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BATF-JUN is critical for IRF4-mediated transcription in T cells

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Abstract

Interferon regulatory factor 4 (IRF4) is an IRF family transcription factor with critical roles in lymphoid development and in regulating the immune response[1,2]. IRF4 binds DNA weakly due to a C-terminal auto-inhibitory domain, but cooperative binding with factors such as PU.1 or SPIB in B cells increases binding affinity[3], allowing IRF4 to regulate genes containing ETS/IRF composite elements (EICEs; 5′-GGAAnnGAAA-3′)[1]. Here, we show that in $CD4^+$ T cells, where PU.1/SPIB expression is low, and in B cells, where PU.1 is well expressed, IRF4 unexpectedly can cooperate with Activator Protein-1 (AP-1) complexes to bind to AP-1/IRF4 composite (TGAnTCA/GAAA) motifs that we denote as AP-1/IRF composite elements (AICEs). Moreover, BATF/Jun family protein complexes cooperate with IRF4 in binding to AICEs in pre-activated $CD4^+$ T cells stimulated with IL-21 and in Th17 differentiated cells. Importantly, BATF binding was diminished in *Irf4*$^{-/-}$ T cells and IRF4 binding was diminished in *Batf*$^{-/-}$ T cells, consistent with functional cooperation between these factors. Moreover, we show that AP-1 and IRF complexes cooperatively promote transcription of the *Il10* gene, which is expressed in Th17 cells and potently regulated by IL-21. These findings reveal that IRF4 can signal via complexes containing ETS or AP-1 motifs depending on the cellular context, thus indicating new approaches for modulating IRF4-dependent transcription.

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Author Contributions: P.L. designed experiments, analyzed data, and wrote the paper. R.S. designed and performed experiments, analyzed data, and wrote the paper. W.L. and L.W. designed and performed experiments and analyzed data. T.L.M. and K.M.M. provided reagents and made valuable suggestions. W.J.L. designed experiments, analyzed data, and wrote the paper.

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Full methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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There are nine mammalian IRF family members, IRF1 to IRF9[1], that collectively exhibit broad actions within and beyond the immune system[2]. IRFs were identified based on their induction by type I interferons (IFNα/β), and some IRFs are induced by Toll-like receptors and other pattern recognition receptors[1,2]. IRF4 expression is restricted to the immune system and is induced in T cells by T-cell receptor stimulation[4,5]. IRFs contain an N-terminal DNA-binding domain that recognizes GAAAnnGAAA motifs, but IRF4 only weakly binds DNA due to its C-terminal auto-inhibitory domain[3]. In B cells, PU.1 or the related factor SPIB relieves auto-inhibition to increase binding affinity, allowing IRF4 to regulate genes expressing composite GGAAnnGAAA ETS/IRF consensus motif elements (EICEs)[3,6], including κ and λ immunoglobulin light chain genes. Whereas PU.1 directly binds to EICEs, efficient IRF4 binding requires phosphorylated, DNA-bound PU.1[1,2]. IRF4 also acts in T cells[7], contributing to development of multiple Th cell subsets[2], with defective Th1[8], Th2[8–11], Th9[12], and Th17[13] differentiation in its absence. Using genome-wide chromatin immunoprecipitation coupled to DNA sequencing (ChIP-Seq), we previously demonstrated that IRF4 cooperates with STAT3 to control IL-21-induced *Prdm1* expression and that these factors globally regulate IL-21-mediated gene expression[14]. Moreover, we found that IRF4 expression is required for normal STAT3 binding *in vivo* and for development of an additional T-cell effector population, namely Tfh cells[14].

As anticipated, analysis of IRF4 ChIP-Seq peaks from B cell libraries we previously generated[14] identified EICEs as the top motif (Fig. 1a, b). In contrast, EICEs were not readily identified in IRF4 ChIP-Seq libraries from activated T cells (Fig. 1c, d) or Th17 cells (Fig. 1e), consistent with T cells expressing much less PU.1 than B cells (Fig. 1f and Supplementary Fig. 1). Instead, examination of the top 1000 peaks (sorted by p-values) from libraries from pre-activated T cells, unstimulated or stimulated with IL-21, or from Th17 cells unexpectedly revealed that the top IRF4 ChIP-Seq motifs were Activator Protein-1 (AP-1) TGA[G/C]TCA motifs (Fig. 1c–e). IRF8, which is like IRF4 also interacts with PU.1[6], bound to some AP-1-containing sites but dominantly bound to canonical IRF motifs with tandem GAAA motifs (5′-GAAA[C/G][T/A]GAAA[G/C]-3′)(Supplementary Fig. 2a, b). To elucidate the IRF4-AP-1 relationship, we examined whether IRF4 GAAA/TTTC core motifs were associated with AP-1 sites, and within IRF4 ChIP-Seq peaks, we found enrichment of these motifs adjacent to or 5 bp away (4 intervening bp) from AP-1 sites (Fig. 1g and Supplementary Fig. 3), in contrast to their overall random distribution (Fig. 1g, blue line), suggesting binding cooperativity for AP-1 and IRF4. We denote these AP1-IRF4 composite elements as AICEs.

