNA and treated with IL-17A for 4 h. Blots are representative of 4 independent experiments. (**D**) Left: qRT-PCR analysis of *Nfkbiz* mRNA expression in primary MEFs transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 24 h. Fold-change data are means ± SEM from 3 independent experiments. Right: Western blot analysis of IκBζ in whole cell lysates from primary MEFs transfected with siRNAs targeting Arid5a or control siRNA and treated with IL-17A for 24 h. Blots are representative of 3 independent experiments. (**E**) Western blot analysis of IκBζ and Myc-tagged Arid5a in lysates from HEK293T cells transfected with empty vector (EV), IκBζ or Flag/Myc-Arid5a. Blots are representative of 3 independent experiments. Quantified band intensity values are means ± SEM from all experiments. (**F**) qRT-PCR analysis of *Nfkbiz* mRNA expression in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control that were pretreated with TNFα for 3 h, and then treated with Actinomycin D and IL-17 for the indicated times. Remaining mRNA compared to time=0 data are means of ± SEM representative of 2 independent experiments. (**G**) Left: RIP assay of *Nfkbiz* mRNA amount by qRT-PCR analysis on IgG or eIF4G immunoprecipitates from cytoplasmic extracts of ST2 cells after transfection with siRNAs targeting Arid5a or control siRNA and treatment with IL-17 for 3 h. Right: qRT-PCR analysis of *Nfkbiz* in 'input' used for eIF4G RIP. Fold-change data are means ± SEM from 3 independent experiments. Data are fold-change means ± SEM representative of 3

independent experiments. Inset: Western blot analysis of eIF4G in cytoplasmic fractions immunoprecipitated with IgG or eIF4G. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test or Dunnett's test (A, B); half-lives (t½) were assessed as described (92) (E).

### 3.2.8 Role of TRAF2 in Arid5a-mediated regulation of IL-17 target genes.

Because Arid5a associated with TRAF2 (**Figure 3.2-A and B**), I postulated that TRAF2 participates in Arid5a-mediated increased expression of IL-17 target genes. ST2 cells were transfected with siRNAs against TRAF2 and/or Arid5a (knockdown efficiency was ~60-70% for Arid5a, and ~70-80% for TRAF2, **Figure 3.9-D and E)**. Silencing of TRAF2 decreased IL-17-induced expression of *Il6*, while knockdown of TRAF2 in combination with Arid5a did not reduce expression of *Il6* compared to either alone **(Figure 3.9-A)**. However, TRAF2 was dispensable for IL-17-induced expression of C/EBPβ and IκBζ **(Figure 3.9-B and C)**. These data suggest that TRAF2 is important for Arid5a-mediated regulation of *Il6* but not of C/EBPβ and IκBζ. TRAF2 has previously been shown to be important for IL-17-induced mRNA stability. Even though TRAF2 inducibly associated with Arid5a and is implicated in promoting HuR-induced stabilization in the IL-17 pathway (120, 122), TRAF2 was dispensable for promoting translation of these TFs C/EBPβ and IκBζ. Consistently, TRAF2 is not found in translationally-active polysomes following IL-17 stimulation (120). Accordingly, Arid5a appears to direct mRNA translation and mRNA stabilization by differing mechanisms. Nonetheless, more studies are required to understand whether association of TRAF2 and Arid5a is important for Arid5a-mediated mRNA stability of IL-17 target genes.

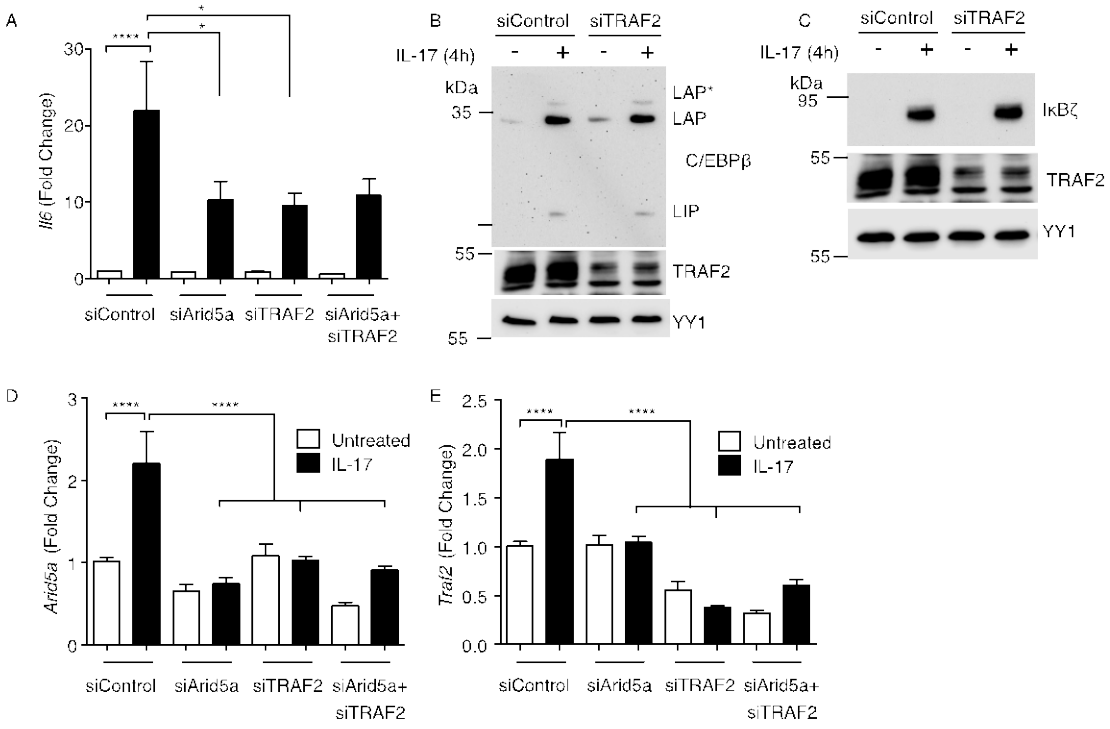


**Figure 3.9: Role of TRAF2 in Arid5a-mediated regulation of IL-17 signaling.**

**(A)** qRT-PCR analysis of *Il6* mRNA expression in ST2 cells transfected with pooled siRNAs targeting Arid5a, TRAF2 or scrambled control and treated with IL-17 for 3 h. Fold-change data are means ± SEM from 3 independent experiments. (**B and C)** Western blot analysis of LAP*, LAP and LIP isoforms of C/EBPβ or of IκBζ performed on nuclear extracts from ST2 cells treated with IL-17A for 4 hours after transfection with siRNAs targeting TRAF2 or control siRNA. Representative blots are from 2 independent experiments. **(D and E)** qRT-PCR analysis of *Arid5a*, and *Traf2* in ST2 cells transfected with pooled siRNAs targeting Arid5a or TRAF2 followed by IL-17 treatment for 3h. Data are fold-change means ± SEM from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

### 3.2.9 Arid5a binds to the 3'UTR of IL-17-induced mRNAs

To determine whether Arid5a binds directly to target mRNAs, ST2 cells were transfected with Flag/Myc-Arid5a, stimulated with IL-17, and nuclear and cytoplasmic fractions of cell lysates were subjected to RIP with Abs against Myc. I found that the association of *Il6, Cxcl1, Cxcl5, Nfkbiz* and *Cebpb,* transcripts with Arid5a was increased in cytoplasmic fractions after IL-17stimulation **(Figure 3.10-A)**. These transcripts were not enriched in control or nuclear fractions **(Figure 3.10-D)**, consistent with the fact that posttranscriptional control of mRNA takes place in the cytoplasm (57). In contrast, *Ccl20 and Csf2* transcripts were not associated with Arid5a, which agrees with our earlier results that Arid5a knockdown does not affect IL-17-dependent expression of these genes **(Figure 3.10-B)**. Together, our data suggest that Arid5a associates specifically with IL-17-induced, cytoplasmically-localized mRNA transcripts.

To verify that Arid5a binds directly to the 3'UTRs of target transcripts, I employed an in vitro RNA pulldown assay. Biotinylated transcripts encoding the 3'UTR sequences of *Il6, Cxcl1* and *Csf2* were generated in vitro and incubated with recombinant Flag/Myc-Arid5a derived from transfected HEK293T cells. Biotinylated transcripts were isolated with streptavidin-conjugated beads and precipitates subjected to immunoblotting with Myc Abs **(Figure 3.10-C)**. Consistent with the earlier RIP data, Arid5a was detected in fractions corresponding to the *Il6, Cxcl1* 3'UTR sequences but not with the *Csf2* 3'UTR **(Figure 3.10-C)**. Thus, Arid5a stabilizes IL-17-induced target mRNAs by binding directly to the 3'UTR.

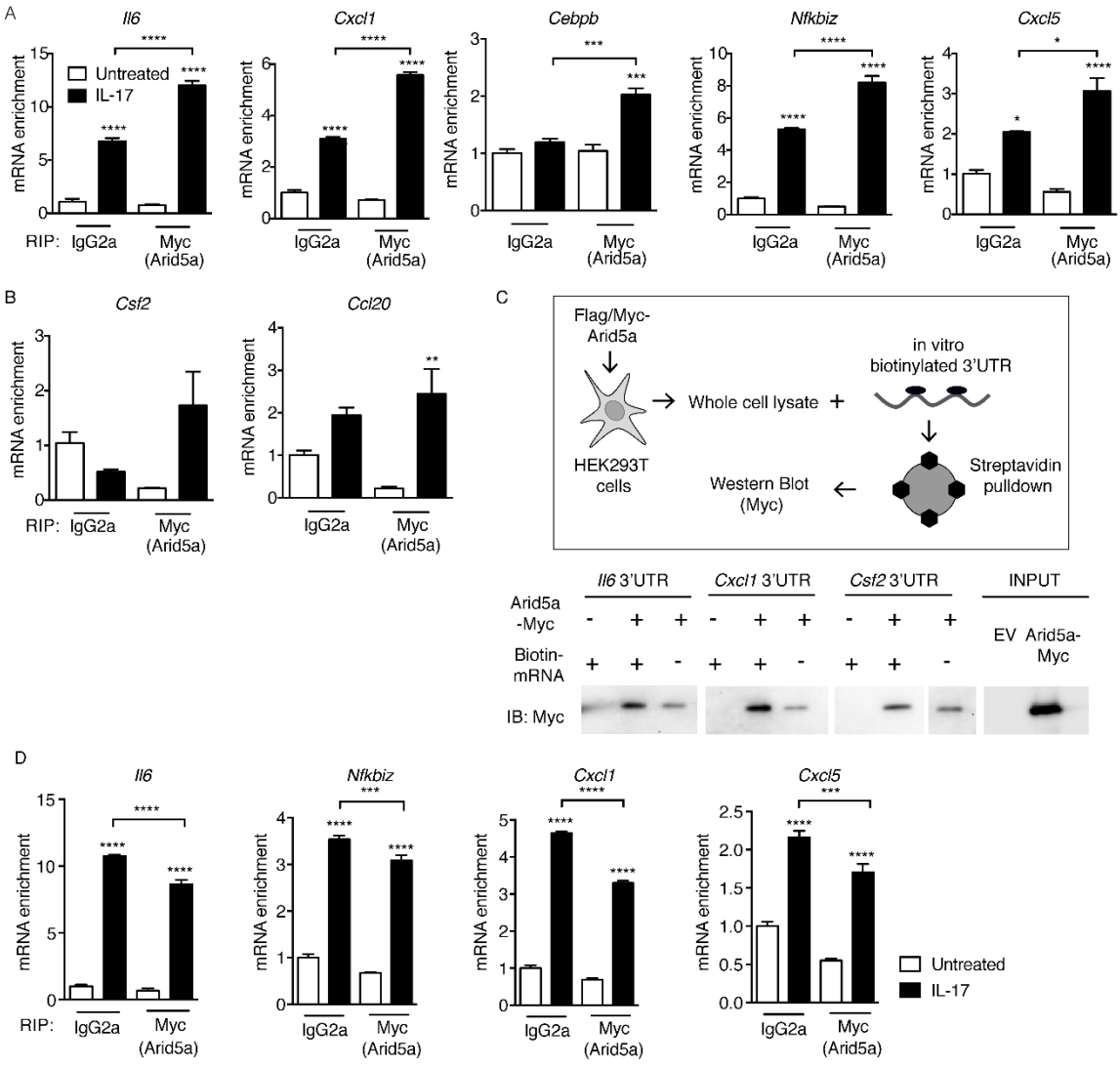


**Figure 3.10: Arid5a binds directly to target mRNA transcripts.**

(**A** to **B**) qRT-PCR analysis of mRNAs from ST2 cytoplasmic extracts transfected with Flag/Myc-Arid5a, treated with IL-17 for 3 h, and subjected to RIP with IgG2a or Myc. Data are fold-change means ± SEM representative of 3 independent experiments. (**C**) *In vitro* RNA pulldown assay (box) of Arid5a-Myc by western blot analysis streptavidin bead immunoprecipitates from lysates of Arid5a-Myc transfected HEK293T cells incubated with the indicated in vitro-generated, biotinylated mRNAs. Data are derived from the same blot, and are

representative of 3 independent experiments (for *Il6*) and 2 independent experiments for *Cxcl1* and *Csf2*. **(D)** qRT-PCR analysis of indicated mRNA enrichment after RNA immunoprecipitation with IgG2a or α-Myc on nuclear extracts of ST2 cells treated with IL-17 for 3h following transfection with Flag/Myc-Arid5a. Fold-change data are means ± SEM representative of 2 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

### 3.2.10 Arid5a promotes expression of IL-17 target genes in primary MEFs and human keratinocytes

Although ST2 cells are generally a good reflection of IL-17-dependent events in other cell types, I also assessed Arid5a function by siRNA knockdown in primary murine MEFs and N/TERT2G immortalized human keratinocytes (KC) (157). N/TERT2G cells maintain normal KC differentiation patterns and are considered to be a good representation of primary human KCs (158). In MEF cells Arid5a deficiency also decreased IL-17-induced expression of *Il6*, *Cxcl1* and *Lcn2* mRNA, and IL-6 and CXCL1 protein levels (**Figure 3.11-A and B**). Similarly, silencing of Arid5a in N/TERT2G cells impaired expression of human *LCN2* and *CXCL1* stimulated by IL-17 (**Figure 3.11-C**). Thus, Arid5a promotes IL-17 responses in multiple cells, including primary murine and human cell types.

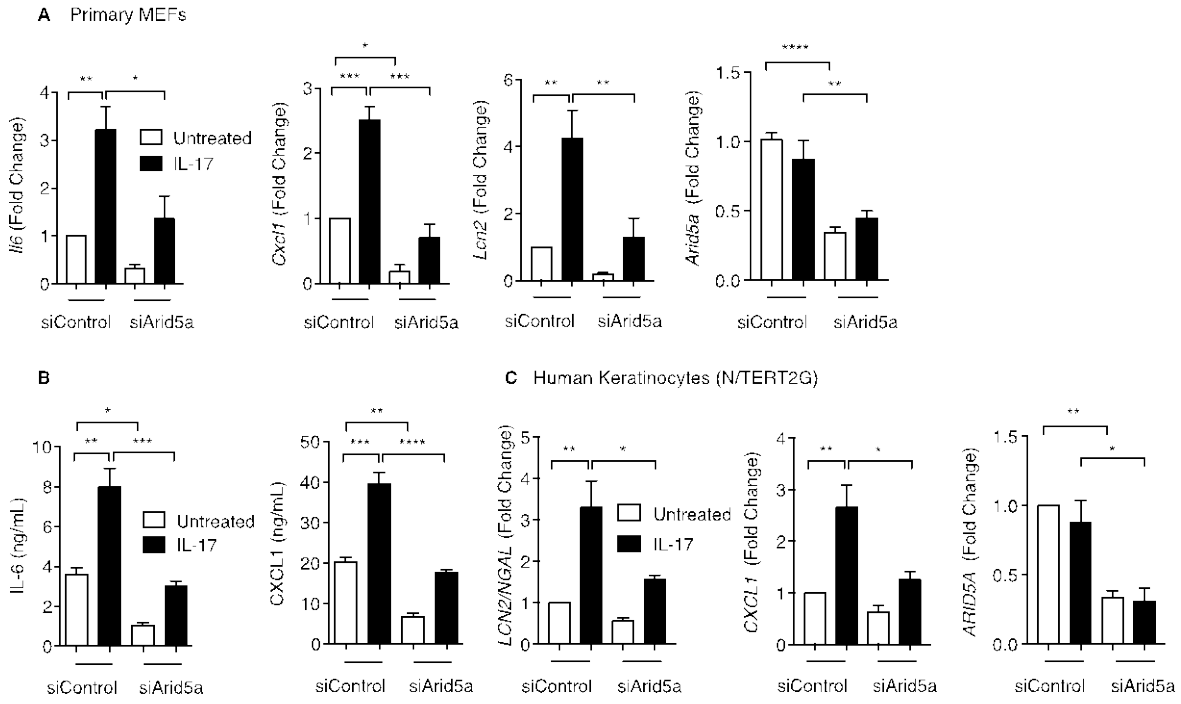


**Figure 3.11: Arid5a promotes responses to IL-17 in primary MEFs and human keratinocytes.**

(**A**) qRT-PCR analysis of primary MEFs transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 24 h. Fold change data are means ± SEM from 3 independent experiments. (**B**) ELISA analysis of conditioned supernatants from MEFs transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 24 h. Fold change data are means ± SEM representative of 2 independent experiments. (**C**) qRT-PCR analysis of human keratinocyte (N/TERT2G) cells transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 5 h. Fold change data are means ± SEM from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

### 3.2.11 ARID family members regulate IL-17 signaling

Arid5a contains an Arid domain, which is essential for its mRNA stability function, and which is shared with other Arid family members (141, 144). This evidence prompted us to assess the role of these Arid-containing proteins in IL-17 signaling. To test this possibility, I knocked down 8 other ARID family members in ST2 cells and treated them with IL-17 for 3 hours. Interestingly, transient knockdown of Arid4a, Arid4b and Arid5b increased IL-17-induced IL-6 mRNA and protein expression **(Figure 3.12-A and B)**. Although silencing of Arid1b and Arid3a decreased *Il6* mRNA expression, these differences were not significant at the level of IL-6 protein. As expected, knockdown of MCPIP1 increased IL-17-induced expression of IL-6, which was used as an experimental control. These results suggest that Arid4a, Arid4b and Arid5b can all negatively regulate IL-17 signal transduction. These proteins have all been associated with various forms of human cancer (143). Functionally, Arid4a, Arid4b and Arid5b are known to associate with histone deacetylase and regulate chromatin remodeling (143, 159). Although transcription is the most well-studied facet of IL-17 signaling, not much is known about the role of IL-17 in chromatin remodeling. Therefore, it would be interesting to further investigate the function of these Arid family members in controlling IL-17-induced gene expression.

