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IL-17 integrates multiple self-reinforcing, feed-forward mechanisms through the RNA-binding protein Arid5a

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Abstract

Interleukin-17A stimulates immunity to fungal pathogens, but also contributes to autoimmune pathology. IL-17 is only a modest activator of transcription in experimental tissue culture settings. However, IL-17 controls post-transcriptional events that enhance the expression of target mRNAs. Here, we showed that the RNA-binding protein (RBP) Arid5a (AT-rich interactive domain-containing protein 5a) integrated multiple IL-17-driven signaling pathways through post-transcriptional control of mRNA. IL-17 induced expression of Arid5a, which was recruited to the adaptor TRAF2. Arid5a stabilized IL-17-induced cytokine transcripts by binding to their 3' untranslated regions and also counteracted mRNA degradation mediated by the endoribonuclease MCPIP1 (Regnase-1). Arid5a inducibly associated with the eukaryotic translation initiation complex and facilitated the translation of the transcription factors (TFs) IκBξ (*Nfkbiz*) and C/EBPβ (*Cebpb*). These TFs in turn transactivated IL-17-dependent promoters. Together these data indicated that Arid5a orchestrates a feed-forward amplification loop, which promoted IL-17 signaling by controlling mRNA stability and translation.

Introduction

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* (1). IL-17 also drives immunopathology in autoimmune and chronic inflammatory conditions (2). 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

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(2). Therefore, understanding the mechanisms by which IL-17 functions has important clinical implications.

IL-17 is produced by Th17 cells and innate lymphocytes (3) and signals through a dimeric receptor composed of IL-17RA and IL-17RC (4). 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 kinase (MAPK) cascades. 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 (4, 5). 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 (4). 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 (6).

IL-17 signaling in culture systems is typically modest compared to potent inflammatory stimuli such as tumor necrosis factor α (TNFα) (2, 7). 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*) (8). 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 synergy is not fully understood, but is mediated in part through cooperative activation of IL-17-induced TFs that activate downstream genes (9, 10). For example, IL-17 induces IκBξ (NF-κB Inhibitor ξ, encoded by *Nfkbiz*) (11), an atypical member of the NF-κB family that facilitates transcription of several IL-17-dependent genes in cooperation with TNFα (12-14). Similarly, C/EBP family members are implicated in functional cooperativity between IL-17 and TNFα (7, 11, 15).

Additionally, IL-17 promotes stabilization of mRNA (16). Many IL-17-induced transcripts possess AU-rich elements (AREs) or other stability-determining sequences in the 3' untranslated region (UTR) (17). IL-17 promotes mRNA stabilization of numerous genes, including *Il6*, C-X-C motif Chemokine Ligand 1 (*Cxcl1*) and *Cxcl5*, among others (16). 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) (18-20). 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*) (15, 21). IL-17 induces *Zc3h12a* expression and promotes its mRNA stability through DDX3X (20, 22). MCPIP1 mediates 3'UTR-mediated mRNA decay of *Il6, Nfkbiz* and other transcripts and thus is a feedback inhibitor of IL-17 signaling (22). 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.

Here, we showed that the RBP AT-rich interactive domain-containing protein 5A (Arid5a) promoted IL-17 signaling. 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 certain IL-17-dependent genes (e.g., *Lcn2*) without impacting mRNA stability. This observation led us to interrogate the impact of Arid5a on IL-17-induced TFs, specifically C/EBPβ and IκBξ. Arid5a stabilized mRNA encoding *Nfkbiz* but not *Cebpb*. Rather, Arid5a strongly enhanced IL-17-induced translation of C/EBPβ and IκBξ proteins, revealing its potential to control translational circuitry.

RESULTS

IL-17 increases Arid5a expression and promotes its association with TRAF2

To identify RBPs that might participate in IL-17 signaling cascades, we screened for IL-17-dependent genes encoding RBPs that were induced during a strongly IL-17-dependent immune response (23), specifically 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 (Fig 1A). This finding paralleled our observations with another RBP, *Zc3h12a* (encoding MCPIP1), which is induced by IL-17 in the tongue during OPC (22). Similarly, IL-17 stimulated the expression of *Arid5a* in the IL-17-responsive stromal cell line, ST2 (Fig 1B) (22). IL-17-induced *Arid5a* expression was blocked by an NF-κB inhibitor, suggesting that its expression is NF-κB-dependent (Fig S1A).

Some IL-17-dependent pathways that lead to mRNA stabilization are initiated through Act1, TRAF2 and TRAF5 (18-20). To test the hypothesis that Arid5a uses similar proximal signaling intermediates, we 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 did not co-IP with Act1 but did associate with TRAF2 (Fig 1C, S1B). To determine if the TRAF2-Arid5a association is IL-17-dependent, ST2 cells were transfected with Flag/Myc-Arid5a, stimulated with IL-17 for 2 h and lysates subjected to IP with Myc Abs. Endogenous TRAF2 co-immunoprecipitated with Arid5a, and the TRAF2-Arid5a interaction peaked at ~15-60 minutes post IL-17 treatment (Fig 1D, Fig S2E). Together, these data show that IL-17 enhances Arid5a expression, which inducibly associates with TRAF2.

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%, Fig S1C, S1D). Cells were treated with IL-17 for 3 h, and expression of prototypical IL-17 target genes was assessed. Knockdown of Arid5a suppressed IL-17-mediated induction of *Il6*

mRNA and secreted IL-6 protein (Fig 2A, Fig S3A). Similar results were obtained for other canonical IL-17 target genes, including CXCL1 and CXCL5 (Fig 2A, Fig S3A). However, not all IL-17-driven genes were detectably regulated by Arid5a, such as *Ccl20* and *Csf2* (Fig 2A). Together, these data show that Arid5a is required for the expression of a subset of IL-17 target genes.

Because Arid5a associated with TRAF2 (Fig 1C-D), we 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, Fig S1E). 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 (Fig 2B). These data are consistent with a model in which Arid5a and TRAF2 act in the same pathway.

TRAF2 promotes mRNA stabilization in the IL-17 pathway through the RBP HuR (encoded by the *Elavll* gene) (19). Double knockdown of Arid5a and HuR did not change or only marginally decreased IL-17-induced expression of *Il6* or *Cxcll* compared to knockdown of Arid5a or HuR alone (Fig S2A). Knockdown efficiency was typically ~60% for Arid5a and >95% for HuR (Fig S2B). These results suggest that Arid5a and HuR are likely to function in the same pathway in IL-17 signal transduction. However, in contrast to Arid5a, IL-17 did not induce expression of HuR (*Elavl*) mRNA (Fig S2C).