Of 14838 IRF4 ChIP-Seq peaks, 5304 bound within genes annotated by RefSeq (Fig. 2a), and analysis of our published Affymetrix array datasets[14] revealed that 2356 of these genes were regulated by IL-21 at 1, 6, or 24 h (Fig. 2b). RNA-Seq analysis revealed markedly lower expression of some of these genes, including *Prdm1* and *Il10*, in IL-21-stimulated *Irf4*$^{-/-}$ than in WT T cells (Fig. 2c), underscoring the importance of IRF4 for their expression. To characterize the IRF4 binding complex in T cells, we analyzed ChIP-Seq libraries from Th17-differentiated cells and IL-21-stimulated pre-activated T cells, focusing on Jun family proteins and BATF, which can heterodimerize with Jun proteins to bind to AP-1 motifs and is critical for Th17 differentiation[15–17], a process promoted by IL-21[18–20].

In Th17 cells, ~54% (11693 out of 21775) of the IRF4 binding sites overlapped with BATF binding sites, and ~65% of the BATF sites overlapped with IRF4, indicating substantial co-localization of these factors (Fig. 2d). As expected, the dominant binding motif for BATF was an AP-1 motif (TGA[G/C]TCA) (Supplementary Fig. 4), and IRF4, BATF, and Jun family proteins co-localized by ChIP-Seq (Fig. 2d and Supplementary Fig. 5a, b). The specificity of these data was indicated by essentially absent ChIP-Seq peaks in the IgG control as well as with anti-IRF4 in *Irf4*$^{-/-}$ cells or anti-BATF in *Batf*$^{-/-}$ cells (Fig. 2e). ChIP-Seq peaks for STAT3 also globally co-localized with IRF4 (Supplementary Fig. 5c), including at the *Prdm1* region previously studied[14] and in the *Il21* promoter and *Il17a* 3′ region (Fig. 2e). Interestingly, ~50% of genes (1167 out of 2356) with co-localization of these transcription factors in Th17 cells were also induced by IL-21 in activated CD4^{+} T cells. STAT3 binds to GAS motifs rather than AP-1 motifs, but its co-localization at AP-1 motifs might be explained by STAT3's ability to physically associate with cJun[21].

To investigate potential cooperative binding between IRF4 and AP-1 complexes, we identified strong IRF4 binding sites containing a GAAA motif adjacent to or 5 bp away from the AP-1 motif (a preferred spacing in Fig. 1g; Supplementary Table 1 lists genes with these sites). We selected sites in the *Il10*, *Ikzf2* (which encodes Helios), and *Ctla4* genes and confirmed co-localization of IRF4, STAT3, BATF, and Jun by ChIP-Seq (Fig. 2f). The *Il10* gene, which is expressed in IL-21-stimulated CD4^{+} T cells, CD8^{+} T cells, and polarized Th17 cells[22,23], contained two IRF4 binding sites with associated AP-1 motifs (Fig. 2f). We performed EMSAs with Th17 nuclear extracts and a probe corresponding to the conserved noncoding sequence, CNS9, located ~9.1 kb 5′ of the *Il10* transcription start site (Fig. 3a, *Il10* **peak 1)**, which is known to be an *Il10* regulatory element[24]. A strong complex formed, but it was reduced when the GAAA motif was mutated and abolished when the AP-1 motif was mutated (Fig. 3b). Supershifting with antibodies revealed that IRF4, JunB, JunD, and BATF were components of the complex (Fig. 3c); these factors also bound to *Il10* peak2 and *Ikzf2* probes (Fig. 3c). As expected, no shift was seen when nuclear extracts were omitted (Supplementary Fig. 6). Antibodies to c-Fos and Fra2 had a minor effect on the *Il10* peak1 and no effect on the *Il10* peak2 and *Ikzf2* complexes (Fig. 3c). In contrast, an AP1 consensus probe complex was not supershifted by anti-IRF4 but was by antibodies to BATF, cFos, and Fra2 (Fig. 3c). We next studied binding to the *Il10* peak1 IRF4 motif in B cells, Th2 cells, and Th9 cells, which all express *Il10.* B cell nuclear extracts formed a complex supershifted by antibodies to IRF4, BATF, and JunB but not PU.1 (Supplementary Fig. 7a), even though anti-PU.1 supershifted a complex formed with an EICE probe from the immunoglobulin λ light chain enhancer (Supplementary Fig. 7b). Thus, EICEs were the most common IRF4-containing complexes in B cells (Fig. 1a, b), but IRF4/AP-1AICEs also formed in these cells (Fig. 1b). Although Th2 and Th9 polarized cells were reported to express PU.1 protein[25,26], RNA-Seq analysis showed little PU.1 mRNA in these cells (Fig. 1f), and EMSAs showed IRF4/BATF/JunB interactions but no PU.1 binding activity (Supplementary Fig. 7c). To determine whether IRF4 and BATF-JUN proteins cooperatively bound to DNA, we used nuclear extracts from 293T cells transfected with various combinations of IRF4, BATF, and JunB or JunD and performed EMSAs with *Il10*, *Ctla4*, and *Ikzf2* probes. Little if any binding activity was observed with extracts from 293T cells expressing IRF4, JunD, or BATF alone, certain pairwise combinations exhibited some binding, but strong binding was