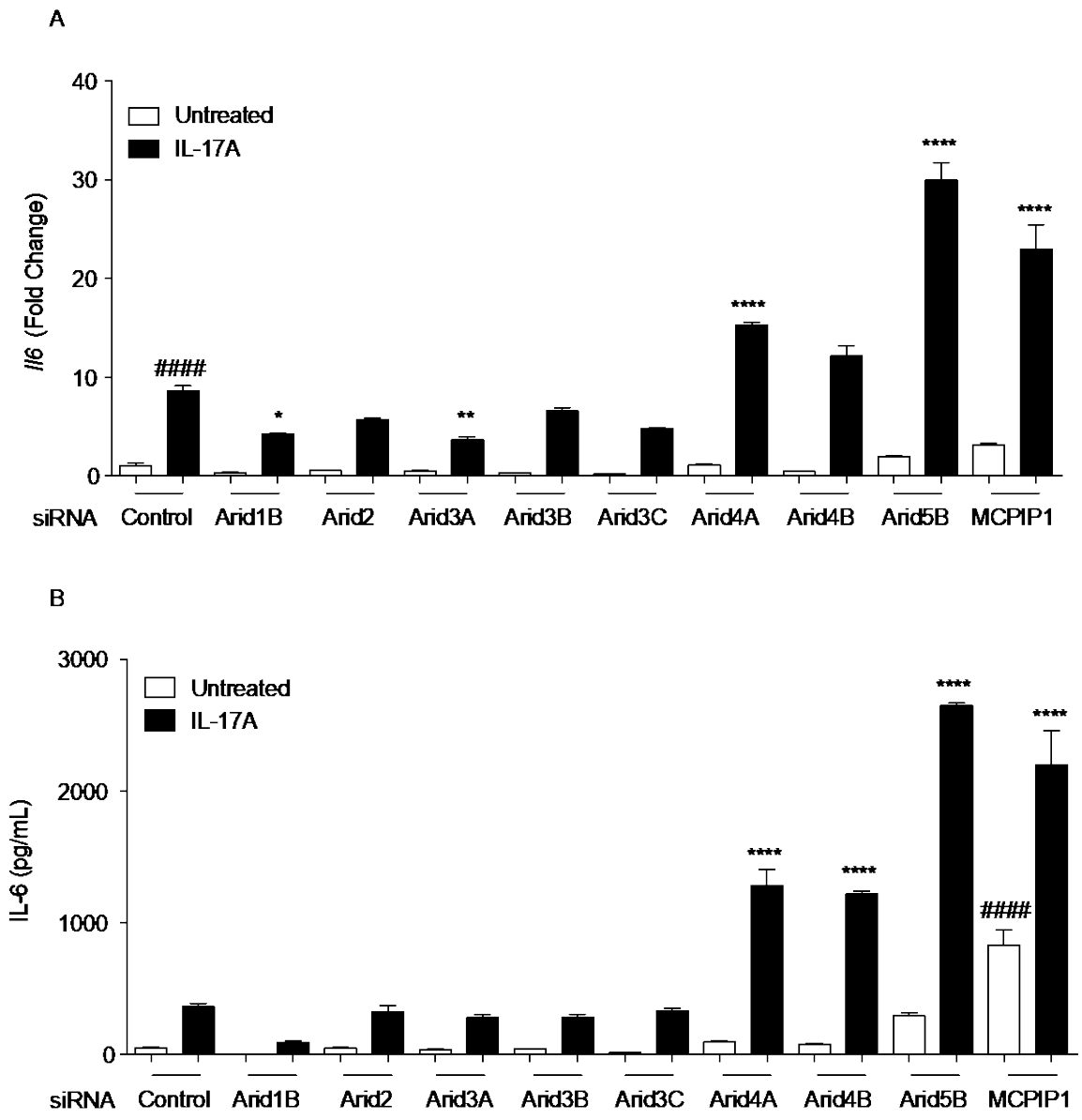


**Figure 3.12: Assessing role of ARID-containing proteins in IL-17 signaling.**

ST2 cells were transfected with indicated pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 3 h. **(A)** *Il6* expression was analyzed by qRT-PCR and **(B)** IL-6 expression was analyzed by ELISA. Fold-change data are means ± SEM from technical triplicates. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

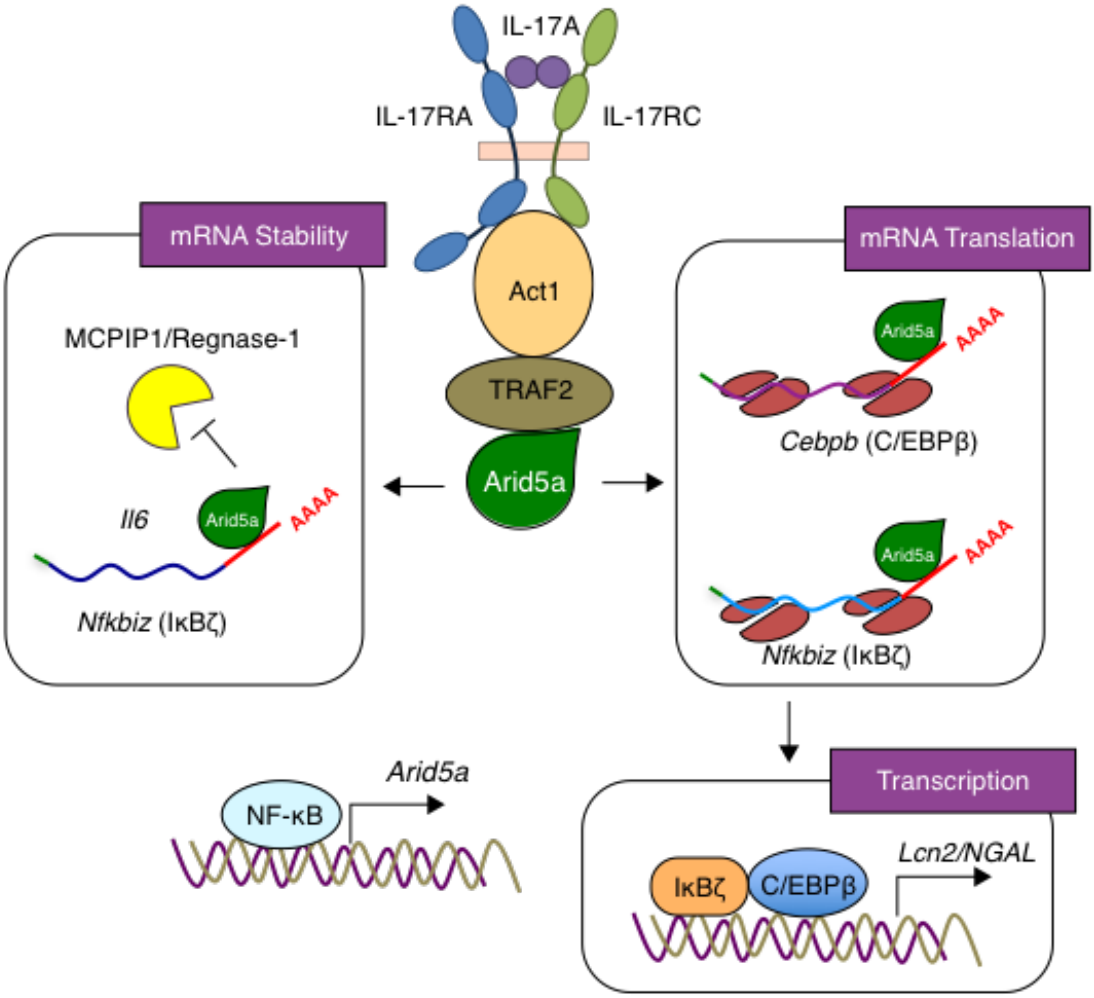


**Figure 3.13: Model of Arid5a in the IL-17R signaling pathway.**

Upon IL-17 stimulation, Arid5a expression is increased and this RNP is recruited to TRAF2. Arid5a promotes the mRNA stability of multiple genes, including cytokines and chemokines as well as the transcription factor *Nfkbiz* (IκBζ). Additionally, Arid5a enhances the translation of *Nfkbiz* and *Cebpb*, transcription factors that in turn regulate downstream genes such as *Lcn2*, which are not intrinsically unstable. Thus, Arid5a is a central player in the post-transcriptional IL-17 signaling cascade.

## 3.3 DISCUSSION

IL-17 signaling predominantly occurs in epithelial and mesenchymal cell types (75), and thus IL-17 functions as a bridge between the immune system and inflamed tissue. The signal transduction mechanisms downstream of IL-17 and related cytokines are still incompletely understood. Here, I identified the RNA binding protein Arid5a as a driver of cellular responses to IL-17 in mouse mesenchymal cells and in human keratinocytes. Arid5a stabilized IL-17-induced mRNA transcripts encoding IL-6 and CXC chemokines., and bound directly to the 3' UTR of multiple target mRNAs. These activities are similar to those described for Arid5a in the TLR4 signaling pathway (144, 147, 160). Additionally, I observed that Arid5a facilitated the translation of two IL-17-dependent transcription factors, C/EBPβ and IκBζ, allowing for increased expression of genes reliant on these TFs such as *Lcn2*. Thus, in these capacities, Arid5a promotes IL-17-driven immunity **(Figure 3.13)**.

The IL-17 family is a distinct subclass of cytokines. Accumulating evidence indicates that the mechanisms by which IL-17-mediates mRNA stabilization are not the same as better-studied pathways (92, 116, 148, 149, 161). For example, IL-17 does not stabilize target mRNAs through the commonly-used RBP tristetraprolin and or the adaptor TRAF6 (116-118, 120). Rather, RBPs such as HuR, DDX3X and Act1 serve to stabilize IL-17-dependent transcripts in a pathway initiated by TRAF2 and TRAF5 (120, 122, 125). Typically, the RNA-stabilizing activity of these RBPs is offset by de-stabilizing RBPs *e.g.,* SF2 (119, 120), allowing for rapid changes in the accumulation of inflammatory mRNAs in response to cues from IL-17 or other stimuli. In an analogous manner, Arid5a function appeared to be offset by the endoribonuclease MCPIP1 (Regnase-1), which is also induced by IL-17 and which degrades many of the same IL-17-dependent mRNA transcripts (98, 127). In the setting of LPS signaling in macrophages,

Arid5a and MCPIP1 bind to an overlapping RNA sequence in the *Il6* 3'UTR (128, 144, 147). Nonetheless, in the IL-17 pathway, not all the targets of Arid5a and MCPIP1 were identical (e.g., *Ccl20*, which is strongly affected by MCPIP1 but not detectably by Arid5a). Thus, Arid5a and MCPIP1 appear to be opposing players in an RBP-mediated signaling cascade triggered by IL-17.

There is considerable evidence linking Arid5a to IL-17-induced pathways in vivo. We saw that Arid5a was induced in an IL-17-dependent manner in the oral mucosa during *C. albicans* infections, although a contribution of Arid5a to fungal host defense has not been directly demonstrated. Arid5a$^{-/-}$ mice are resistant to EAE (144), an IL-17-dependent model of autoimmunity (9). Mice lacking either IL-17A or Arid5a show similar resistance to bleomycin-induced lung injury (162-164). Additionally, Arid5a is linked to Th17 cell differentiation through stabilization of *Il6*, *Stat3* and *Ox40* in APCs or T cells (144, 145, 147, 160, 165), and thus may enhance IL-17-dependent responses by virtue of increasing IL-17 levels in the inflamed environment.

IL-17 acts almost exclusively on non-hematopoietic cells (75), so factors such as Arid5a or MCPIP1 that target both Th17 cells and IL-17 signaling could potentially function in one or both contexts (98, 127). In this regard, the cell type(s) where Arid5a functions in EAE or bleomycin injury have not been determined but in a model of IL-17-driven psoriasis, MCPIP1 acts entirely within the non-hematopoietic compartment (166). More studies will be needed to delineate the specific cell types in which Arid5a (or MCPIP1) contribute to autoimmune responses.

Our data revealed a role for Arid5a in increasing expression of the TFs C/EBPβ and IκBζ, which play vital roles in IL-17-driven disease. For example, *Arid5a*$^{-/-}$, *Cebpb*$^{-/-}$ and *Nfkbiz*$^{-}$

$^{-/-}$ mice are all refractory to EAE (144, 147, 167, 168), and *Nfkbiz*$^{-/-}$ mice are also resistant to imiquimod-induced inflammation, a model of psoriasis-like IL-17-dependent skin inflammation (155). Once induced, C/EBPβ and IκBζ promote expression of genes encoded by mRNAs that are not themselves intrinsically unstable, such as the canonical IL-17 target gene *Lcn2* (91). IL-17 enhanced *Cebpb* and *Nfkbiz* mRNA, albeit modestly (76, 95), whereas concomitant protein expression of both TFs was profoundly increased. However, the mechanisms underlying translational control of these proteins have been elusive (19, 108, 152, 156). I observed that Arid5a associated with the translation initiation complex and that Arid5a knockdown impaired translation of both C/EBPβ and IκBζ. Arid5a is highly expressed in the nucleus, but TLR4 signaling induce its translocation to the cytoplasm (169). Although it is not known if IL-17 similarly alters Arid5a subcellular localization, interaction of Arid5a with its target mRNAs was observed only in cytoplasmic, not nuclear extracts. In future, co-localization studies could be performed to fully investigate the subcellular localization of Arid5a during IL-17 treatment. Collectively, these findings show that by inducing expression of these key TFs, Arid5a amplifies the IL-17-driven signaling program.

Our results show that Arid5a binds to transcripts of IL-17 target genes such as *Il6, Cxcl1, Cxcl5, Nfkbiz* and *Cebpb.* Consistent with previous reports, I observed that Arid5a binds to the 3'UTR of *Il6* and *Cxcl1* (144). Whether Arid5a also binds to similar AREs of other IL-17 target genes needs to be further investigated. Since I found that Arid5a participated in mRNA translation by associating with eIF4G at the 5' cap of mRNAs, it is likely that Arid5a binds to the 5'UTR of *Cebpb* and *Nfkbiz.* Similarly, it would be beneficial to assess the functional domains on Arid5a required for mRNA stabilization of IL-17 target genes. Previous studies have shown that the Arid domain of Arid5a is important for its mRNA binding capacity (144, 145).

Therefore, assessing whether the Arid domain is also required for stabilization of IL-17 target gene transcripts would be interesting. We and others have shown that Arid5a directly binds to target mRNAs (144, 145, 170). However, in a physiological context it is likely that Arid5a-mRNA interactions are rather complex with several other RBPs present in the same RNP (ribonucleoprotein) complex. This possibility should be further investigated using mass spectrometry, and will open many avenues for future studies.

Blocking antibodies to IL-17 are remarkably effective in treating psoriasis and are under evaluation for other autoimmune conditions (36, 171). Hence, defining the molecular basis of IL-17 signal transduction may inform therapeutic strategies for diseases where IL-17 is implicated (172). Exploiting RNA is particularly attractive given the potential for exquisite specificity in targeting otherwise "un-druggable" molecules. There are emerging therapeutic approaches directed at RNA or RBPs (173). For example, oligonucleotide "aptamers" representing an Act1 recognition site in the *Cxcl1* 3'UTR were effective in pre-clinical models of autoimmunity (122). In addition, Arid5a was reported to be a target of the anti-psychotic drug chlorpromazine (144), suggesting a possible option for diseases involving IL-17. Hence, gaining a mechanistic understanding of how RNA expression is regulated could lead to rational design of new therapies.