In the IL-17 and other inflammatory signaling pathways, competition among RBPs helps determine the overall amount of target mRNA transcripts (4, 18, 19). IL-17 induces the endoribonuclease MCPIP1, which remains elevated during prolonged stimulation and binds to a similar RNA binding site as Arid5a (22, 24). Because the net effect of IL-17 is to enhance expression of mRNAs that can be inhibited by MCPIP1, *e.g, Il6* or *Lcn2* (Fig 2C), 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, we knocked down Arid5a and MCPIP1 together (efficiency ~80%, Fig S1F) and assessed *Il6* and *Lcn2* expression. As reported, MCPIP1 knockdown enhanced IL-17-induced expression of *Il6* and *Lcn2* mRNA (Fig 2D) and IL-6 protein (Fig S3B), but knockdown of Arid5a partially offset the inhibitory effect of MCPIP1 deficiency (Fig 2D, Fig S3B). Knockdown of Arid5a also suppressed IL-17-induced the abundance of LCN2 protein (Fig S3A). Therefore, Arid5a counteracts the activity of MCPIP1 stimulated by IL-17.

IL-17 increases *Il6* expression both through acting on its proximal promoter and also by stabilizing its mRNA transcript (7, 25, 26). However, we 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 (Fig S1G). To determine whether Arid5a promoted IL-17-dependent stability of *Il6* mRNA, ST2 cells were transfected with siRNAs against Arid5a and primed with TNFα for 3 h 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, we assessed the half-life ($ty_{½}$) of 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 decay of *Il6* transcripts (26), but *Il6* stabilization was

impaired upon Arid5a knockdown (Fig 2E, Fig S8A). Arid5a similarly stabilized *Cxcll* and *Cxcl5* transcripts (Fig 2E, Fig S3C, Fig S8B). When we co-transfected HEK293T cells with Arid5a and a luciferase reporter fused to the *Il6* 3'UTR (27), expression of Arid5a increased luciferase activity (Fig 2F). Control expression of either Act1 enhanced *Il6* 3'UTR reporter activity whereas MCPIP1 suppressed activity, as expected (28, 29). Thus, these data suggest that Arid5a stabilizes IL-17 target mRNA transcripts encoding inflammatory cytokines and chemokines through interaction with transcript 3' UTRs.

Arid5a promotes translation of C/EBPβ and IκBξ

Unlike *Il6* transcripts, IL-17 does not alter the mRNA stability of *Lcn2* (6). Moreover, Arid5a knockdown did not increase the half-life of *Lcn2* mRNA (Fig S3E). Even so, when Flag/Myc-Arid5a was co-expressed with a luciferase reporter driven by the mouse *Lcn2* promoter (6), luciferase activity was significantly increased (Fig 3A). 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 (6, 10), and Arid5a failed to activate a *Lcn2* promoter with a C/EBP binding site mutation (Fig 3A) (6). In response to IL-17, *Cebpb* expression increased 2-4-fold (Fig 3B), consistent with prior observations (7, 30-32) 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 (Fig 3C). By western blot, we 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 (30). We found that silencing of Arid5a strongly inhibited IL-17-induced expression of all C/EBPβ protein isoforms (Fig 3D). However, TRAF2 was dispensable for IL-17-induced expression of C/EBPβ (Fig S2D). Furthermore, neither IL-17 stimulation nor Arid5a knockdown affected the mRNA stability of *Cebpb* transcripts (Fig S3D). Thus, Arid5a appears to promote C/EBPβ protein abundance but not mRNA expression.

These data suggested that Arid5a may increase C/EBPβ translation. Therefore, we employed RNA-immunoprecipitation (RIP) assays to determine whether Arid5a affects the occupancy of *Cebpb* within the eukaryotic eIF4F translation initiation complex (Fig 3E) (33). 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 (34). We found that *Cebpb* transcripts were enriched in the eIF4G IP fraction after IL-17 stimulation, consistent with increased translation (Fig 3E, Fig S7A). Similarly, *Il6* and *Cxcl1* transcripts were also enriched, which was expected because their corresponding proteins are increased in abundance in conditioned media following IL-17 stimulation (Fig S5A, Fig S7C). Moreover, Arid5a knockdown impaired enrichment of *Cebpb, Il6* and *Cxcl1* transcripts in the eIF4F complex, supporting a role for Arid5a in promoting translation of C/EBPβ and other target proteins. Total *Cebpb* mRNAs in the cytoplasmic lysate used as the input for RIP were not affected by Arid5a knockdown (Fig S5B). To determine whether Arid5a itself interacts with the translation initiation complex, we transfected ST2 cells with Flag/Myc-Arid5a and stimulated these cells with IL-17 for 2 h. When lysates were immunoprecipitated with Myc

Abs, we found that endogenous eIF4G co-immunoprecipitated with Arid5a in an IL-17-dependent manner (Fig 3F, Fig S2F). These data indicate that Arid5a promotes IL-17-induced translation of C/EBPβ by interacting with eIF4F translation initiation complex.

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 IκBξ (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 (6, 10, 15). Arid5a did not activate a luciferase construct driven by the *Lcn2* promoter mutated at its NF-κB binding site (Fig 3A), which suggested that Arid5a may enhance expression of IκBξ. We found that IL-17 enhanced expression of IκBξ both at mRNA and protein levels (Fig 4A-B), consistent with prior reports (15, 22, 35, 36). Arid5a silencing only modestly inhibited IL-17-induced mRNA expression of *Nfkbiz* in ST2 cells and had no effect in primary MEFs (Fig 4C-D). In contrast to *Cebpb* transcripts, Arid5a knockdown impaired IL-17- induced stabilization of *Nfkbiz* mRNA (Fig 4F, Fig S8C). However, Arid5a knockdown reduced IL-17-induced IκBξ protein abundance in both ST2 cells and MEFs (Fig 4C-D, Fig S4A-B). Consistently, we found that co-expression of Arid5a with IκBξ in HEK293T cells increased IκBξ abundance (Fig 4E), and TRAF2 was not required for IL-17-induced expression of IκBξ (Fig S2D). As with *Cebpb* transcripts, *Nfkbiz* mRNA was less abundant in eIF4G RIP fractions following Arid5a knockdown, which suggested that Arid5a promotes *Nfkbiz* translation (Fig 4G, Fig S4B, Fig S7B). Thus, our results showed Arid5a enhanced both mRNA stability and translation of IκBξ. Collectively, these data suggest that Arid5a indirectly stimulates target genes that are activated by the IL-17-inducible TFs C/EBPβ and IκBξ.