seen with extracts containing all three proteins, indicating cooperative binding to these sites (Fig. 3d, *left*); this was also observed when JunB was substituted for JunD (Fig. 3d, *right*). Cooperative binding was indicated by slower mobility, particularly of the *Il10* peak1 probe (Fig. 3d). Although mobility changes for other probes were less evident, even on 4% or 7% gels (not shown), supershifting experiments confirmed that IRF4, JunD, and BATF were present in complexes formed with each probe (Fig. 3e).

To examine the functional significance of the *Il10* IRF4 motif, we first analyzed *Il10* mRNA expression in *Irf4*$^{-/-}$ T cells and found much lower *Il10* mRNA in response to IL-21, anti-CD3/anti-CD28, or IL-21 + anti-CD3/anti-CD28 than was observed in WT cells ($P < .02$ at both time points; Fig. 4a). Correspondingly, *Il10* luciferase reporter activity was potently induced by IL-21 or anti-CD3/anti-CD28, and more so by IL-21 + anti-CD3/anti-CD28, but expression was diminished when the GAAAIRF4 motif or associated AP-1 site was mutated ($P < .02$; Fig. 4b). Moreover, IRF4, JunB, JunD, and BATF each bound to a WT probe spanning this region, but binding was diminished when *Irf4*$^{-/-}$ nuclear extracts were used (Fig. 4c). Moreover, in ChIP-Seq experiments, there was markedly decreased binding of IRF4 in *Batf*$^{-/-}$ T cells and of BATF and Jun in *Irf4*$^{-/-}$ T cells (Fig. 4d) at the *Il10* locus but also globally (Fig. 4e), including for example at the *Il17a* gene (Fig. 4f), consistent with defective *Il17a* expression and Th17 differentiation in *Irf4*$^{-/-}$[13] and *Batf*$^{-/-}$[15] T cells. These results indicate cooperative binding and transcriptional activation by IRF4 and BATF/Jun.

IRF4 is a pleiotropic IRF family transcription factor with broad immunological actions. Its critical role in regulating Ig genes involves functional cooperation with the largely B-cell restricted factor PU.1. We now demonstrate that in T cells, where PU.1 expression is low, IRF4 instead functionally cooperates with AP-1 family proteins to act via AICEs, with functional cooperation with BATF and Jun family proteins in pre-activated T cells stimulated with IL-21 as well as in Th2, Th9, and Th17 polarized cells. Interestingly, a number of genes we selected for analysis (*Il10*, *Ctla4*, *Il17a*, *Prdm1*, and *Ikzf2*) were functionally grouped in a study of Th2 inhibitory effector cells during chronic inflammation as preferentially expressed in IL-10^{+} versus IL-10^{-} cells[27]; it will be interesting to determine whether IRF4/AP1-dependent gene expression helps to explain these observations. Although IRF4 and BATF cooperatively bound in the context of AICEs, it was unclear if they associated in the absence of these sites. In T cells, we could co-precipitate BATF and Jun (Supplementary Fig. 8), but we only co-precipitated IRF4 and Jun in a single experiment and could not co-precipitate BATF and IRF4. Thus, if a direct interaction occurs, it may be relatively weak, but the dramatic decrease of BATF binding in *Irf4*$^{-/-}$ and of IRF4 in *Batf*$^{-/-}$ cells indicates cooperative binding to AICEs. The ability of IRF4 to act via two types of complexes-- PU.1/IRF4 EICEs in B cells and AP-1/IRF4 AICEs in T cells and to some degree in B cells-- highlights mechanisms for IRF4-mediated transcriptional activation. The identification of the IRF4/AP-1 connection suggests new approaches may be employed to selectively target certain actions of IRF4, potentially allowing ways to manipulate the immune response in a cell-type restricted fashion.