# 4.0 THE RNA-BINDING PROTEIN IMP2 DRIVES INTERLEUKIN-17 RECEPTOR-MEDIATED AUTOIMMUNITY

## 4.1 BACKGROUND

Although IL-17 is crucial for protection against extracellular pathogens, it is also strongly associated with immunopathology and autoimmune conditions (174, 175). The inflammatory role of IL-17 has been well-established in several autoimmune diseases including multiple sclerosis (MS) and autoimmune glomerulonephritis (AGN) (176, 177). MS is a chronic inflammatory disease of the central nervous system (CNS), characterized by leukocyte infiltration in the CNS, inflammation, and ultimate demyelination and damage of axons (178). Studies using experimental autoimmune encephalomyelitis (EAE), have shown that mice deficient in IL-17, IL-17RA or Act1 are resistant to EAE (85, 179, 180). Moreover, patients with MS have higher levels of IL-17 mRNA in CNS-infiltrating T cells, cells of cerebrospinal fluid and the peripheral blood (181-183)

Autoimmune glomerulonephritis (AGN) is an autoimmune disorder caused by the generation of antibodies against autoantigen alpha-3 chain of type IV collagen, which are present in the glomerular basement membrane of the kidney (176). Pathogenesis of AGN involves infiltration of inflammatory cells into the renal parenchyma, resulting in disruption of glomerular barrier integrity and renal fibrosis. Proinflammatory cytokines, including IL-17, have emerged as

key players in the pathogenesis of AGN. In a mouse model of AGN, IL-17 expression is increased in the kidney after induction of AGN and IL-17RA deficient mice are resistant to disease (184). Similarly, studies using the FcγRIIb$^{-/-}$ mouse model of lupus have shown that mice lacking Act1 are resistant to glomerulonephritis (185). Thus, IL-17 signaling plays a critical role during pathogenesis of some autoimmune diseases, including mouse models of EAE and AGN.

Multiple RBPs have been described that either promote or inhibit IL-17-induced mRNA expression (4). As described in chapter 3, I identified a new RBP Arid5a (AT-rich interactive domain containing protein 5A) that promotes mRNA stability and translation of key IL-17-dependent transcription factors and inflammatory genes. To make a more comprehensive functional assessment of Arid5a in IL-17 biology, I performed RNA-seq analysis on ST2 cells, following Arid5a knockdown with or without IL-17 treatment. One gene identified in this screen was insulin growth factor 2 mRNA binding protein 2 (*Igf2bp2*, also abbreviated as *Imp2*). *Igf2bp2* encodes the RBP IMP2, which has never been previously linked to IL-17. Therefore, I assessed the role of IMP2 in IL-17 signal transduction.

IMP2 belongs to a family of three insulin growth factor 2 mRNA binding proteins (IMP1-3), named for their ability to bind to the 5'UTR of human *IGF2* (insulin growth factor 2) mRNA (186, 187). IMP1-3 share 59% amino acid sequence homology (187). Although expression of *Imp1* and *Imp3* is mostly terminated before birth, *Imp2* is widely expressed in various adult mouse and human tissues (https://www.proteinatlas.org/ENSG00000073792-IGF2BP2/tissue) (187, 188). The IMP2 protein structure consists of six characteristic RNA-binding domains, including two RNA recognition motifs (RRM1 and RRM2) at the N terminus, and four hnRNP (heterogenous ribonuclear protein) K-homology domains (KH1-4) at the C-terminus **(Figure 4.1)**. IMP2 shares RNA-binding motifs with many other RBPs such as Vg1

RBP/Vera, IMP1, IMP3, CRD-BP, KOC, ZBP-1, which are hence classified as VICKZ proteins (189, 190). The precise function of these RNA-binding domains on IMP2 is not well understood. The structure of IMP2 was inferred from biochemical studies performed on other RBPs that share similar RNA-binding motifs (191). Some studies suggest that KH domains are important for RNA binding function and RRMs contribute to stabilization of protein-mRNA complexes (192, 193). IMP2 exists in two isoforms with molecular weights 65kDa and 58kDa, which are generated by alternative translation. The 58kDa isoform lacks RRM1, suggesting that these isoforms could be functionally different (194). Nonetheless, we still do not fully understand the functions of the different protein motifs present in IMP2.


**Figure 4.1: Different functional domains of IMP2**

The IMP2 protein structure is composed of six different RNA-binding domains. Two RNA recognition motifs (RRM1 and RRM2) are present at the N terminus. Four hnRNP (heterogenous ribonuclear protein) K-homology domains namely (KH1-4) are present at the C-terminus.

IMP2 plays important roles in cell metabolism and cancer progression by regulating diverse post-transcriptional mechanisms such as RNA splicing, export, stability and translation (190). According to GWAS, SNPs (single nucleotide polymorphisms) in intron 2 of *Imp2/Igf2bp2* gene are associated with a risk for type 2 diabetes (195-197). According to a recent study, *Imp2*-deficient mice resist obesity. Specifically, IMP2 was shown to inhibit translation of mRNAs encoding *Ucp1* (uncoupling protein-1), a mitochondrial protein that regulates

thermogenesis, in brown adipocytes. Consequently, elevated levels of UCP1 protein in brown adipocytes deficient in Imp2 results in resistance to diet-induced obesity (198). IMP2 has also been shown to promote gene expression by inhibiting function of microRNAs (miRNA) and long non-coding RNAs (lncRNAs) that would otherwise mediate gene silencing (190, 199). In glioblastoma stem cells, IMP2 promotes expression of mRNA of genes involved in maintenance of these cancer stem cells by counteracting let-7 miRNA (200). In myoblasts, a lncRNA known as *LncMyoD* binds to IMP2 and blocks IMP-2-mediated translation of proliferative genes *N-Ras* and *c-Myc*, and hence promotes skeletal muscle differentiation (201). Hence, IMP2 can both inhibit and promote expression of its target mRNAs through various post-transcriptional mechanisms.

In this study, I made the unexpected observation that IMP2 positively regulates IL-17 signal transduction. Gene silencing of IMP2 impaired IL-17-induced expression of inflammatory genes in primary mouse embryonic fibroblasts (MEFs). Similarly, *Imp2*$^{-/-}$ MEFs were compromised in IL-17 signaling compared to WT MEFs. Physiologically, *Imp2*$^{-/-}$ mice were less susceptible to EAE, a mouse model of multiple sclerosis, and developed less severe AGN compared to their WT littermate controls. However, *Imp2*$^{-/-}$ mice were not susceptible to oral candidiasis and were not resistant to imiquimod-driven psoriasis, suggesting specificity in IMP2-mediated gene regulation. Findings presented in this thesis suggests that IMP2 is a novel mediator of IL-17-induced inflammation and autoimmunity.

## 4.2 RESULTS

### 4.2.1 IMP2 is a novel IL-17-induced RNA-binding protein

As discussed above, I identified IMP2 as an Arid5a regulated IL-17-dependent RBP from RNA-seq analysis on ST2 cells, following Arid5a knockdown ± IL-17 **(Figure 4.2-A)**. Knockdown of Arid5a decreased IL-17-induced expression of *Imp2* **(Figure 4.2-B)**. In ST2 cells, *Imp2* mRNA expression was enhanced within an hour of IL-17 treatment, and remained elevated approximately 10-fold for at least six hours **(Figure 4.2-C)**. Transcription factors NF-κB and HMGA2 (high mobility group A2) are known to cooperatively activate *Imp2* transcription (202). Therefore, I asked whether IL-17-induced expression of *Imp2* is NF-κB-dependent. Indeed, upregulation of *Imp2* expression following IL-17 treatment in ST2 cells was blocked by an NF-κB inhibitor (**Figure 4.2-D**). These results indicate that *Imp2* is an IL-17-induced and Arid5a-regulated RBP.

I next assessed the expression of *Imp2* in response to IL-17 in primary cells. Surprisingly, IL-17 did not enhance *Imp2* mRNA in primary MEFs **(Figure 4.2-E)**. To understand this variation of IL-17-mediated *Imp2* expression in different cell types, I compared the mRNA levels of *Imp2* relative to *Gapdh* between ST2 cells and MEFs. Primary MEFs expressed significantly higher levels of *Imp2* mRNA than ST2 cells, even at a basal level **(Figure 4.2-F)**. Moreover, IMP2 protein was detected only in MEFs but not in ST2 cells. Furthermore, IL-17 did not promote IMP2 protein expression in primary MEFs **(Figure 4.2-G and H)**. These results suggest cell-specific IL-17-induced upregulation of *Imp2,* and present an interesting conundrum. Since ST2 cells expressed significantly lower basal levels of *Imp2*, I speculate that minor effects of IL-

17 on *Imp2* transcription are nonetheless functionally significant **(Figure 4.2-F)**. However, such effects may be saturated in MEFs due to a much higher expression of *Imp2* at baseline. Another explanation could come from the perspective of cell-specific epigenetic regulation of the *Imp2* locus. That is, *Imp2* chromatin may be more open in ST2 cells than in MEFs. There could be differences in histone modifications in *Imp2* chromatin in ST2 cells vs MEFs. Nevertheless, it will be vital to validate whether IMP2 protein is expressed in ST2 cells, perhaps by immunoblotting for IMP2 using much larger amounts of ST2 cells. Alternative tools to detect intracellular proteins such as immunofluorescence or imaging flow cytometry (Amnis) can be attempted to detect IMP2 in ST2 cells. Thus far, these results indicate that the effect of IL-17 on *Imp2* gene induction varies between cell types. However, more studies are required to validate that ST2 cells express IMP2.

Since I showed Arid5a stabilizes mRNAs of IL-17-dependent genes in chapter 3, I hypothesized that Arid5a promotes *Imp2* mRNA stability. To test this idea, ST2 cells were transfected with siRNAs against Arid5a, and primed with TNFα for three hours to induce *Imp2* expression without activating IL-17 signaling. Cells were then treated with actinomycin D (ActD) to block active transcription following IL-17 stimulation. Expression of *Imp2* transcripts was assessed over 90 minutes. Arid5a knockdown did not affect mRNA stabilization of *Imp2* **(Figure 4.3-A)**. Although IL-17 treatment seemed to have prevented degradation of *Imp2* mRNA, the increase of remaining *Imp2* transcripts over 100% after blocking transcription questions the efficiency of actinomycin D treatment in these experiments. Furthermore, it is important to repeat these experiments in primary MEFs where IMP2 is more abundant. In chapter 3, I showed that Arid5a can also transactivate the promoters of an IL-17-dependent gene (*Lcn2*) by enhancing expression of transcription factors in the IL-17 pathway. This raised the

possibility that Arid5a activates the *Imp2* promoter. Indeed, co-expression of Flag/Myc-Arid5a with a luciferase reporter driven by the mouse *Imp2* proximal promoter resulted in modest increase of luciferase activity **(Figure 4.3-D)**. These data suggest that Arid5a activates the *Imp2* promoter but does not stabilize its transcripts. The proximal promoter of *Imp2* contains many putative transcription factor binding sites (TFBS) **(Figure 4.3-B and C)**. Since Arid5a is known to act as a transcriptional coactivator, Arid5a could possibly cooperate with any of these TFs to drive *Imp2* transcription. It would be interesting to see whether mutating any of these sites would impair Arid5a-induced *Imp2* promoter activity.

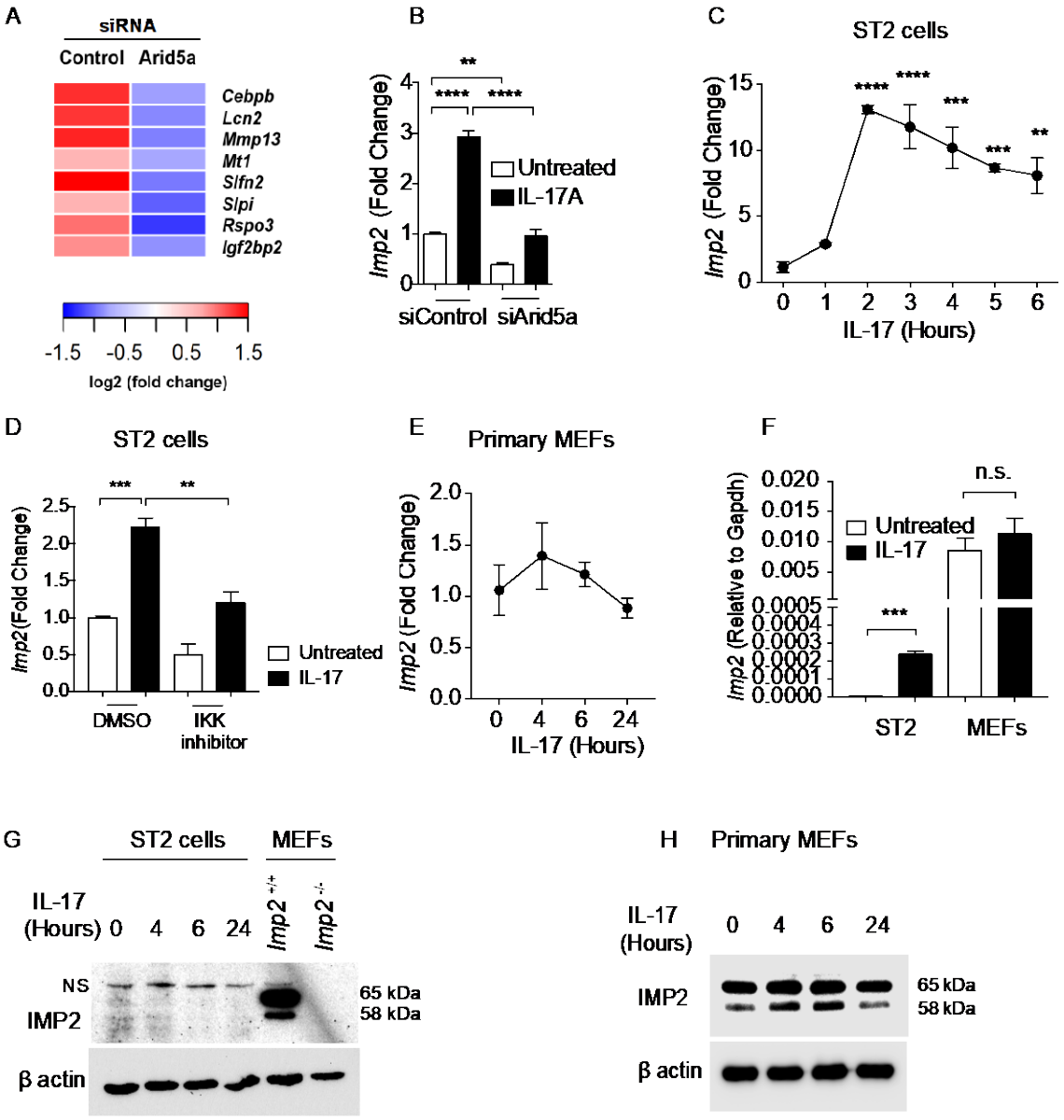


**Figure 4.2: IMP2 is a novel IL-17-induced RNA-binding protein.**

**(A)** Murine ST2 cells were transfected with pooled siRNAs targeting Arid5a or with control siRNA and treated with IL-17 for 3 hours. Expression of indicated genes was assessed by RNA sequencing. (**B)** *Imp2* expression was independently assessed by qPCR after Arid5a knockdown ± IL-17. Fold-change relative to unstimulated samples transfected with control siRNA is shown. (**C)** ST2 cells were treated with IL-17 for a period of 6 hours, and *Imp2* expression was measured

by qRT-PCR. Fold-change relative to unstimulated samples is shown. (**D)** ST2 cells were pre-treated with 10uM IKK inhibitor VII for 30 minutes following IL-17 treatment for 3 h. *Imp2* expression was measured by qRT-PCR. Fold-change relative to unstimulated samples pre-treated with DMSO only is shown. (**E)** Primary MEFs were treated with IL-17 for 24 hours, and *Imp2* mRNA expression was measured by qRT-PCR. Fold-change relative to unstimulated samples is shown. (**F)** ST2 cells or primary MEFs were treated with IL-17 for 3 hours and 4 hours respectively, *Imp2* gene expression was measured by qRT-PCR. Expression of *Imp2* relative to *Gapdh* is shown. (**G and H)** ST2 cells or primary MEFs were treated with IL-17 for the indicated times and IMP2 expression was assessed by immunoblot. Blots are representative of at least 2 independent experiments. Fold change data are means ± SEM representative of 2-3 independent experiments. **P<0.01, ***P<0.001, ****P<0.0001, n.s. = not significant by ANOVA with post hoc Tukey's test (A, D, F) or Dunnett's test (C and E).