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. We found that the association of *Il6, Cxcl1, Cxcl5, Nfkbiz* and *Cebpb*, transcripts with Arid5a was increased in cytoplasmic fractions after IL-17stimulation (Fig 5A, Fig S6). These transcripts were not enriched in control or nuclear fractions (Fig S5C-H), consistent with the fact that posttranscriptional control of mRNA takes place in the cytoplasm (17). In contrast, *Ccl20 and Csf2* transcripts were associated with Arid5a, which agrees with our earlier results that Arid5a knockdown does not affect IL-17-dependent expression of these genes (Fig 5B, Fig S6, see also Fig 2A). 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, we 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 (Fig 5C). 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 (Fig 5C). Thus, Arid5a stabilizes IL-17-induced target mRNAs by binding directly to the 3'UTR.

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, we also assessed Arid5a function by siRNA knockdown in primary murine MEFs and N/TERT2G immortalized human keratinocytes (KC) (37). N/TERT2G cells maintain normal KC differentiation patterns and are considered to be a good representation of primary human KCs (38). In MEF cells Arid5a deficiency also decreased IL-17-induced expression of *Il6, Cxcl1* and *Lcn2* mRNA, and IL-6 and CXCL1 protein abundance (Fig 6, **A** and **B**). Similarly, silencing of Arid5a in N/TERT2G cells impaired expression of human *LCN2* and *CXCL1* stimulated by IL-17 (Fig 6C). Thus, Arid5a promotes IL-17 responses in multiple cells, including primary murine and human cell types.

DISCUSSION

IL-17 signaling dominantly occurs in epithelial and mesenchymal cell types (39), 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, we identified the RNA binding protein Arid5a as a driver of cellular responses to IL-17 in mouse mesenchymal cells and in human keratinocytes. Arid5a bound directly to the 3' UTR of multiple target mRNAs and stabilized IL-17-induced mRNA transcripts encoding IL-6 and CXC chemokines. These activities are similar to those described for Arid5a in the TLR4 signaling pathway (24, 40). Additionally, we observed that Arid5a facilitated the translation of two IL-17-dependent transcription factors, C/EBPβ and IκBξ, which allowed for increased expression of genes reliant on these TFs such as *Lcn2*. Thus, in these capacities, Arid5a promotes IL-17-driven immunity.

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 (25, 26, 41-43). For example, IL-17 does not stabilize target mRNAs through the commonly-used RBP tristetraprolin and or the adaptor TRAF6 (19, 43-45). Rather, RBPs such as HuR, DDX3X and Act1 serve to stabilize IL-17-dependent transcripts in a pathway initiated by TRAF2 and TRAF5 (19, 20, 28). Typically, the RNA-stabilizing activity of these RBPs is offset by de-stabilizing RBPs *e.g.*, SF2 (18, 19), which allows 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 (21, 22). In the setting of LPS signaling in macrophages, Arid5a and MCPIP1 bind to an overlapping RNA binding sequence on the *Il6* 3'UTR (24, 46). Nonetheless, in the IL-17 pathway, not all the targets of Arid5a and MCPIP1 were identical (e.g., *Ccl20*, which was strongly affected by MCPIP1 but not detectably altered 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 (24), an IL-17-dependent model of autoimmunity (47). Mice lacking either IL-17A or Arid5a show similar resistance to bleomycininduced lung injury (48-50). Additionally, Arid5a is linked to Th17 cell differentiation through stabilization of *Il6, Stat3* and *Ox40* in APCs or T cells (24, 40, 51, 52), and thus may enhance IL-17-dependent responses by virtue of increasing IL-17 expression in the inflamed environment.

IL-17 acts almost exclusively on non-hematopoietic cells (39), so factors such as Arid5a or MCPIP1 that target both Th17 cells and IL-17 signaling could potentially function in one or both contexts (21, 22). 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 (53). 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 IκBξ, which play vital roles in IL-17-driven disease. For example, *Arid5a*$^{-/-}$, Cebpb$^{-/-}$ and *Nfkbiz*$^{-/-}$ mice are all refractory to EAE (24, 54, 55), and *Nfkbiz*$^{-/-}$ mice are also resistant to imiquimodinduced inflammation, a model representing psoriasis-like IL-17-dependent skin inflammation (35). Once induced, C/EBPβ and IκBξ promote expression of genes encoded by mRNAs that are not themselves intrinsically unstable, such as the canonical IL-17 target gene *Lcn2* (6). IL-17 enhanced *Cebpb* and *Nfkbiz* mRNA, albeit modestly (7, 56), whereas concomitant protein expression of both TFs was profoundly increased. However, the mechanisms underlying translational control of these proteins have been elusive (30, 32, 36, 57). We observed that Arid5a associated with the translation initiation complex and that Arid5a knockdown impaired translation of both C/EBPβ and IκBξ. Even though TRAF2 inducibly associated with Arid5a and is implicated in promoting HuR-induced stabilization in the IL-17 pathway (19, 28), TRAF2 was dispensable for promoting translation of these TFs. Consistently, TRAF2 is not found in translationally-active polysomes following IL-17 stimulation (19). Accordingly, Arid5a appears to direct mRNA translation and mRNA stabilization by differing mechanisms. Arid5a is highly expressed in the nucleus, but TLR4 signaling induces its translocation to the cytoplasm (58). Although it is not known if IL-17 similarly alters Arid5a subcellular localization, Arid5a occupancy with its target mRNAs occurred only in cytoplasmic, not nuclear extracts. Collectively, these findings show that by inducing expression of these key TFs, Arid5a amplifies the IL-17-driven signaling program.

Blocking IL-17 is remarkably effective in treating psoriasis and is under evaluation for other autoimmune conditions (59, 60). Hence, defining the molecular basis of IL-17 signal transduction may inform therapeutic strategies for diseases where IL-17 is implicated (61). 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 (62). For example, oligonucleotide “aptamers” representing an Act1 recognition site in the *Cxcl1* 3’UTR were effective in pre-clinical models of autoimmunity (28). Arid5a was reported to be a target of the anti-psychotic drug chlorpromazine (10), 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.

Materials and METHODS

Cell culture

ST2, HEK293T and mouse embryonic fibroblasts (MEFs) were cultured in α-MEM (Sigma Aldrich, St. Louis MO) with L-glutamine, antibiotics and 10% FBS (11). N/TERT2G cells were cultured in KC-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. Murine IL-17 was used at 200 ng/mL, human IL-17 at 100 ng/mL and murine TNFα at 5μg/mL (Peprotech, Rocky Hill, NJ). The IKK inhibitor VII (EMD Millipore, Burlington MA) was used at 10 μM, and Actinomycin D (Sigma, St Louis MO) at 5μg/mL.