METHODS

Cells and Cell Culture

T and B cells were isolated using kits (Miltenyi) and cultured in RPMI-1640 medium containing 10% fetal bovine serum. Cells were pre-activated with plate-bound anti-CD3 (2 μg/ml) + soluble anti-CD28 (1 μg/ml) for 3 days, rested overnight and stimulated with IL-21 (100 ng/ml) for 1 h (ChIP-Seq) or 4 h (EMSAs). For Th17 polarization, cells were subjected to 2 rounds of polarization with anti-CD3 + anti-CD28 for 4 days in the presence of IL-6 (10 ng/ml), TGF-β (2 ng/ml), anti-IFN-γ (10 μg/ml), and anti-IL-4 (10 μg/ml). Unlike the $CD4^+$ T cells, Th17 polarized cells were not stimulated with IL-21.

Mice

WT, *Batf*$^{-/-}$ and *Irf4*$^{-/-}$ mice were 6–8 weeks old C57BL/6 background mice of mixed gender. All experiments with mice were performed under protocols approved by the NHLBI Animal Care and Use Committee, and followed NIH guidelines for use of animals in intramural research.

ChIP-Seq Experiments

We used chromatin from ~2×10^7 cells, which corresponds to ~100 ng of DNA, for each ChIP-Seq library and antibodies to IRF4 (Santa Cruz, sc-6059), STAT3 (Invitrogen), BATF, cJUN (Abcam, ab31419), JunB (Santa Cruz, sc-73), and JunD (Santa Cruz, sc-74). The ChIPed DNA fragments were blunt-ended, ligated to adaptors, and sequenced using an Illumina 1/2G Genome Analyzer and HiSeq2000 platform to obtain reads of 25–50 bp, depending on the platform. Sequenced reads were aligned to the mouse genome (NCBI36/mm8, Feb. 2006 assembly) with Bowtie 0.12.4[30]; only uniquely mapped reads were retained. Uniquely mapped reads and non-redundant reads numbers for each library are listed in Supplementary Table 2. The output of Bowtie was converted to BED files, which represent the genomic coordinates of each read. Reads were mapped into non-overlapping 200 bp windows, and the location of reads on positive (negative) strand was shifted ±75 bp from its 5′ start to determine the approximate center of the DNA fragment associated with the reads. With these locations, the reads in each 200 bp summary window were counted. BedGraph files were generated and viewed using the UCSC genome browser, and we aligned the BATF, IRF4, JUN, and STAT3 binding sites in IL-21 stimulated $CD4^+$ T cells or Th17 cells. Some data were also performed in cells from *Batf*$^{-/-}$ or *Irf4*$^{-/-}$ mice as well as from WT mice. Because each antibody presumably has a different binding affinity, we scaled libraries that used the same antibodies to normalize binding strength, but libraries from different antibodies were not scaled.

Database of Genes

RefSeq gene database (mm 8 revision) was downloaded from the UCSC genome browser; 24,769 genes were used for RNA-Seq analysis and genome-wide binding site distribution analysis.

Identification of Binding Sites

ChIP-Seq experiments were performed to identify transcription factor binding sites in splenic B cells, $CD4^+$ T cells and Th17 cells. We used MACS 1.3.7.1[31] to call binding sites (peaks) relative to a control IgG library as input control. The P-value threshold was set as 1e–10. To call a peak, the total number of reads in each peak region need to be >20 with FDR < 0.1. Only non-redundant reads were analyzed for peak calling.

De novo Motif Discovery

Due to the computational complexity, for each library we selected the top 1000 peaks with lowest p-values, extracted 100 bp of DNA sequence centered on the "summit" for each peak, and performed *de novo* motif analysis using MEME[32] to characterize the IRF4/IRF8 consensus binding motifs in B cells, T cells, as well as Th17 cells. Motif discovery was also performed for other transcription factors, including BATF, STAT3, c-Jun, JunB, and JunD. Where indicated, the five most significant motifs are shown; motifs were sorted by consensus E-values[4] or by motif occurring frequencies.