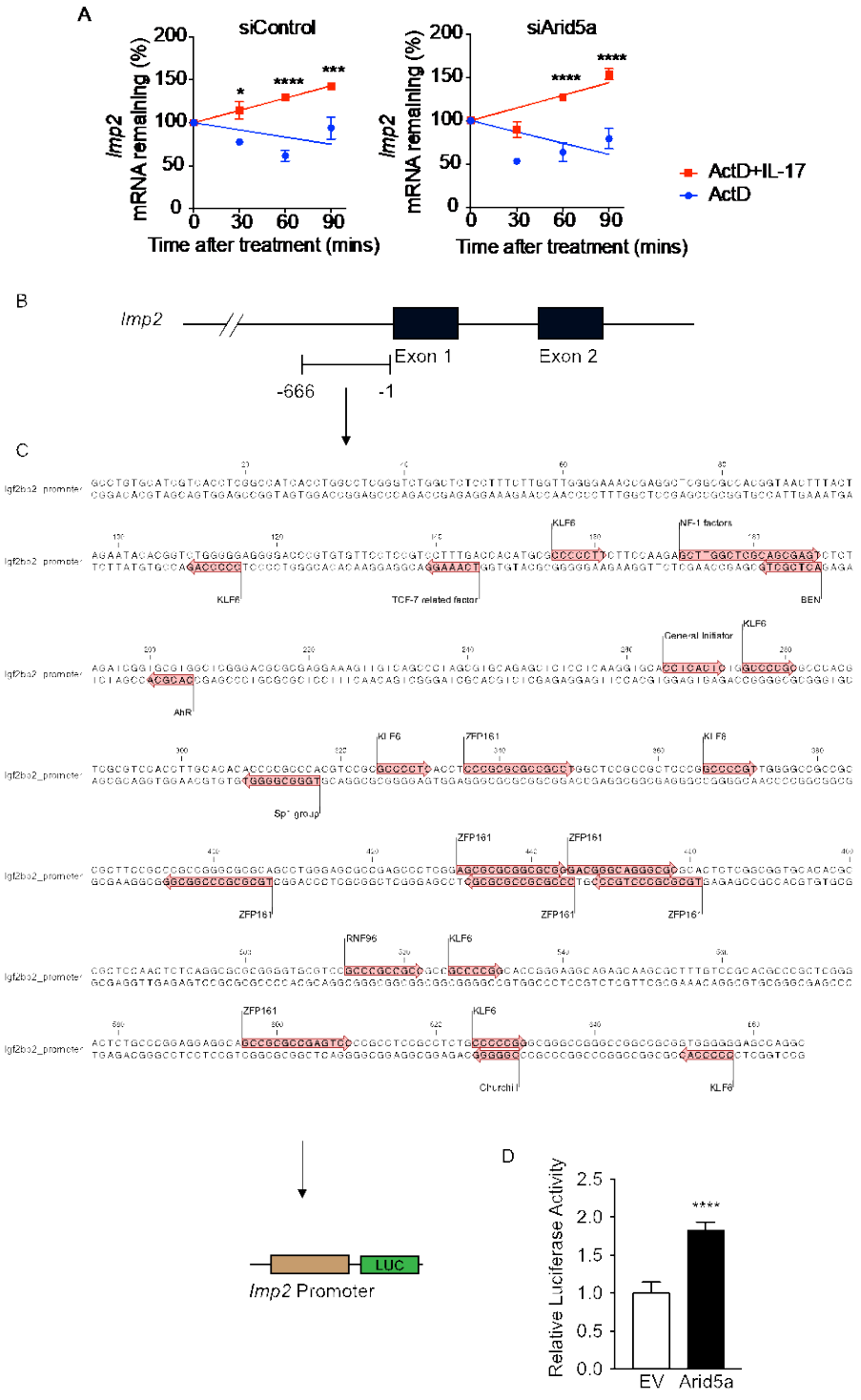


**Figure 4.3: Arid5a activates Imp2 promoter.**

**(A)** ST2 cells were transfected with pooled siRNA against Arid5a or control siRNA. Cells were treated with TNFα for 3 h, washed, and then treated with Act D ± IL-17 for 0, 30, 60 or 90

minutes. Expression of *Imp2* was assessed by qPCR. Remaining mRNA % was compared to time = 0 minutes. Data were analyzed by ANOVA with post hoc Tukey's test. **(B)** Schematic diagram of mouse *Imp2* gene. First two exons are shown as black boxes. 666 bp upstream of transcription start site (TSS) was cloned and inserted into pGL3-basic vector. **(C)** Putative transcription factor binding sites present on the proximal promoter of *Imp2* generated by using CLC workbench (**D)** HEK293T cells were transfected with a luciferase reporter driven by *Imp2* proximal promoter together with empty vector (EV) or Flag/Myc-Arid5a. After 24 h, luciferase activity was assessed. Fold-change relative to samples transfected with EV is shown. Fold change data are means ± SEM representative of 2 independent experiments. **P<0.01, ***P<0.001, ****P<0.0001, n.s. = not significant by ANOVA with post hoc Tukey's test (A) or paired Student t-test (D).

### 4.2.2 IMP2 promotes expression of IL-17-dependent genes

To delineate the role of IMP2 in IL-17 signal transduction, primary MEFs were transfected with siRNAs against IMP2 or control siRNA (knockdown efficiency was typically >90%) (**Figure 4.4-B and C**). Cells were treated with IL-17 for 24 hours, and expression of several prototypical IL-17 target genes was assessed. Knockdown of IMP2 almost completely suppressed IL-17-induced expression of *Il6, Cxcl1, Csf2* and *Cxcl5*(**Figure 4.4-A and C**). I then assessed the responsiveness of *Imp2* deficient MEFs to IL-17. Compared to $Imp2^{+/+}$ (WT) MEFs, $Imp2^{-/-}$ MEFs expressed lower levels of *Il6, Cxcl1 and Cxcl5,* but not *Csf2,* in response to IL-17 treatment (**Figure 4.5-A to C**). $Imp2^{-/-}$ MEFs also produced significantly lower levels of IL-6 and CXCL5 protein (**Figure 4.5-A**). To validate that $Imp2^{-/-}$ MEFs are not genetically deficient in IL-17 receptor or key intermediates of IL-17 signaling, I measured the mRNA levels of *Il17ra*,

*Traf3ip2* (Act1) and *Traf6* in these MEFs. WT and *Imp2*$^{-/-}$ MEFs expressed similar levels of *Il17ra*, *Traf3ip2* and *Traf6* (**Figure 4.5-D and E**). Similarly, WT and *Imp2*$^{-/-}$ MEFs exhibited similar levels of IL-17RA surface expression (**Figure 4.5-F**). Unexpectedly, tail fibroblasts generated from adult *Imp2*$^{-/-}$ mice were not compromised in IL-17 signaling (**Figure 4.5-G**). Although, WT and *Imp2*$^{-/-}$ tail fibroblasts were generated from littermate controls, the results obtained could be because primary cells from two different mice may respond to IL-17 at different magnitude. Therefore, comparing IL-17 responsiveness between WT and *Imp2*$^{-/-}$ MEFs or adult tail fibroblasts may not be an ideal approach. Currently, I am performing IMP2 knockdown experiments in adult tail fibroblasts to assess whether silencing of IMP2 in these cells decreases IL-17 signaling. These data indicate that IMP2 positively regulates some but not all IL-17 target genes.

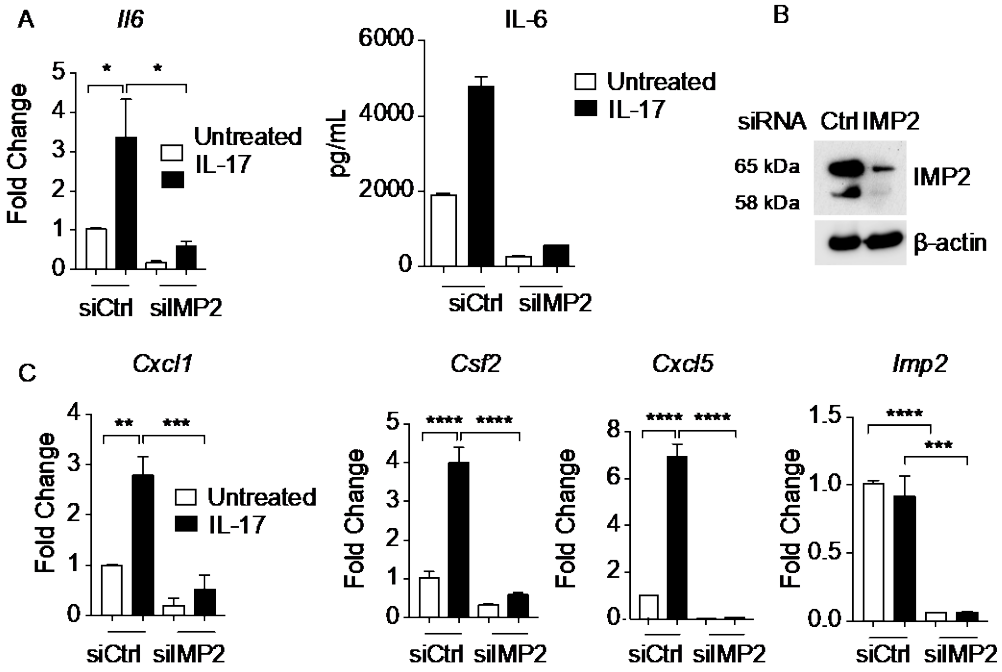


**Figure 4.4: IMP2 positively regulates IL-17 signaling.**

**(A)** Primary MEFs were transfected with pooled siRNAs targeting IMP2 or control siRNA. Cells were treated with IL-17 for 24 hours and expression of IL-6 was assessed by qPCR or ELISA. Fold-change relative to unstimulated samples transfected with control siRNA is shown. Fold change data are means ± SEM pooled from 3 independent experiments for *Il6* qPCR and representative of 2 experiments for IL-6 ELISA. (**B)** Whole cell lysates from MEFs transfected with either control siRNA or siRNA against IMP2 were subjected to immunoblot with anti-IMP2 antibody. **(C)** Primary MEFs were transfected with pooled siRNAs targeting IMP2 or control siRNA. Cells were treated with IL-17 for 24 hours and expression of indicated genes were assessed by qPCR. Fold-change relative to unstimulated samples transfected with control siRNA is shown. Fold change data are means ± SEM representative of 2-3 experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by ANOVA with post hoc Tukey's test.

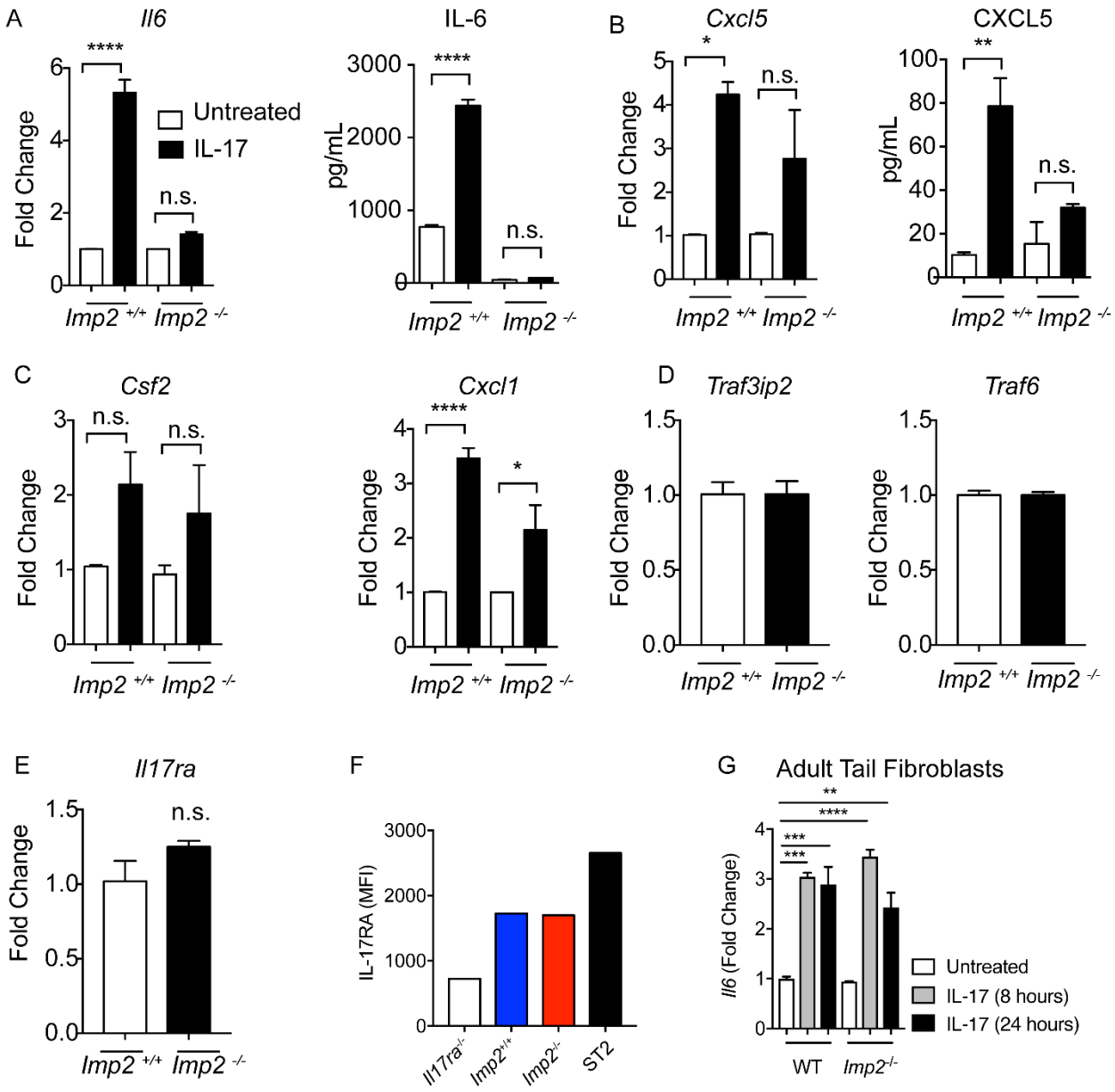


**Figure 4.5: IMP2 deficient primary MEFs are compromised in IL-17 signaling.**

**(A to C)** *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ MEFs were treated with IL-17 for 24 hours and expression of the indicated genes was assessed by qPCR or ELISA. Fold-change relative to unstimulated samples transfected with control siRNA is shown. Fold change data are means ± SEM pooled from 3 independent experiments for qPCR and representative of 2 experiments for ELISA. *P<0.05,

**P<0.01, ****P<0.0001by ANOVA with post hoc Tukey's test. (**D and E)** Expression of *Traf3ip2* (Act1), *Traf6* and *Il17ra* was assessed by qPCR in *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ MEFs. Fold change data are means ± SEM pooled from 1 experiment. **(F)** *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ MEFs, ST2 cells and *Il17ra*$^{-/-}$ MEFs were stained for IL-17RA and mean fluorescence intensity was measured by flow cytometry. Data are representative of 1 experiment. **(G)** *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ adult tail fibroblasts were treated with IL-17 for 24 hours and expression of *Il6* was assessed by qPCR. Fold-change relative to unstimulated samples transfected with control siRNA is shown. Fold change data are means ± SEM pooled from 1 experiment.

### 4.2.3 IMP2 regulates IL-17-dependent NF-κB activation

IL-17 activates NF-κB and MAPK pathways including ERK, p38 and JNK to induce inflammatory gene expression (4). Since IMP2 deficiency led to decreased expression of multiple IL-17 target genes, I considered that lack of IMP2 might inhibit activation of IL-17-driven NF-κB and MAPK pathways. To test this possibility, *Imp2*$^{+/+}$ and *Imp2*$^{-/-}$ primary MEFs were treated with IL-17 over a 60 minute time course, and activation of NF-κB, ERK, p38 and JNK was assessed by immunoblot. Interestingly, *Imp*$^{-/-}$ MEFs were defective in IL-17-induced phosphorylation of IκBα. While phosphorylation of ERK, p38 and JNK was not affected **(Figure 4.6-A to D)**. Activation of NF-κB is regulated at multiple levels. As discussed in chapter 1, after the binding of IL-17 to its receptor, adaptor proteins such as Act1 and TRAF6 are recruited to the IL-17 receptor. TRAF6 is then ubiquitinated, promoting recruitment of the TAK1/TAB2/TAB3 complex, which itself activates an IKK complex composed of IKKα, IKKβ and IKKγ (also known as NEMO). This series of events results in IKK-mediated

phosphorylation and proteosomal degradation of IκBα. Since IκBα is a constitutive inhibitor of NF-κB, degradation of IκBα leads to nuclear translocation of NF-κB and gene transcription (4). So far, I have only assessed the effect of *Imp2* on phosphorylation of IκBα. My preliminary data suggest that IMP2 plays a role in regulating IL-17-induced NF-κB, but not MAPK, activation. However, it is important to confirm that IMP2 promotes NF-κB activation by evaluating the impact of IMP2 deficiency on other forms of NF-κB regulation. Does IMP2 deficiency affect phosphorylation of NF-κB p65? Is nuclear localization of NF-κB impaired in the absence of IMP2? These are important questions that need to be answered.