Mice

Oral candidiasis was performed by sublingual inoculation of *C. albicans* (CAF2-1) for 75 min under anesthesia (23). RNA was prepared from tongue after dissociation on a GentleMACS (Miltenyi Biotec, Cambridge MA) with M-tubes. Mice were age-matched on the C57BL/6 background and both sexes were used. WT mice were from The Jackson Laboratory (Bar Harbor, ME). *Il17ra*$^{-/-}$ mice were from Amgen. 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.

siRNA, plasmids and qPCR

ON-TARGETplus SMARTpool siRNAs targeting Arid5a, HuR, TRAF2 and MCPIP1 were from Dharmacon (Lafayette, CO). For RNA silencing, ST2 cells and MEFs were seeded overnight in antibiotic-free α-MEM, and N/TERT2G cells were seeded in KC-serum free media (Gibco) with supplements. Transfection was performed 18 h later with 50nM siRNAs with DharmaFECT Reagent 1. Culture media was replaced after 24 h, and IL-17 administered 24 h later. Flag/Myc- Arid5a was from OriGene (Rockville MD). All other constructs were described (6, 22, 27, 63). Transfections of ST2 cells and MEFS were performed with Fugene HD (Promega, MadisonWI) or $CaPO_4$ (HEK293T cells). RNA was prepared with RNeasy Kits (Qiagen, Valencia CA). cDNA was synthesized by Superscript III First Strand Kits (ThermoFisher, Waltham MA). Quantitative real-time PCR (qPCR) was performed with the SYBR Green FastMix (Quanta Biosciences, Beverly MA) and analyzed on a 7300 ABI Real Time instrument. Primers were from QuantiTect Primer Assays (Qiagen).

ELISA, antibodies, IP and Luciferase assays

ELISA kits were from eBioscience (ThermoFisher). Antibodies used for western blots and coimmunoprecipitation were: Arid5a, FLAG (Sigma Aldrich), β actin-HRP, α Tubulin-HRP (Abcam, Cambridge UK), IκBξ, Myc (Cell Signaling, Beverly MA), C/EBPβ (BioLegend, San Diego CA), IκBα, MCPIP1, TRAF2, Act1, YY1 (Santa Cruz Biotechnology, Santa Cruz CA). Protein G or Protein A beads (Roche) were used to pull-down antibodies during

co-immunoprecipitation. Luciferase assays used the Dual-Luciferase Reporter Assay System (Promega) (6).

RNA immunoprecipitation and Biotinylated-RNA pulldown

Cytoplasmic extracts were isolated with polysome lysis buffer for Myc RIP (100mM KCL, 5mM $MgCl_2$, 10mM Hepes pH 7.0, 0.5% NP-40, 1mM DTT) with 100 U/mL RNAse Out (Invitrogen) or CHAPS lysis buffer for eIF4G RIP (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 extracts were isolated as described (64). Lysates were pre-cleared with isotype control Abs (Abcam) and Protein G agarose beads (Roche) or Protein G-conjugated magnetic dynabeads for eIF4G RIP (Life Technologies), and immunoprecipitated with Myc-or eIF4G Abs (Cell Signaling) (34). Immunoprecipitates were incubated with Protein G beads or Protein Gconjugated magnetic dynabeads for eIF4G RIP. Total RNA was extracted with acid phenol. Biotinylation: 3'UTR motifs from *Il6, Cxcl1, Csf2* (GeneArt, Thermo Scientific) were subcloned in pCR2.1. RNA was synthesized with biotinylated CTP (Enzo Life Sciences, Farmingdale NY) in a TranscriptAid T7 High Yield Transcription Kit (ThermoFisher) and mixed with lysates from HEK293T cells transfected with Flag-MycArid5a, followed by streptavidin Dynabeads M-280 (ThermoFisher). Fractions were isolated by magnetic separation.

Statistics

ANOVA with post hoc Tukey's analysis or Student's *t* test was used to assess statistical significance, with $P<0.05$ considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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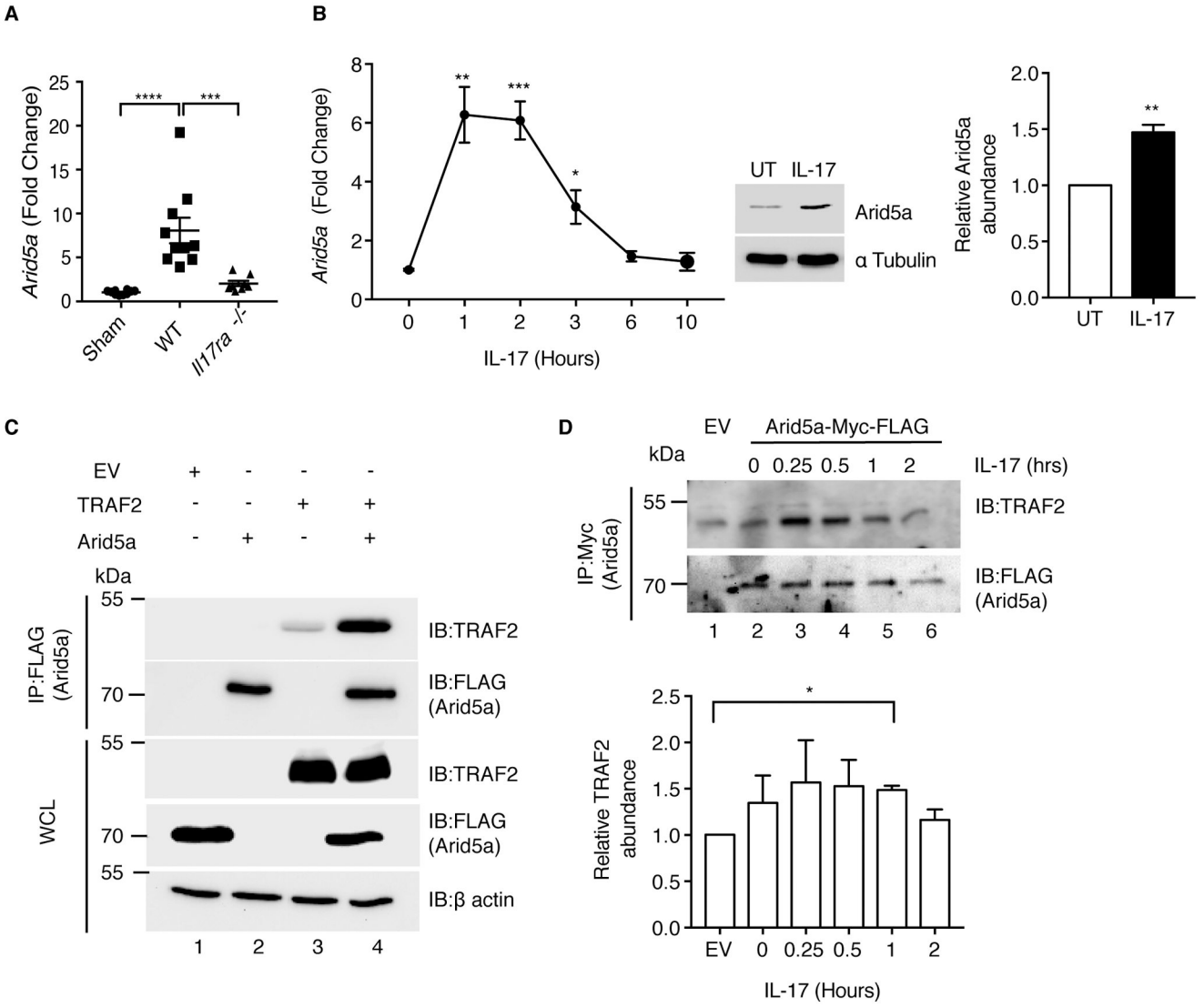