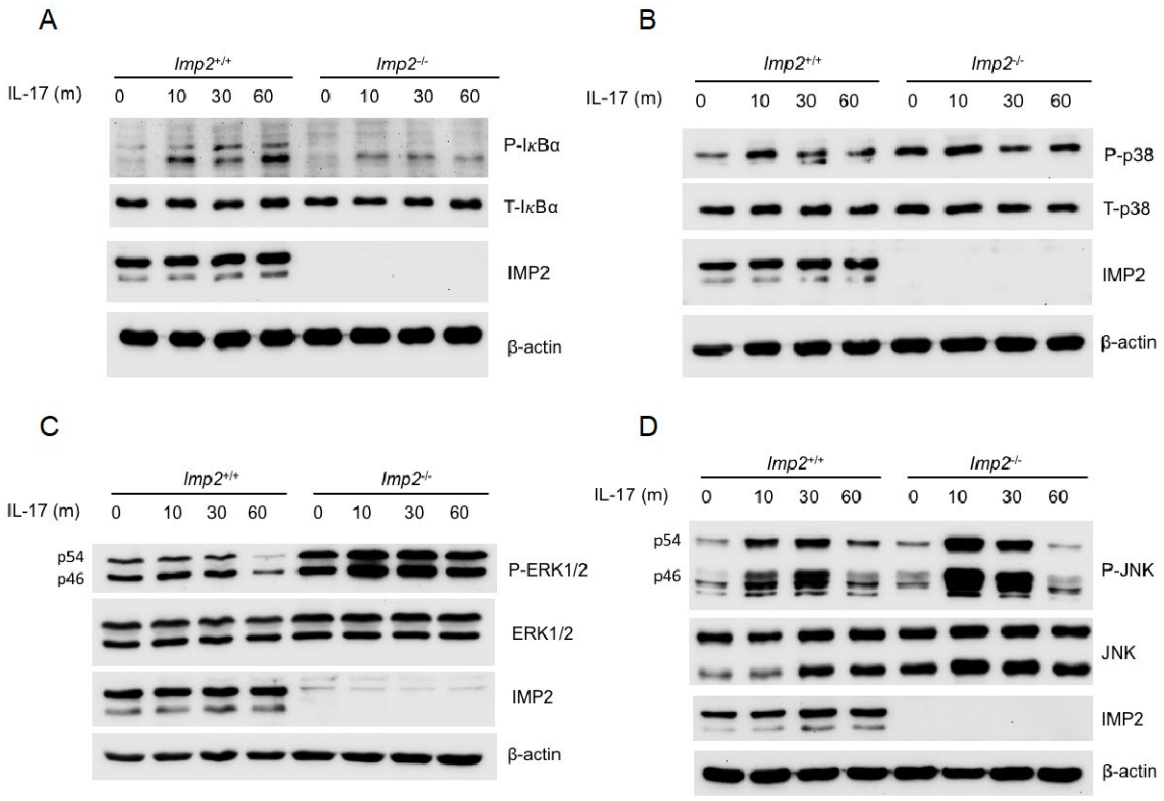


**Figure 4.6: IMP2 promotes IL-17-induced NF-kB activation**

**(A)** *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ MEFs were treated with IL-17 for 10, 30 and 60 minutes. Expression of p-IκBα and total IκBα; (**B**) p-p38 and total p38; (**C**) p-ERK1/2 and total ERK1/2; (**D**) p-JNK and total JNK were assessed by immunoblot. Blots are representative of two independent experiments.

### 4.2.4 IMP2 is an important driver of EAE and AGN pathogenesis

Since *Imp2* deficiency impaired IL-17 signaling in MEFs, it was logical to postulate that IMP2 is a driver of IL-17-dependent autoimmune inflammation *in vivo*. To test this hypothesis, I assessed the effect of *Imp2* deficiency on the pathogenesis of EAE. As noted, IL-17 signaling is known to be essential during EAE disease pathogenesis (179). Male *Imp2*$^{-/-}$ mice and sex-matched WT littermate controls were co-housed a week before experiment to normalize microbiota. On day 0, mice were immunized subcutaneously with myelin oligodendrocyte glycoprotein (MOG) peptide (MOG35-55) emulsified with complete Freund's adjuvant (CFA). Mice were also given two doses of pertussis toxin intraperitoneally on day 0 and day 2. *Il17ra*$^{-/-}$ mice, which are refractory to EAE, were used as negative controls. As expected, WT mice developed severe EAE, while *Il17ra*$^{-/-}$ mice were resistant to EAE. Strikingly, *Imp2*$^{-/-}$ were less susceptible to EAE, as indicated by lower clinical scores than littermate *Imp2*$^{+/+}$ controls **(Figure 4.7-A)**. However, the time of disease onset for *Imp2*$^{-/-}$ mice was similar to that of littermate controls. These results indicate that *Imp2* is an important mediator of IL-17-dependent autoimmune inflammation in EAE.

In the EAE model, MOG-specific effector Th17 cells are generated in the periphery, cross the blood brain barrier and enter the CNS. In the CNS, these autoimmune T cells are reactivated by APCs, resulting in production of Th17-related cytokines (203). To determine whether IMP2 affects generation of Th17 cells during EAE, I assessed Th17 cells in the lymph node and spleen of *Imp2*$^{+/+}$ and *Imp2*$^{-/-}$ mice on day 10 (onset of disease) after EAE immunization. Deficiency of *Imp2* did not affect the percentages of IL-17-producing Th17 cells in the lymph node but there was a slight increase in IL-17-producing Th17 cells in the spleen of *Imp2*$^{-/-}$ mice. Percentages of GM-CSF-producing Th17 cells and IFNγ-producing Th1 cells were

comparable between *Imp2*$^{+/+}$ and *Imp2*$^{-/-}$ mice in both lymph node and spleen **(Figure 4.7-B and C).** To confirm that IMP2 does not affect Th17 generation, I assessed the effect of IMP2 in Th17 differentiation *in vitro*. Naïve CD4$^{+}$ T cells from *Imp2*$^{+/+}$ and *Imp2*$^{-/-}$ mice were stimulated for three days with plate bound anti-CD3 and anti-CD28 under Th0 or Th17 conditions (IL-6, TGF-β, IL-1β and IL-23). As shown, *Imp2* deficiency did not affect percentages of *in vitro*-generated CD4$^{+}$IL-17$^{+}$ Th17 cells and IL-17 production **(Figure 4.8-A to C)**. Interestingly, according to ImmGen, *Imp2* is expressed at very minimal levels in most immune cells except for some subsets of pro-B cells. However, *Imp2* is mostly expressed in multiple subsets of stem cells and stromal cells (www.immgen.org). Consistently with that, IMP2 protein was not detectable in CD4$^{+}$ T cells cultured either in Th0 or Th17 conditions **(Figure 4.8-D)**. These results validate that *Imp2* deficiency does not affect Th17 cell generation *in vivo* or *in vitro*.

During EAE, CNS-resident non-hematopoietic cells such as astrocytes, oligodendrocytes, etc. respond to IL-17 by producing inflammatory cytokines and chemokines. These inflammatory effectors lead to infiltration of neutrophils, ultimately resulting in CNS inflammation (204), so I assessed levels of IL-17 dependent inflammatory genes in the spinal cord of WT and *Imp2*$^{-/-}$ mice on day 15. Spinal cords of *Imp2*$^{-/-}$ mice had reduced levels of *Csf2*, *Ccl20* and *Ccl5,* but not of *Il6, Cxcl1, Cxcl5, Csf3, Cxcl2* and *Il17a* **(Figure 4.9)**. These results are consistent with a model in which IMP2 drives EAE pathogenesis by positively regulating IL-17 receptor-mediated signaling rather than by affecting Th17 cell generation.

Autoimmune glomerulonephritis (AGN) is another autoimmune disease in which IL-17-mediated inflammation has been shown to be pathogenic (184, 185). To investigate whether IMP2 plays a role during AGN disease pathogenesis, I assessed the effect of *Imp2* deficiency in experimental autoimmune glomerulonephritis (EAGN). During EAGN, kidney injury is induced

by generating an autoimmune response against rabbit anti-mouse glomerular basement membrane (GBM) serum. Studies suggests that EAGN recapitulates many fundamental features of human autoimmune kidney disease (e.g. lupus nephritis) in humans (205). Therefore, I hypothesized that compromised IL-17 signaling due to *Imp2* deficiency would lead to reduced EAGN disease severity. Female *Imp2*$^{-/-}$ mice and sex-matched littermate controls were co-housed for a week before the start of the experiment. On day -3, mice were injected i.p. with rabbit IgG emulsified with complete Freund's adjuvant (CFA) to generate anti-rabbit antibodies in the host. Control mice were injected with PBS emulsified with CFA. On day 0, all mice were injected with rabbit anti-mouse GBM serum intravenously. On day 14, mice were sacrificed, and blood and kidneys were harvested. As expected, WT mice exhibited significantly higher blood urea nitrogen (BUN) and serum creatinine levels that control mice, indicating impaired kidney function. As previously reported, *Act1*$^{-/-}$ mice were resistant to AGN (185). Interestingly, *Imp2*$^{-/-}$ mice had significantly lower levels of BUN and serum creatinine levels than WT mice **(Figure 4.10 A and B)**. Assessment of H&E-stained kidney sections of nephritic WT mice showed significant glomerular damage evident by hypercellularity, neutrophil infiltration and damage of glomerular barrier integrity. However, glomeruli of *Imp2*$^{-/-}$ mice showed less severe tissue damage **(Figure 4.10 C)**. Moreover, blinded scoring of renal pathology on H&E stained kidney sections showed lower percentages of abnormal glomeruli in *Imp2*$^{-/-}$ mice, compared to their littermate controls **(Figure 4.10 D)**. Collectively, these results indicate that IMP2 is an important mediator of AGN disease pathogenesis.

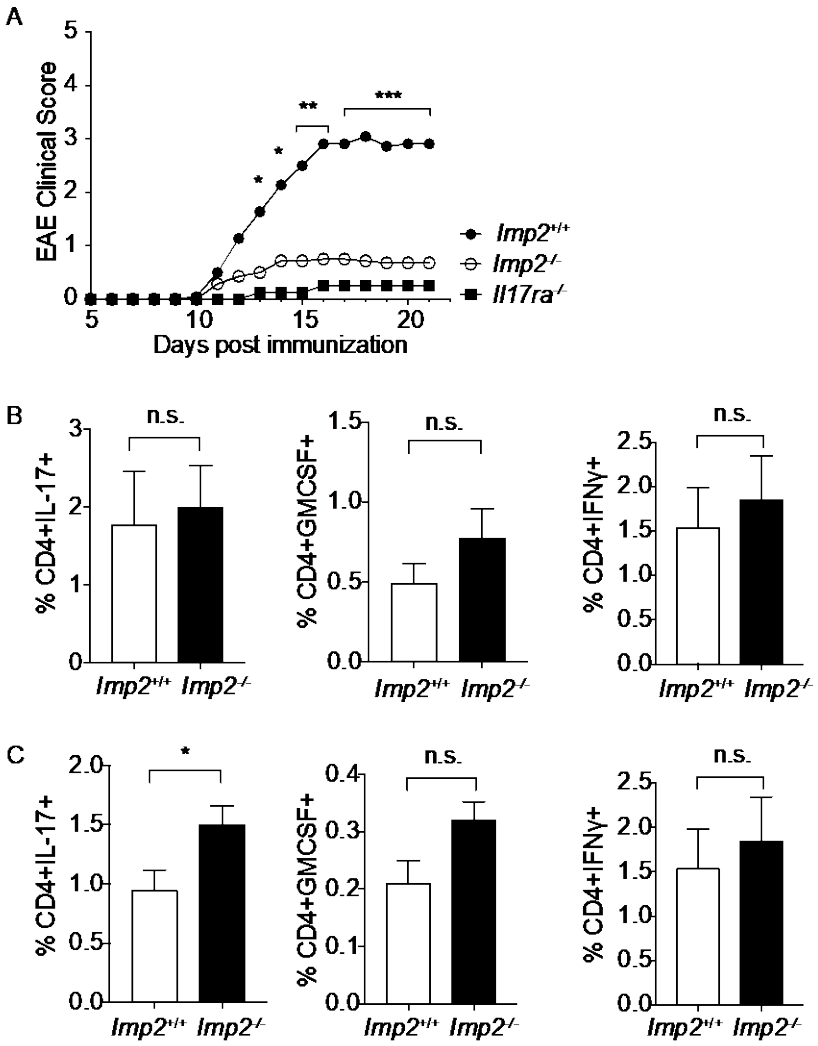


**Figure 4.7: IMP2 deficient mice are refractory to EAE**

**(A)** *Imp2*$^{+/+}$ (n=11), *Imp2*$^{-/-}$ (n=14) and *Il17ra*$^{-/-}$ (n=4) mice were immunized s.c. with MOG35-55 emulsified with CFA. Mice were injected i.p. with pertussis toxin on day 0 and 2. Clinical scores were blindly assessed daily until day 21. Data are pooled from 3 independent experiments. Data are presented as mean clinical score. *P<0.05, **P<0.01, ***P<0.001 by Mann-Whitney test. (**B)** Lymph nodes and **(C)** spleens were harvested on day 10 of EAE and cells were stimulated with PMA/ionomycin for 4 hours. Cells were stained for CD4, IL-17, GM-CSF and IFNγ, and quantified by flow cytometry. *P<0.05, n.s. = not significant by unpaired Student t-

test.

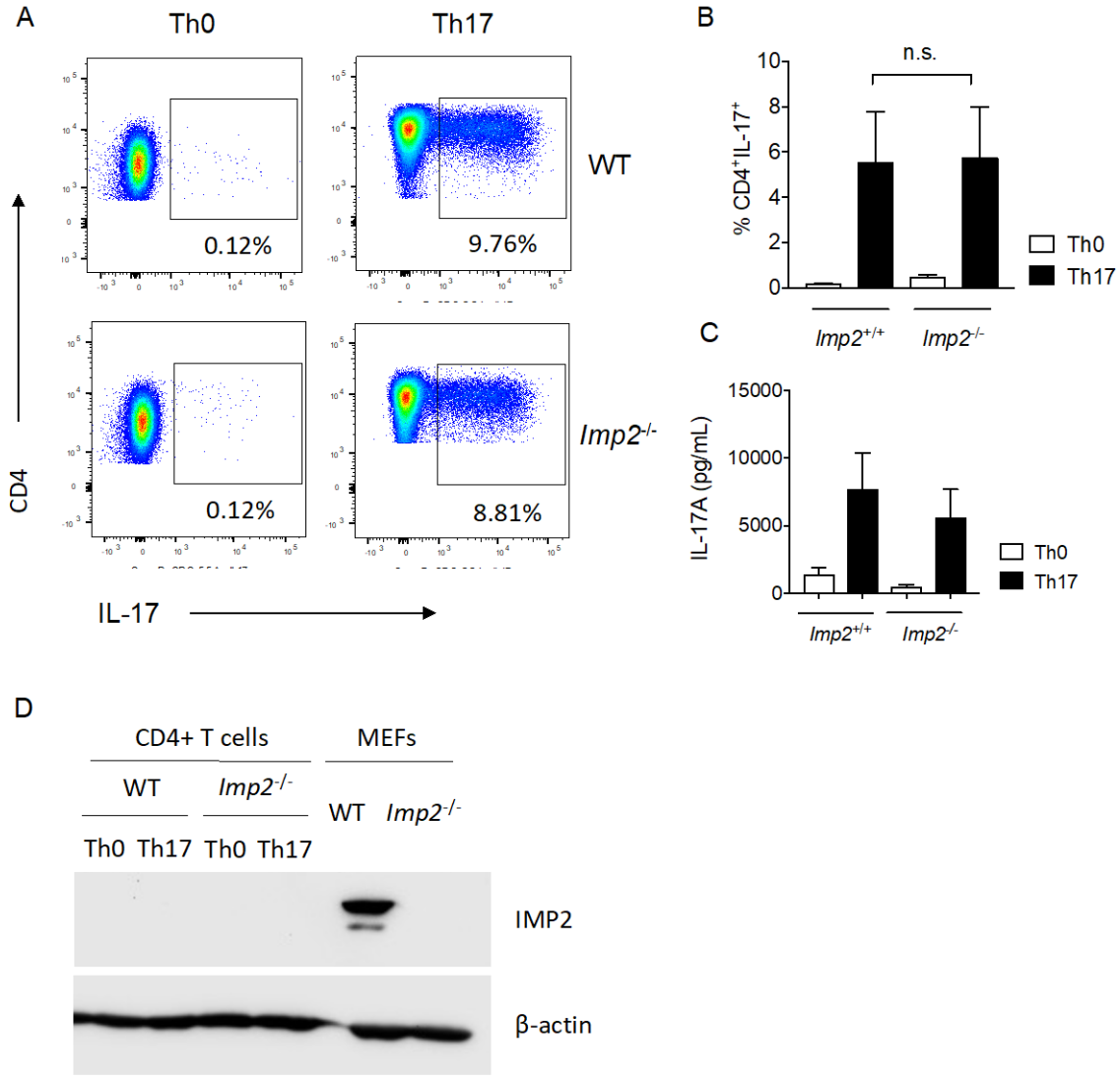


**Figure 4.8: IMP2 is dispensable for *in vitro* Th17 generation.**

Naive CD4$^+$ T cells were isolated from spleens of *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ mice. Cells were stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 under Th0 or Th17 (IL-6, IL-23, TGFβ and IL-1β) conditions. After 3 days cells were stimulated with PMA/ionomycin for 4 hours. Cells were then stained for CD4, IL-17 and IFNγ and quantified by flow cytometry. **(A)** Plots are representative of 3 mice for each group from 2 independent experiments and **(B)** data were

pooled from 3 independent experiments. **(C)** ELISA was performed on supernatants collected after 72 hours before PMA/ionomycin treatment. *P<0.05, **P<0.01, ***P<0.001, n.s. = not significant by ANOVA with post-hoc Tukey test. n= 3 mice per group. (**D)** Western blot for IMP2 was performed on whole cell lysates from *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ MEFs or CD4 T cells conditioned with Th0 or Th17 cytokines.

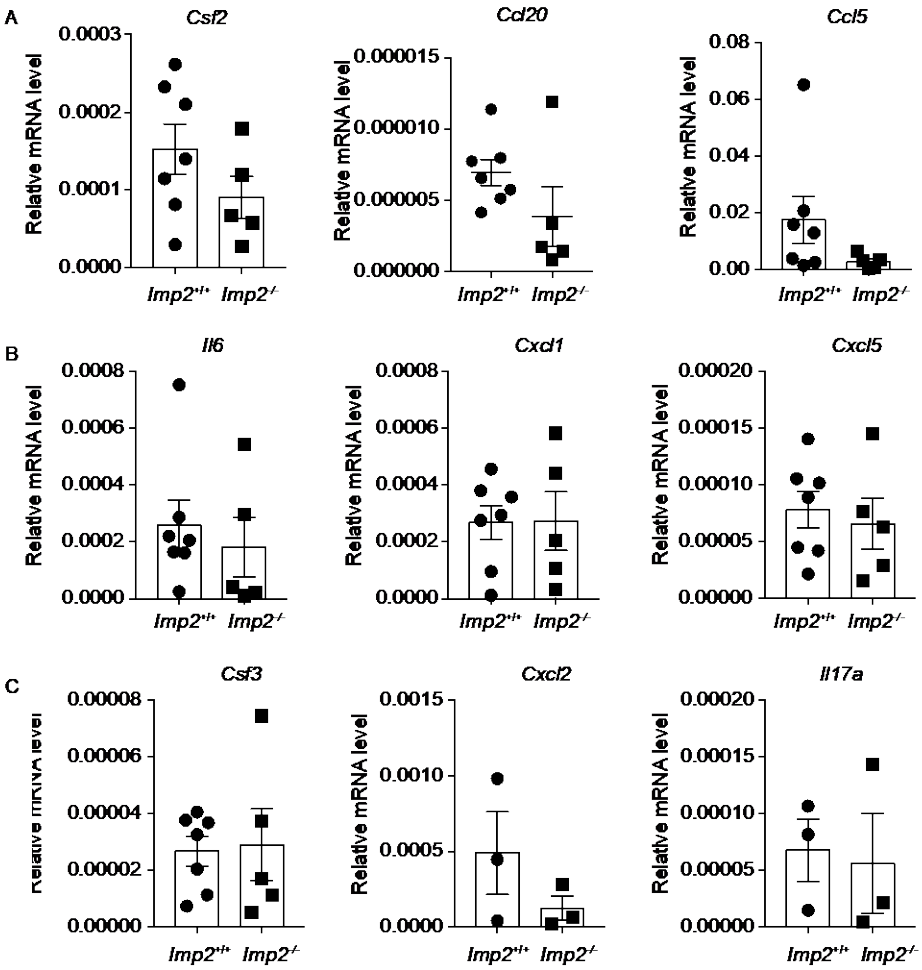


**Figure 4.9: Spinal cords of IMP2 deficient mice express reduced levels of a subset of inflammatory mediators during EAE**

**(A to C)** Spinal cords from *Imp2*$^{+/+}$ (n=7) and *Imp2*$^{-/-}$ (n=5) mice subjected to EAE were harvested on day 15 (peak of disease). Expression of indicated genes was assessed by qPCR. Data are pooled from two independent experiments and expression as relative to *Gapdh* is shown.

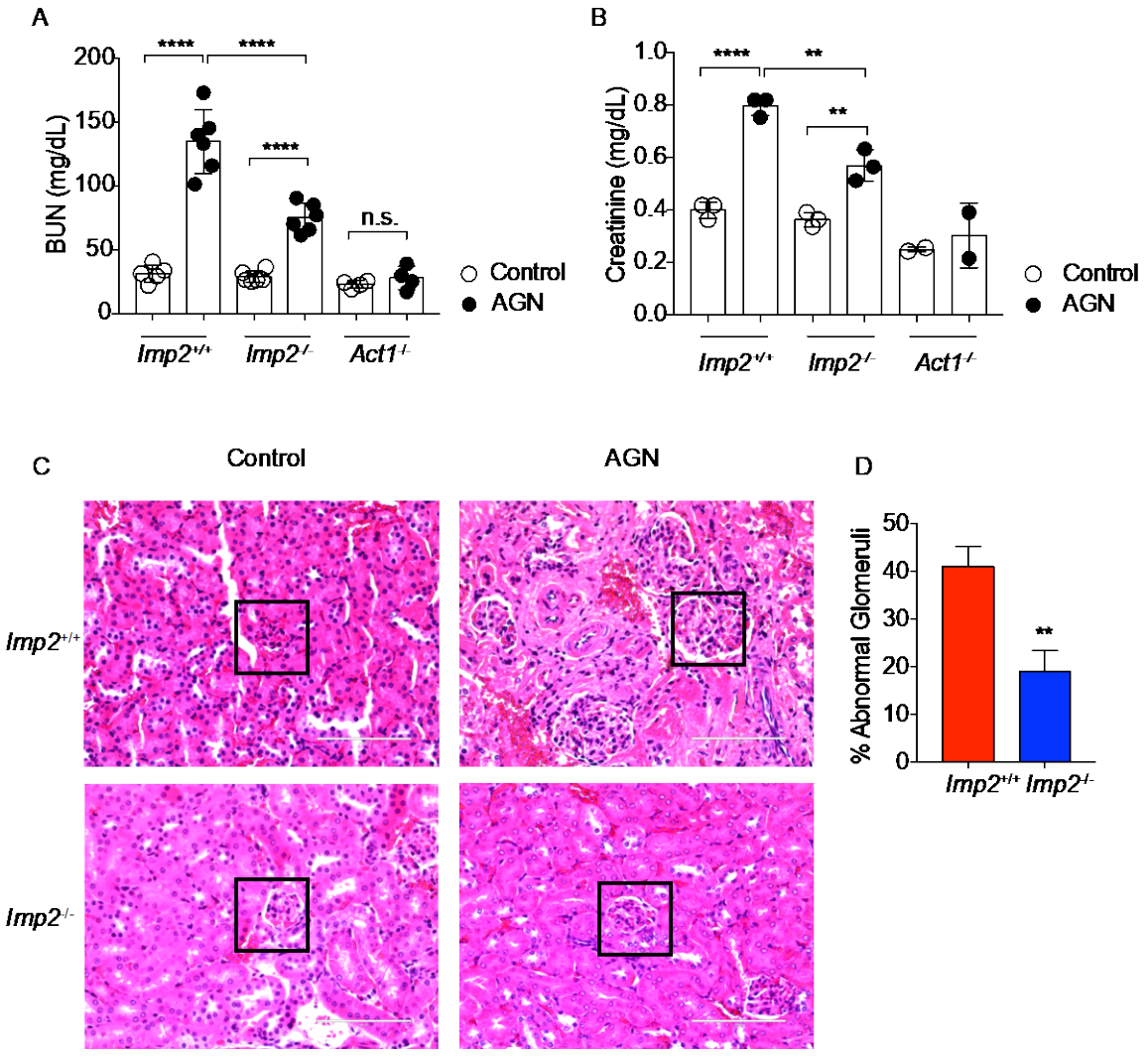


**Figure 4.10: IMP2 deficient mice are less susceptible to AGN.**

**(A and B)** *Imp2*$^{+/+}$ (n=6), *Imp2*$^{-/-}$ (n=6) and *Act1*$^{-/-}$ (n=4) mice were immunized with rabbit IgG emulsified with complete Freund's adjuvant (CFA) intraperitoneally on day -3. On day 0, mice were injected with rabbit anti-mouse GBM serum intravenously. On day 14, blood serum was harvested and blood urea nitrogen (BUN) levels and serum creatinine were measured. Data are pooled from two independent experiments. **P<0.01, ****P<0.0001, n.s. = not significant by ANOVA with post-hoc Tukey test. **(C)** Representative photographs of H&E stained kidney sections of *Imp2*$^{+/+}$ and *Imp2*$^{-/-}$ mice on day 14 of AGN (original magnification, 400X). **(D)**

Blinded evaluation of renal pathology was performed on H&E stained kidney sections of *Imp2*$^{+/+}$ and *Imp2*$^{-/-}$ mice on day 14 of AGN, and percent abnormal glomeruli was calculated.

### 4.2.5 IMP2 functions dominantly in the non-hematopoietic compartment to mediate autoimmune glomerulonephritis

Both IL-17-producing cells and downstream IL-17 receptor signaling have been shown to contribute to the pathogenesis of AGN (176, 184). IL-23 deficient mice, which have impaired Th17 generation, develop less severe glomerulonephritis and show decreased kidney infiltration of Th17 cells (206). Similarly, *Il17a*$^{-/-}$, *Il17ra*$^{-/-}$ and *Act1*$^{-/-}$ mice exhibit reduced kidney damage during experimental glomerulonephritis (184, 185, 206). To distinguish between IMP2 function in hematopoietic versus non-hematopoietic compartments, bone marrow (BM) chimeric mice were created with *Imp2* deficiency in either immune or non-immune cells. The resulting BM chimeric mice were subjected to EAGN. As expected, WT mice receiving WT BM showed higher levels of BUN after EAGN induction compared to control mice **(Figure 4.11, also see Figure 4.10-A)**. Moreover, WT mice receiving *Imp2*$^{-/-}$ BM also showed similar levels of BUN compared to WT mice receiving WT BM. However, *Imp2*$^{-/-}$ mice receiving WT BM were resistant to EAGN, and *Imp2*$^{-/-}$ mice receiving *Imp2*$^{-/-}$ BM were also refractory to EAGN. These data indicate that IMP2 functions dominantly in non-hematopoietic compartment during AGN.

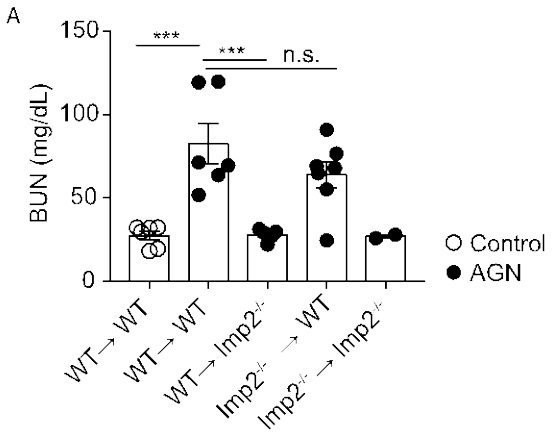


**Figure 4.11: IMP2 dominantly functions in non-hematopoietic compartment during AGN.**

**(A)** *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ mice were lethally irradiated and reconstituted with bone marrow from *Imp2*$^{+/+}$ or *Imp2*$^{-/-}$ mice. After 6 weeks, mice were subjected to AGN as described in Figure 4.9. On day 14, blood serum was harvested and blood urea nitrogen (BUN) levels were measured. Data are pooled from two independent experiments. ***P<0.001, n.s. = not significant by ANOVA with post-hoc Tukey test.

### 4.2.6 IMP2 appears to be dispensable for IL-17-mediated innate immunity

IL-17 confers protection against acute oral *Candida albicans* infections by inducing expression of various inflammatory effectors (134, 207). We have previously performed microarray analysis on tongues of mice infected with *C. albicans* to identify genes upregulated by *C. albicans* in an IL-17-dependent manner (207). Since then we have validated the biological significance of severalof these IL-17-induced genes in IL-17 signal transduction and in immunity against *C. albicans (208, 209)*. Upon re-evaluation of these archival microarray data, I identified the RBP *Imp2* as an IL-17-depenent gene induced during oral candidiasis (OPC) **(Figure 4.12-A).** Based

on this evidence, I hypothesized that reduced IL-17 signaling in *Imp2*$^{-/-}$ mice would result in impaired fungal clearance during oral candidiasis. We have previously shown that WT but not *Il17ra*$^{-/-}$ mice are able to clear infection by day five (207). To test whether IMP2 plays a role in IL-17-induced innate immunity against *C. albicans*, I infected WT, *Imp2*$^{-/-}$, *Imp2*$^{+/-}$ and *Il17ra*$^{-/-}$ mice with *C. albicans* sub-lingually and assessed daily weight changes and tongue fungal burden on day five post-infection. Initially, all mice lost weight after infection. However, WT, *Imp2*$^{-/-}$ and *Imp2*$^{+/-}$ but not *Il17ra*$^{-/-}$ mice regained their original body weights five days post-infection **(Figure 4.12-B)**. As expected, *Il17ra*$^{-/-}$ mice had significantly higher fungal burden compared to WT mice. However, both *Imp2*$^{-/-}$ and *Imp2*$^{+/-}$ mice were able to clear the fungus similarly to WT mice, by day five post-infection **(Figure 4.12-C)**. These results suggest that IMP2 is dispensable for protection against *C. albicans* during acute oral candidiasis.

IL-17 is one of the key inflammatory cytokines implicated in development of psoriasis. Mice deficient in IL-17RA or Act1 are resistant to the imiquimod (IMQ)-induced mouse model of psoriatis plaque formation (166, 210). Therefore, I postulated that *Imp2*$^{-/-}$ mice would be less susceptible to imiquimod (IMQ)-induced dermatitis, as a result of their impaired IL-17 signaling. IMQ is a TLR7/8 agonist, and IMQ-induced dermatitis results in IL-17A and IL-23-dependent skin inflammation and neutrophil infiltration, recapitulating many features of psoriasis in humans (210, 211). *Imp2*$^{+/+}$, *Imp2*$^{-/-}$ and *Il17ra*$^{-/-}$ mice were subjected to topical application of IMQ. After five days of IMQ treatment, both WT and *Imp2*$^{-/-}$ mice showed increased skin scaling, epidermal thickening and erythema **(Figure 4.13-A)**. However, *Il17ra*$^{-/-}$ mice showed much lower skin inflammation indicating lower disease severity. Similarly, neutrophil infiltration in the skin was comparable in both WT and *Imp2*$^{-/-}$ mice **(Figure 4.13-B and C)**. However, *Il17ra*$^{-/-}$ mice had

significantly reduced levels of neutrophils in skin. These results indicate that IMP2 is not required for the development of psoriasis.