Figure 1. IL-17 increases the abundance of Arid5a, which inducibly associates with TRAF2.

(**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. Quantified band intensity values are means ± SEM from all experiments. (**C**) 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 (fig. S1B). (**D**) 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 (fig. S2E). 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), Dunnett's test (B, left), or paired Student t-test (B, right).

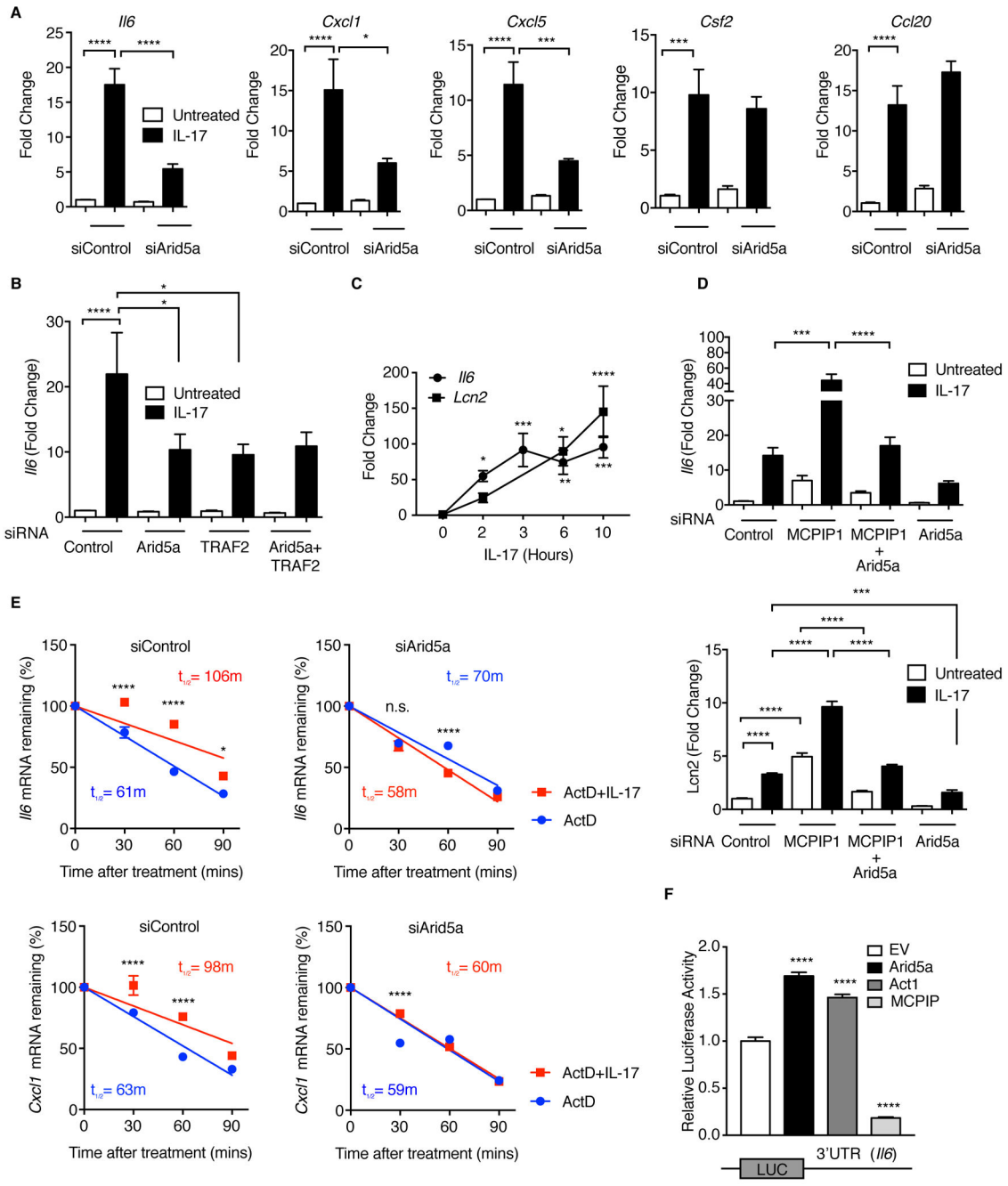

Figure 2. Arid5a promotes cellular responses to IL-17.

(**A**) qRT-PCR analysis of the indicated mRNAs 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. (**B**) 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. (**C**) 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. (**D**) qRT-PCR analysis of *Il6* or *Lcn2* 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. (**E**) qRT-PCR analysis of *Il6* or *Cxcll* 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. (**F**) Luciferase assay of *Il6* 3'UTR activity in HEK293T cells at 24 hours after transfect with a luciferase reporter and empty vector (EV), Flag/Myc-Arid5a, Act1-Myc, or MCPIP1 and analyzed after 24 h. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test (A, B, D and F) or Dunnett's test (C); half-lives ($t_{½}$) were determined using equations that defined decay kinetics as shown by colored lines in the graph, as described (42) (E).

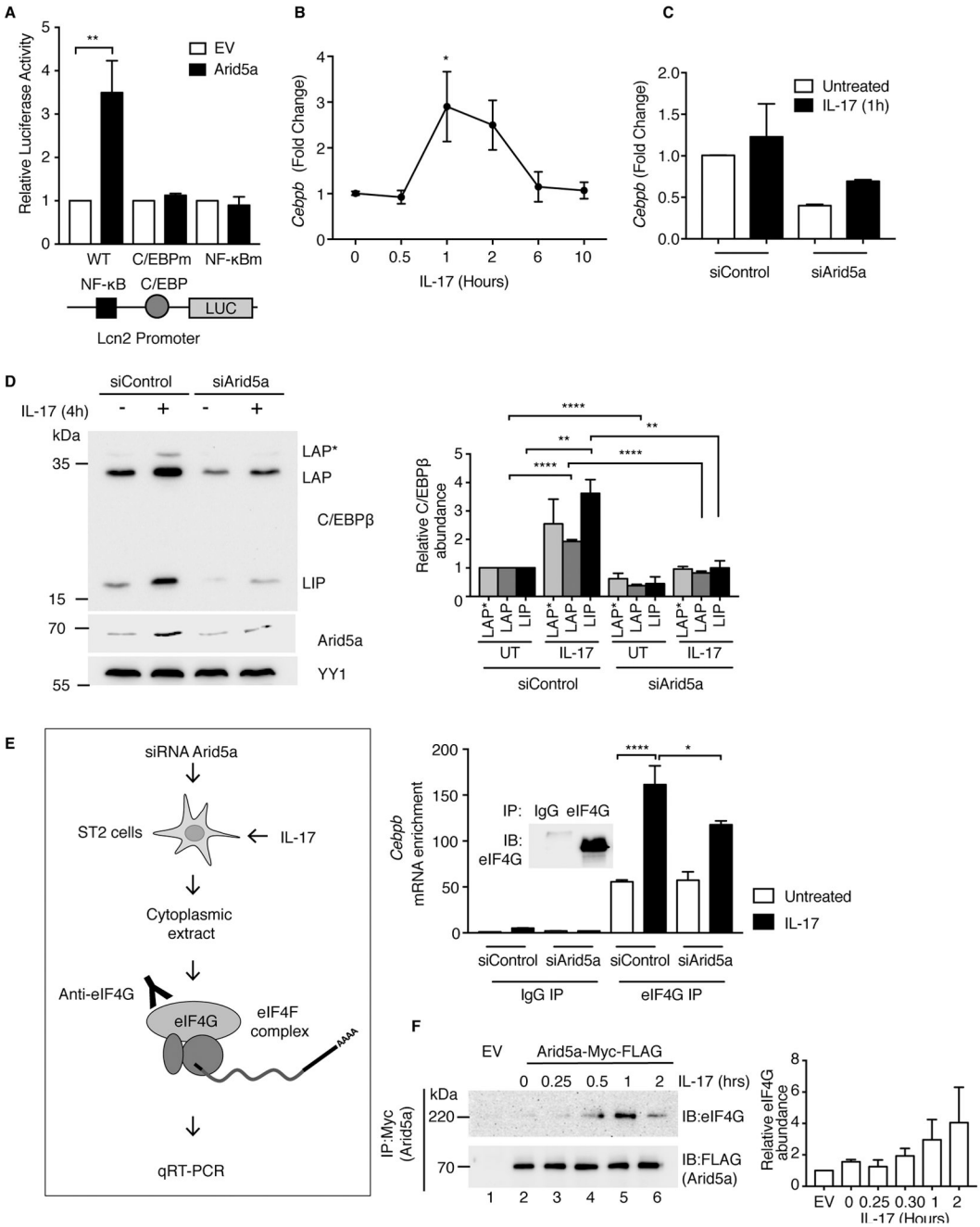

Figure 3. Arid5a mediates translation of C/EBPβ mRNA.

(**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 4 h. Blots are representative of 3 independent experiments. Quantified band intensity values are means ± SEM from all experiments. (**E**) RIP assay (left) 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. (**F**) 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 (fig. S2F). 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 (A, C to F) or Dunnett's test (B).