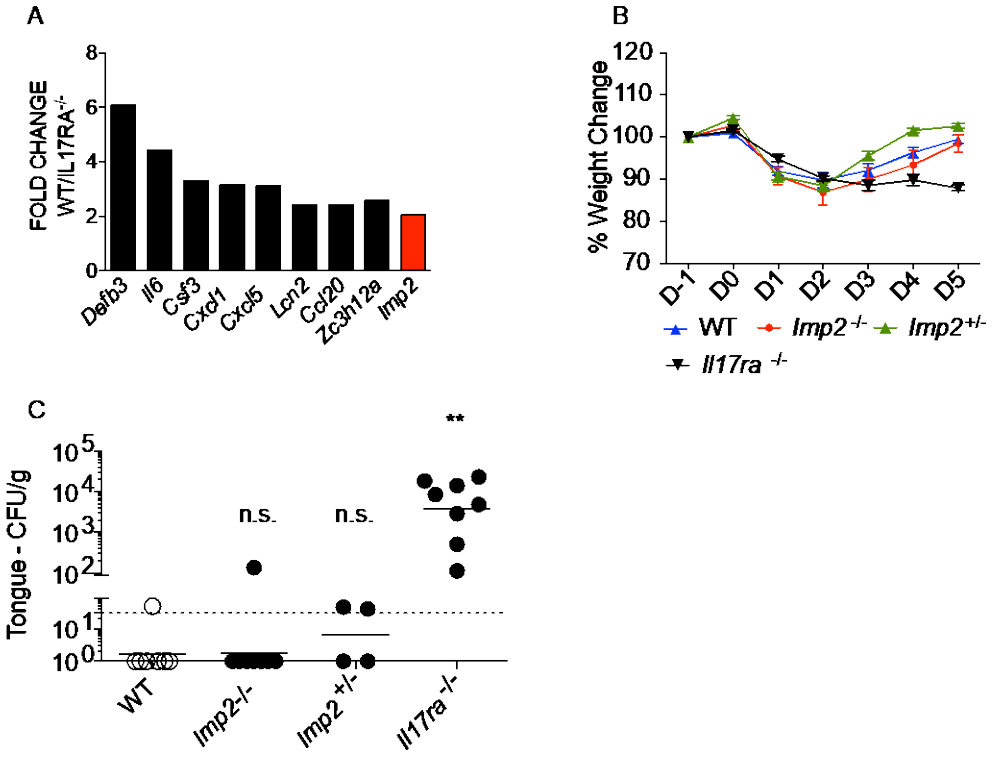


**Figure 4.12: IMP2 is dispensable for protection against OPC.**

**(A)** WT or *Il17ra*$^{-/-}$ mice were challenged sublingually with *C. albicans* (CAF2-1). Tongues of mice were harvested at day 0 or 24 hours post-infection, and cDNA was hybridized to Affymetrix genechips. Data shown are fold change of genes induced in WT mouse tongue compared with *Il17ra*$^{-/-}$ tongue 24 hours post infection (n=2 from two independent experiments) (207). **(B)** WT (n=7), *Imp2*$^{-/-}$ (n=8), *Imp2*$^{+/-}$ (n=4) or *Il17ra*$^{-/-}$ (n=8) mice were challenged sublingually with *C. albicans* (CAF2-1). All mice were weighed daily and percent weight change compared to day -1 is indicated. (**C**) On day 5, tongues were harvested, homogenized and plated in YPD agar plates in triplicates. Fungal burden is shown as CFU/gram. Data are pooled from two independent experiments. **P<0.01, n.s. = not significant by ANOVA with post-hoc Tukey test.

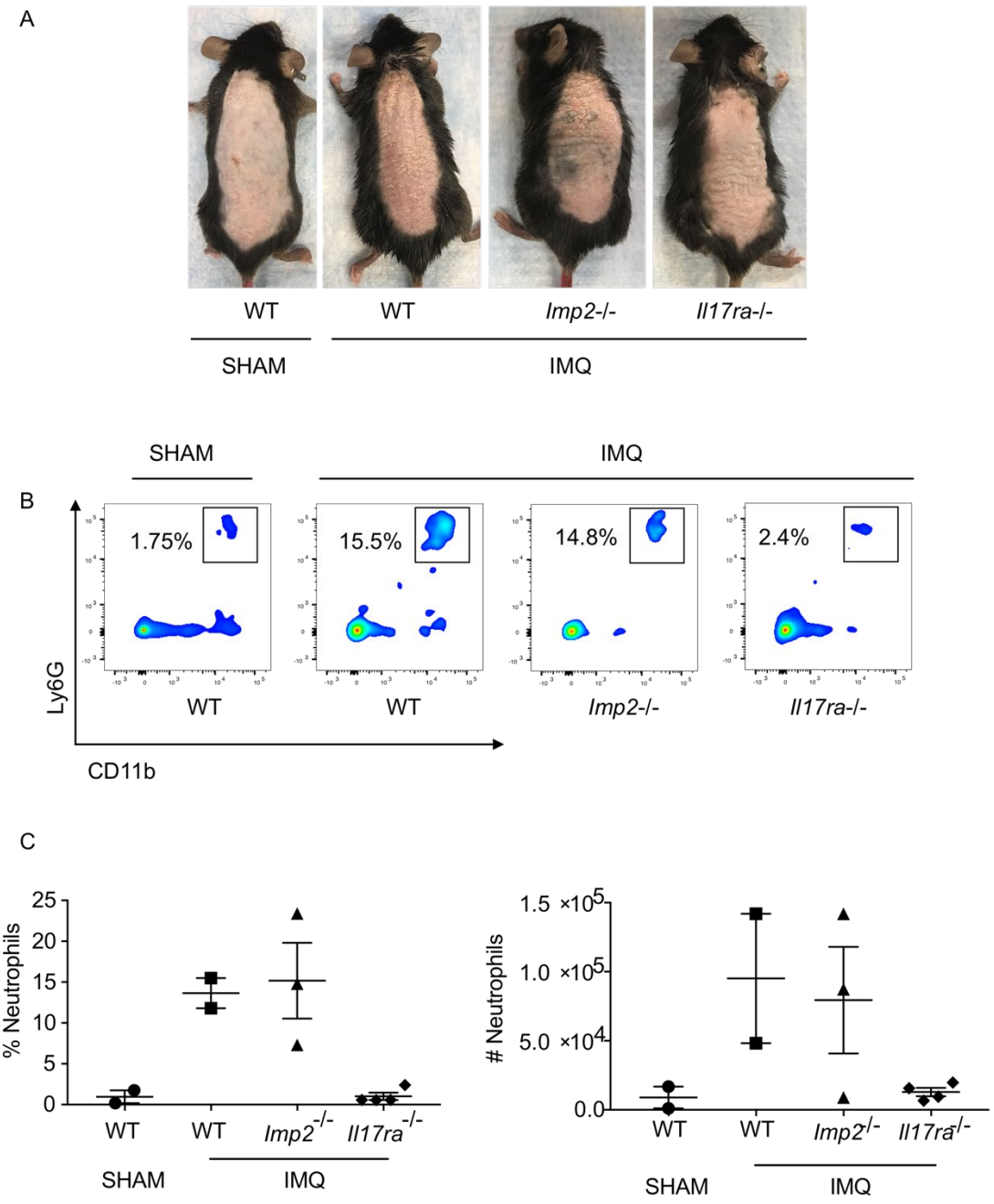


**Figure 4.13: IMP2 is dispensable for IMQ-induced dermatitis.**

**(A)** WT (n=4), *Imp2*$^{-/-}$ (n=3) or *Il17ra*$^{-/-}$ (n=4) mice were treated topically either with mock cream or with imiquimod (IMQ) for 5 days. Representative photographs of gross skin pathology on day 6 is shown. **(B)** Skin infiltration of neutrophils was quantified by flow cytometry. Cells

were harvested from skin and stained for CD45, CD68, Ly6G and CD11b. **(C-D)** Percentage and absolute number of $Ly6G^{+}CD11b^{+}$ neutrophils gated on $CD45^{+}CD68^{-}$ total skin cells are shown.

## 4.3 DISCUSSION

Post-transcriptional control of gene expression is an important facet of IL-17 receptor-mediated inflammation (175). Multiple RBPs such as HuR, Regnase-1/MCPIP1, Act1, Arid5a and DDX3X, have been found to orchestrate the mRNA stability of genes downstream of IL-17 signaling (120, 122, 125, 208). Here, I have identified a new RBP that promotes expression of IL-17-induced inflammatory genes. In primary MEFs, IMP2 deficiency resulted in decreased expression of IL-17 target genes in at least some settings. However, data from RNA-immunoprecipitation (RIP) assay showed that IMP2 does not detectably bind to transcripts of those IL-17 target genes affected by IMP2 deficiency **(Appendix A, Figure 0.1)**. Although RIP assays were performed using the same techniques applied in Chapter 3, lack of a positive control (i.e. transcript that is known to bind to IMP2 in our system) calls into question the limit of detection of this anti-IMP2 RIP assays **(Appendix A, Figure 0.1)**. Therefore, it is important to repeat these experiments using more sensitive approach such as UV crosslinking IMP2-mRNA interactions (212). Moreover, the Rabbit anti-IMP2 antibody (Cell Signaling, 1472S) that I used to perform RIP assays only detects the 65kDa protein isoform of murine IMP2 but not the 58kDa isoform. It is possible that the RNA-binding capacity of IMP2 vary between different isoforms. Surprisingly, nothing is known about functional differences between IMP2 isoforms (194). Thus, this is an interesting question and could be addressed by comparing data from RIP performed using antibodies that detects only one or both isoforms of IMP2.

To understand IMP2 function in IL-17 biology, I explored the role of IMP2 in regulating expression of proximal or distal mediators of IL-17 signaling. Unexpectedly, MEFs lacking IMP2 were compromised in IL-17R-induced phosphorylation of IκBα. However, IMP2 did not affect phosphorylation of ERK, p38 or JNK. Moreover, IMP2 deficient MEFs had intact expression of IL-17RA, Act1 and TRAF6, major intermediates of IL-17 pathway. Thus, IMP2 appears to play a role in promoting NF-κB activation in the IL-17 pathway. However, as discussed earlier, it will be important to assess the impact of IMP2 on other forms of NF-κB regulation. Although these findings suggest that IMP2 controls NF-κB activation, I have not yet determined the specific mechanism but several possibilities exist. In the IL-17 pathway, microRNAs have been identified that affect NF-κB signaling. For example, miR-23b targets TAB2, TAB3 and IKK-α, upstream regulators of NF-κB, and hence inhibits IL-17-induced NF-κB activation (100). Since IMP2 has been previously shown to compete with microRNAs to stabilize mRNA transcripts, one can speculate that IMP2 counteracts miR-23b-mediated suppression of TAB2, TAB3 and IKK-α expression. To test this possibility, expression of miR-23b target genes such as TAB2, TAB3 and IKK-α can be compared between WT and *Imp*$^{-/-}$ MEFs. However, an unbiased approach such as RNA-sequencing to comprehensively assess genes regulated by IMP2 is likely the best way to identify IMP2 targets in the IL-17 pathway. Once IMP2 targets are identified, bioinformatics analysis comparing genes affected by IMP2 and various RNA destabilizing factors that are implicated in IL-17 signaling pathway would also help delineate IMP2-mediated regulation of IL-17 signaling.

IMP2 controls post-transcriptional gene expression by affecting various aspects of RNA metabolism (191). IMP2 is known to interact with the destabilizing RBP ARE/poly(U)-binding/degradation factor 1 (AUF1) (213). Studies suggest that IL-17 inhibits expression of

AUF1, resulting in increased expression of AUF-1 target mRNAs such as inducible nitric oxide synthase (iNOS), during synergistic conditions with IFNγ and TNFα (214). Therefore, it is conceivable that IMP2 promotes mRNA stability of IL-17 target genes by interacting with AUF1 and preventing AUF-1 mediated mRNA decay. On the contrary, it is possible that IMP2 cooperates with AUF1 and degrades mRNAs of inhibitors of IL-17 signaling.

The role of Th17 cells in promoting autoimmunity has been extensively studied (33). However, comparitively little is known about how downstream IL-17R signaling mediates the inflammation associated with various autoimmune diseases, especially at the post-transcriptional level. Here, I have identified IMP2 as a novel RBP that plays a critical role in pathogenesis of IL-17-dependent models of autoimmunity such as EAE and AGN. Our data indicate that IMP2 functions downstream of IL-17 receptor and not in Th17 cells. IMP2 was undetectable in Th17 cells and IMP2 was dispensable for Th17 generation both *in vitro* and *in vivo*. Furthermore, data from bone marrow chimera experiments suggests that IMP2 primarily functions in non-hematopoietic compartment to mediate kidney damage during AGN. Therefore, these findings support the model that IMP2 functions downstream of IL-17 receptor to mediate autoimmune inflammation.

Surprisingly, IMP2 was not required for pathogenesis of all IL-17-dependent mouse models of diseases. *Imp2*$^{-/-}$ mice were resistant to acute OPC and were not protected from IMQ-driven dermatitis. These results raise the important question whether IMP2 regulates specific sets of genes which are critical for EAE and AGN pathogenesis but not for IMQ-driven dermatitis and acute OPC. These findings are consistent with previous reports that IL-6, one of the major IMP2 target genes, is required for EAE development but is dispensable during acute OPC (215, 216). Moreover, IL-6 blockade does not alleviate human psoriasis (148). Here an important

question is whether IMP2 mediates inflammation *in vivo* through IL-6. Moreover, although IMP2 is widely expressed in different tissue compartments (188), it is possible that functional properties of IMP2 is tissue-specific. Therefore, these findings suggest that IMP2 contributes to pathogenesis of some but not all IL-17-dependent diseases.

IMP2 has been implicated in regulating adipocyte metabolism by controlling expression of UCP1, a mitochondrial protein important for thermogenesis (198). Previously our lab showed that IL-17 signals in adipocytes and controls adipogenesis by regulating expression of pro-adipogenic transcription factors (217). More recently, IL-17R signaling was shown promote mitochondrial activity and glucose uptake in stromal cells such as fibroblastic reticular cells (FRCs) resulting in their proliferation and expansion (unpublished data, personal communication with Mandy McGeachy). However, the precise mechanism by which IL-17R signaling promotes cellular metabolism is not well-understood. All these findings indicate a potential role of IMP2 in controlling IL-17-induced cellular metabolism in mesenchymal cells. Thus, assessing the impact of IMP2 deficiency on IL-17-mediated metabolic activity could explain why lack of IMP2 results in reduced IL-17-dependent inflammation.

Control of post-transcriptional gene regulatory circuits is an efficient and rapid way to control inflammation (173, 218). Therefore, direct targeting of gene transcripts has been a topic of therapeutic interest in recent years. Here I have reported a previously undescribed role of the RBP IMP2 in driving IL-17R-mediated inflammation and autoimmunity. Understanding the precise molecular mechanism by which IMP2 functions will advance our knowledge of basic principles by which RBPs function during an immune response, and could help design novel therapeutics for autoimmune diseases.

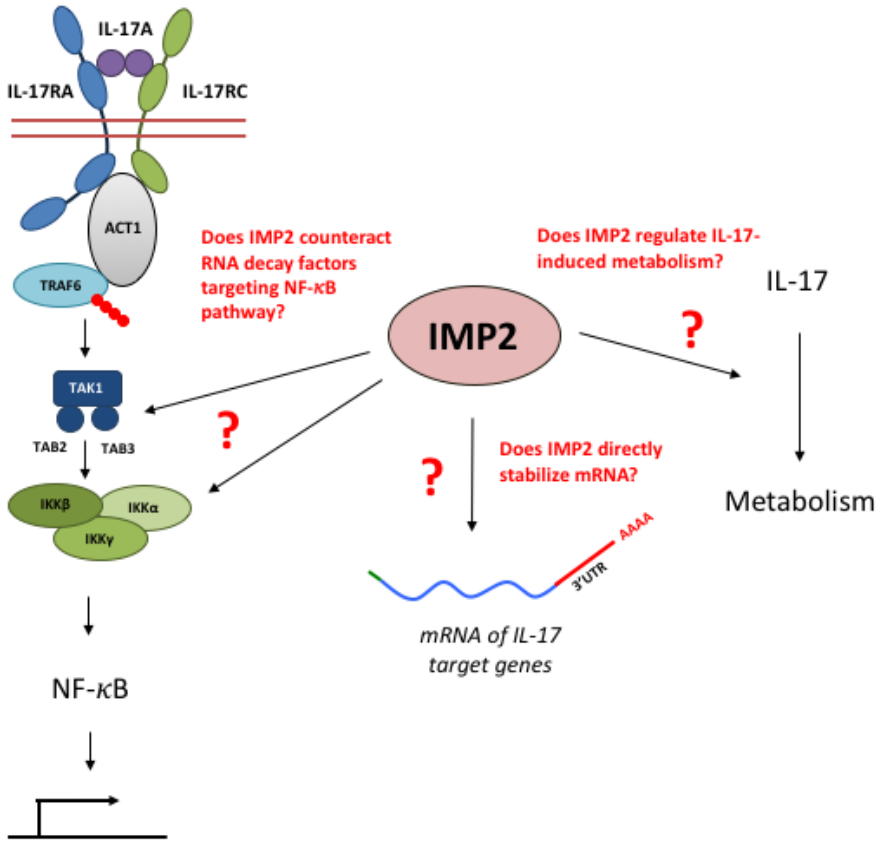


**Figure 4.14: Schematic diagram representing potential mechanisms by which IMP2 regulates IL-17-mediated inflammation.**

IMP2 enhances IL-17-mediated inflammation and autoimmunity. The direct mechanism by which IMP2 regulates IL-17 signal transduction is not unknown. This diagram represents proposed mechanisms by which IMP2 controls downstream IL-17 receptor signaling, which are discussion in details in section 4.3.

# 5.0 CONCLUSION AND FUTURE DIRECTIONS

## 5.1 SUMMARY

In this dissertation, I have identified two RBPs that promote IL-17 receptor signal transduction. In chapter 3, I demonstrated that the RBP Arid5a positively regulates IL-17R signaling. IL-17 stimulation upregulated Arid5a expression, whereupon Arid5a was rapidly recruited to TRAF2. Arid5a stabilized multiple IL-17-induced cytokine and chemokine mRNA transcripts, including *Il6, Cxcl1* and *Cxcl5*, by direct binding. In some cases, Arid5a counteracted mRNA degradation mediated by the endoribonuclease MCPIP1 (Regnase-1). Additionally, Arid5a enhanced IL-17-induced expression of the transcription factors IκBζ (*Nfkbiz*) and C/EBPβ (*Cebpb*), which could then transactivate IL-17-dependent promoters. Arid5a facilitated mRNA stability and translation of IκBζ, and induced mRNA translation of C/EBPβ. Together, these data reveal a previously undescribed role of Arid5a in promoting IL-17-mediated inflammation in non-hematopoietic cells.