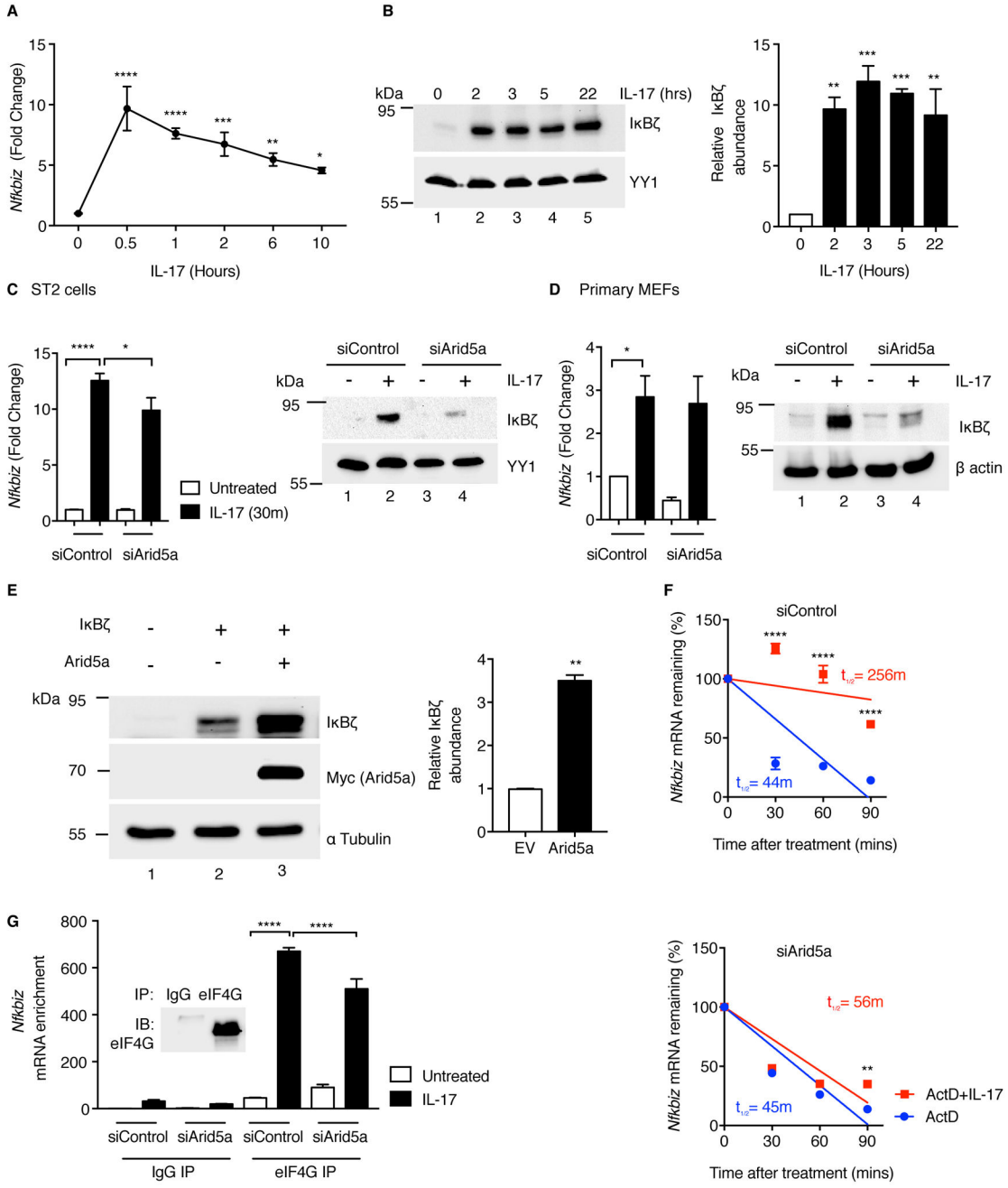

Figure 4. Arid5a mediates translation of IκBξ.

(**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 IκBξ 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 IκBξ 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 IκBξ 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 IκBξ and Myc-tagged Arid5a in lysates from HEK293T cells transfected with empty vector (EV), IκBξ 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**) 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. 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 (42) (E).

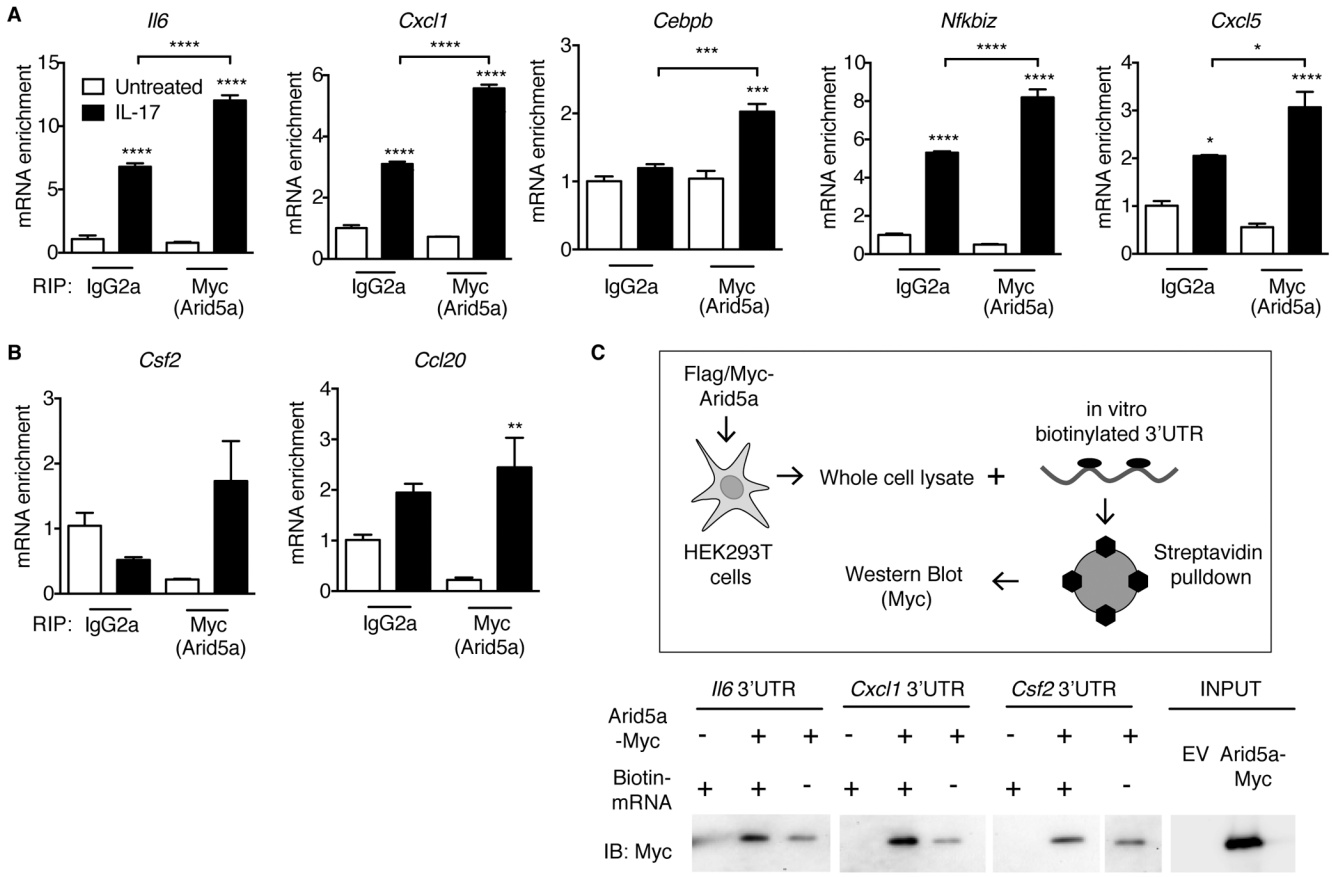

Figure 5. Arid5a binds directly to target mRNA transcripts.

(**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 *Cxcll* and *Csf2*. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001 by ANOVA with post hoc Tukey's test.

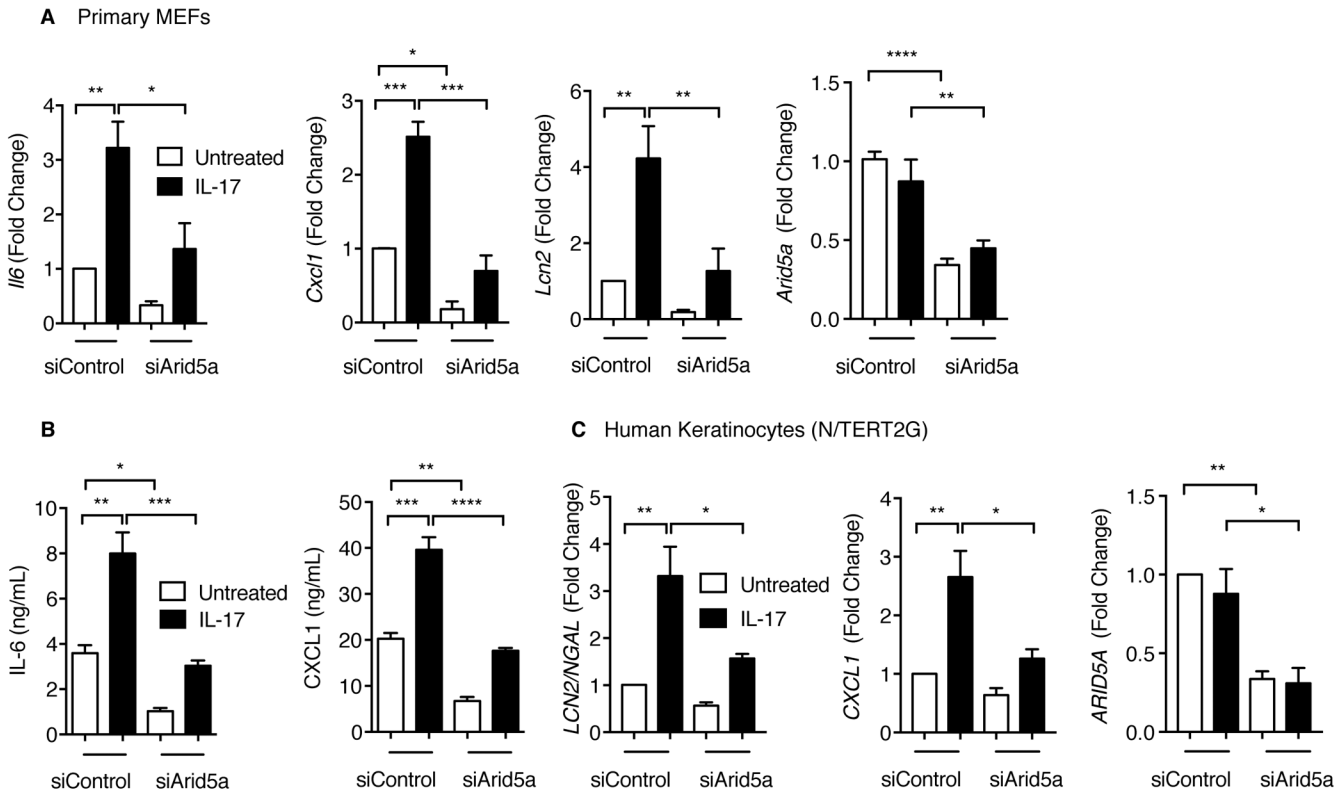

Figure 6. Arid5a promotes responses to IL-17 in primary MEFs and human keratinocytes.

(**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.

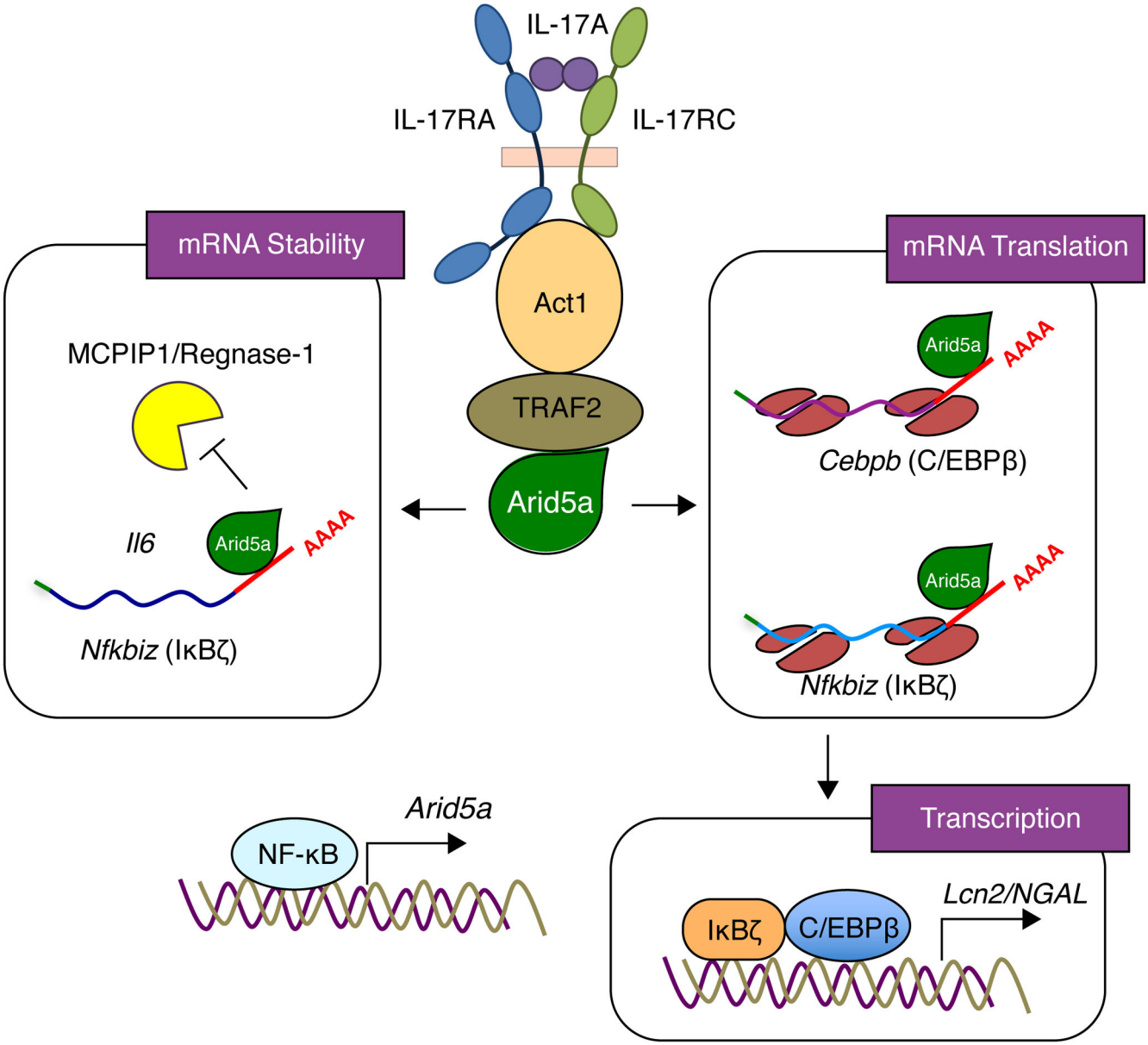

Figure 7. Model of Arid5a in the IL-17R signaling pathway.

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* (IκBξ). 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.