In Chapter 4, I identified another RBP known as IMP2 through an RNA-seq screening on IL-17 treated ST2 cells following Arid5a knockdown. IL-17 signaling induced expression of *Imp2* mRNA in ST2 cells in an Arid5a-dependent manner. *Imp2* deficiency in primary MEFs resulted in decreased expression of IL-17-induced inflammatory effectors. Mechanistically, *Imp2*$^{-/-}$ MEFs showed impaired IL-17-induced phosphorylation of IκBα, suggesting that IMP2

regulates NF-kB activation downstream of IL-17. *Imp2*$^{-/-}$ mice were almost refractory to IL-17-induced autoimmune model of experimental autoimmune encephalomyelitis (EAE), and showed less severe experimental autoimmune glomerulonephritis (EAGN). In EAE, *Imp2* deficiency did not affect Th17 cell generation *in vivo* or *in vitro*. However, *Imp2*$^{-/-}$ spinal cords expressed lower levels of IL-17 regulated genes. In EAGN, *Imp2*$^{-/-}$ mice showed reduced kidney damage. Using radiation chimeras, I found that IMP2 functions dominantly in non-hematopoietic compartment to induce kidney damage during EAGN. These results are consistent with a model in which IMP2, a novel RBP in the IL-17 pathway, positively regulates IL-17 mediated signaling rather than affecting Th17 cell differentiation.

## 5.2 UNIFYING DISCUSSION, OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Control of gene expression is the fundamental mechanism by which all cellular processes including inflammatory responses are regulated. When the immune system is triggered by an insult during an infection or injury, cytokines and chemokines are produced by the local tissue and resident cells. These messenger molecules then further recruit other immune cells to the site of injury/infection. Efforts to understand the underlying molecular mechanisms of such immune responses have often focused on assessing changes in global gene expression in immune cells in the setting of an immune response. Hence, most of these studies regard transcription as the principle factor in influencing gene expression, whereas post-transcriptional mechanisms of gene regulation are often overlooked. Moreover, comparatively little is known about cytokine-induced post-transcriptional gene regulation in the non-immune compartment. Therefore, in this thesis, I

have assessed the post-transcriptional regulation of inflammatory responses downstream of IL-17 receptor signaling in non-hematopoietic cells mediated by RBPs Arid5a and IMP2.

IL-17 is known to cooperate with many diverse stimuli, especially TNF-α. mRNA stability has also been implicated as one of the major mechanisms that induces synergy between IL-17 and TNF-α (4). When the human lung epithelial cell line (A549 cells) is treated with IL-17 and TNFα, IL-17-induced mRNA stabilization of *Nfkbiz* (IκBζ) promotes *LCN2* expression (93). Since the data presented here indicates that Arid5a enhances *LCN2* expression in a human keratinocyte cell line, it is conceivable that Arid5a orchestrates synergy between IL-17 and TNFα through IκBζ, though this was not tested here. The interplay between Arid5a and MCPIP1 in the IL-17 pathway is another area that needs further investigation. Given that MCPIP1 also targets mRNAs of IκBζ and C/EBPβ (208, 219), it is plausible that Arid5a counteracts the degradation of these transcripts by MCPIP1 promoting their stabilization and translation. Furthermore, MCPIP1 can associate with ribosomes and degrade translationally active mRNAs including that of IκBζ (220). Hence, it would be interesting to assess whether Arid5a co-localizes with MCPIP1 within polysomes during IL-17 stimulation. Results obtained from such studies would explain why IκBζ protein is stably expressed during continuous IL-17 treatment (Fig 3.8-B).

Earlier studies showing the involvement of Arid5a in Th17-mediated autoimmunity have suggested that Arid5a stabilizes *Il6* and *Stat3* transcripts in macrophages and Th17 cells, respectively (144). Here, I have reported a previously undescribed role of Arid5a in mediating downstream IL-17 signaling. This new finding opens a series of exciting questions regarding the tissue specific roles of Arid5a. Since IL-17 mostly acts on non-hematopoietic cells, understanding whether Arid5a functions primarily in immune or non-immune compartment is

important. Similarly, IL-17 signaling is essential in mediating several autoimmune conditions. Therefore, future studies could be done to investigate the biological function of Arid5a in mouse models of imiquimod-induced dermatitis (representing psoriasis) and experimental autoimmune glomerulonephritis (representing lupus nephritis and glomerular disease). Furthermore, since Arid5a was induced upon *C. albicans* infection in an IL-17 receptor-dependent manner **(Fig 4.12-A)**, it would be intriguing to test whether Arid5a mediates protection against *C. albicans*.

While the use of biologics targeting individual immune signaling pathways have proven to be effective to treat several autoimmune conditions, not all patients respond equally to such treatment strategies (4, 221). Some patients have to undergo several rounds of treatment until the best strategy is identified. These approaches are not cost-effective and ultimately lower patient's quality of life. Therefore, drug targets that can inhibit multiple cytokine pathway at once could be desirable. Arid5a acts downstream of Th17/IL-17, LPS and IFNγ signaling pathways (144, 145, 170). Designing small molecule inhibitors that constrain the proinflammatory properties of Arid5a would be beneficial for treating inflammatory conditions where Th17/IL-17, LPS and IFNγ are all implicated. Nevertheless, small molecule inhibitors could have potential off target effects and may not be useful when specificity is needed.

An alternative approach to attain drug specificity is the use of RNA-based medications (222). Oligonucleotides, protein coding *in vitro* transcribed (IVT) mRNA and RNA interference (RNAi) are emerging classes of RNA-based drugs that have gained a lot of attention in the recent years (173, 218). Since the early 1990s, when the notion of using mRNA as a drug was first envisioned, the field of mRNA therapeutics has advanced rapidly (223). Multiple RNA-targeted drugs are already in the market, and many are being tested as vaccinations for infectious diseases or for cancer immunotherapy and are at various clinical trials stages. (173, 218). However, due to

the intrinsic unstable nature of mRNA, effective *in vivo* delivery of mRNA molecules is still a big challenge. Therefore, researchers are constantly developing new tools and techniques to effectively deliver mRNA *in vivo*. A deeper understanding of the mechanisms underlying the stabilization of mRNA, mRNA-binding proteins and their binding sequences will help advance the field of mRNA pharmacology (218). Studies like the ones presented in this thesis provide insights on how mRNAs are stabilized by RBPs such as Arid5a. Although, I have not investigated the specific Arid5a-binding elements on its target mRNA, our results may open up future opportunities to delineate cis-regulatory elements on Arid5a target transcripts. Once such sequences are determined, oligonucleotides that can inhibit Arid5a-mRNA interaction could be designed to dampen immune responses for treating inflammatory conditions. Moreover, knowledge of regulatory elements on Arid5a target transcripts may be helpful for engineering IVT mRNA that are protected from RNA-decay elements without interfering with their coding potential.

IMP2 is another RBP identified in this thesis as a highly novel IL-17 signaling intermediate. To understand the mechanism by which IMP2 promotes IL-17-mediated inflammation we are currently analyzing data obtained from RNA-seq analysis performed on primary MEFs after knocking down IMP2 in the presence and absence of IL-17. A recent study has shown that IMP2 recognizes $N^6$-methyladenosine ($m^6A$) modification on mRNA and promotes stability (224). Such modifications are naturally found in eukaryotic mRNAs, and are incorporated into IVT mRNAs to increase stability and to prevent deleterious immune-stimulatory effects of such mRNA drugs (218). Once we identify IMP2 target mRNAs in IL-17 pathway from RNA-sequencing, it would be interesting to assess whether IMP2 stabilizes these mRNA transcripts by recognizing the consensus GG($m^6A$)C sequences (224).

Another remaining question is how Arid5a regulates IMP2 expression in response to IL-17. I have found that Arid5a activates the proximal promoter of *Imp2*. However, the TFs HMGA2 and NF-κB cooperatively activate *Imp2* transcription by binding to a region located in the first intron of *Imp2.* Interestingly, this intronic region contains multiple AT-rich sites (202). Since Arid5a is known to bind to AT-rich regions on DNA, and has been shown to act as a transcriptional co-activator (141, 142), it is plausible to predict that Arid5a potentially binds to this region and acts as an enhancer to activate *Imp2* promoter. Therefore, better understanding of Arid5a-mediated regulation of *Imp2* expression is needed.

As discussed in chapter 4, *Imp2*$^{-/-}$ mice resist obesity. IMP2 reduces thermogenesis by repressing translation of uncoupling protein 1 (UCP1), a mitochondrial protein essential for thermogenesis, in adipose tissue and mice are leaner than normal (198). Previously, our lab and others have shown that IL-17 is a negative regulator of adipogenesis and glucose metabolism, and accordingly mice lacking IL-17A show increased body weight (217, 225). A recent report has comprehensively studied the role of IL-17 in regulating thermogenesis. This study has shown that IL-17 produced by γδ T cells promote thermogenesis in adipose tissue, and mice lacking γδ T cells or IL-17A have lower levels of UCP1 (226). This implies that downstream IL-17 signaling could directly contribute to regulation of thermogenesis. Therefore, we have crossed *Il17ra*$^{-/-}$ mice with *Imp2*$^{-/-}$ mice to determine whether *Imp2* deficiency reverses the over-weight phenotype of *Il17ra*$^{-/-}$ mice. We are currently evaluating weight change over time of *Il17ra*$^{-/-}$ *Imp2*$^{-/-}$ mice and comparing it to that of *Il17ra*$^{-/-}$ mice. Results obtained from this study would provide new insights into the role of IL-17 receptor signaling in regulating thermogenesis and metabolism and set the stage for future studies.

Identification of transcripts targeted by RBP Arid5a broadens the spectrum of genes regulated by IL-17 at the post-transcriptional level. Although one goal of exploring signaling intermediates is to identify drug targets for patient benefit, such studies are equally important to appreciate the basic fundamentals of cytokine biology. Since IL-17 shares many signaling intermediates with other immune pathways (4), principles presented here likely apply to other signal transduction pathways/system.

## 5.3 CONCLUDING REMARKS

The work presented in this dissertation has expanded our current understanding of molecular mechanisms underlying IL-17-mediated signaling and inflammation. Findings presented here have uncovered new RBPs in the IL-17 pathway that controls mRNA stability and translation through diverse post-transcriptional mechanisms. The novel function of Arid5a as an RBP controlling inflammatory processes was reported very recently in immune cells (144). Here, we have extended the role of Arid5a in regulating downstream IL-17 signaling in non-hematopoietic compartment. Furthermore, we have made a comprehensive assessment of genes regulated by Arid5a. Identification of IMP2, as an Arid5a regulated IL-17 induced RBP has been an exciting discovery. To the best of our knowledge, this is the first study describing the role of IMP2 in promoting autoimmunity. In conclusion, post-transcriptional control of gene expression is an essential component of IL-17 signaling and has a promising future from a therapeutic perspective.

# APPENDIX

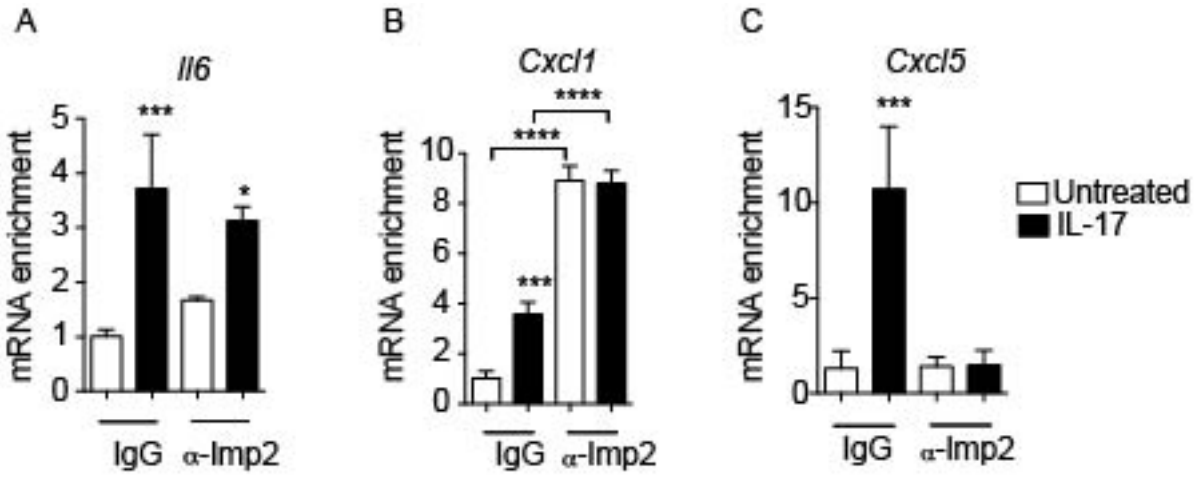


**Figure A.1: IMP2 does not bind to Il6, Cxcl1 and Cxcl5 transcripts.**

(**A** to **C**) qRT-PCR analysis of *Il6*, *Cxcl1* and *Cxcl5* from primary MEFs treated with IL-17 for 24 hours, and subjected to RIP with IgG or anti-IMP2. Data are fold-change means ± SEM representative of 1 experiment.

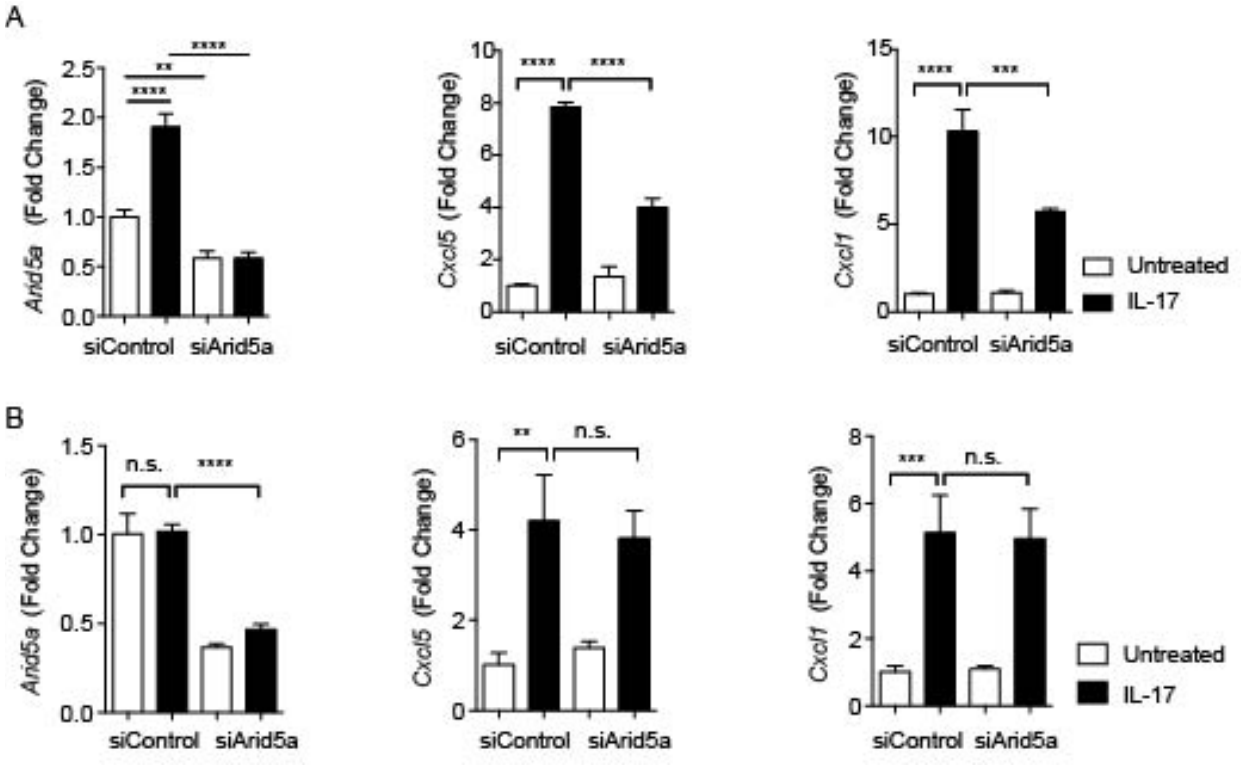


**Figure A.2: Arid5a-mediated regulation of Cxcl1 and Cxcl5 in ST2 cells.**

qRT-PCR analysis of the indicated genes in ST2 cells transfected with pooled siRNAs targeting Arid5a or scrambled control and treated with IL-17 for 3 h. Fold-change data are means ± SEM from 3 technical replicates within an experiment. **(A)** *Arid5a* knockdown efficiency ~ 70%, **(B)** *Arid5a* knockdown efficiency ~ 50%.

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