Motif Scanning Analysis

For the motif scanning analysis related to Fig. 1g, AP-1 motifs from Fig. 1d were centered and 100 bp of DNA sequence 5′ and 3′ were analyzed for the proximal IRF motif, GAAA/TTTC. Matched motif hits were counted at each nucleotide position and then plotted using a histogram, with breaks set at 200.

Genome-wide Distribution Analysis

The 5′ UTR, 3′ UTR, introns, exons and intergenic regions were defined according to the RefSeq database. Promoter regions were defined as regions extending 15 kb 5′ of the transcription start site. Peaks up to 5 kb 3′ of the transcription end site were considered as binding within the gene body.

Reporter assays

$CD4^+$ T cells were activated for 24 h with anti-CD3 + anti-CD28, washed, rested overnight, and 10^7 cells electroporated with 20 μg reporter plasmid and 1μg pRLTK in 0.2 ml RPMI using 960 μF and 250V. Cells were immediately stimulated with IL-21, anti-CD3/anti-CD28, or IL-21 + anti-CD3/anti-CD28. Dual luciferase assays were performed 7 h later (Promega). Shown is luciferase activity relative to the control pRLTK activity.

Electrophoretic mobility shift assays

Nuclear extracts were prepared as described[14]. Binding reactions contained 5 μg extract, 1.5 μg poly dI:dC, and 30,000 cpm of ^{32}P-labeled probe. For supershift analysis, extracts were pre-incubated for 20 min on ice with antibodies to IRF4 (M-17), JunB (N-17), JunD (329), BATF (WW8), c-Fos (4), Fra2 (H103), PU.1 (T-21) (Santa Cruz Biotechnologies). Reactions were electrophoresed on 5% polyacrylamide gels in 0.5 x TBE buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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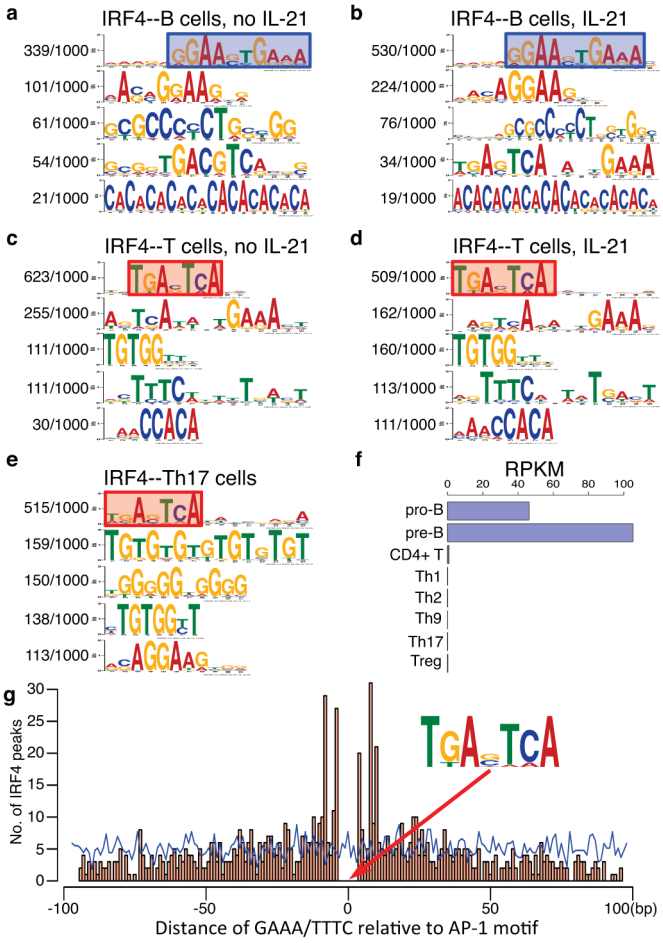

Figure 1. Preferential IRF4 binding to AP-1 motifs in T cells

a–e, IRF4 motifs in B cells not stimulated **(a)** or stimulated **(b)** with IL-21, in pre-activated T cells not stimulated **(c)** or stimulated **(d)** with IL-21 for 1 h, or in Th17 cells **(e). f**, *Sfpi1*/PU.1 mRNA expression in the indicated populations based on RNA-Seq**. g,** TTTC/GAAA motif distribution relative to the TGAnTCAAP-1 motif in 1000 sequences with strong IRF4 binding, and the number of IRF4 ChIP-Seq peaks at each spacing. The blue line shows the TTTC/GAAA distribution in 1000 random sequences; motif frequency was ~0.5%.

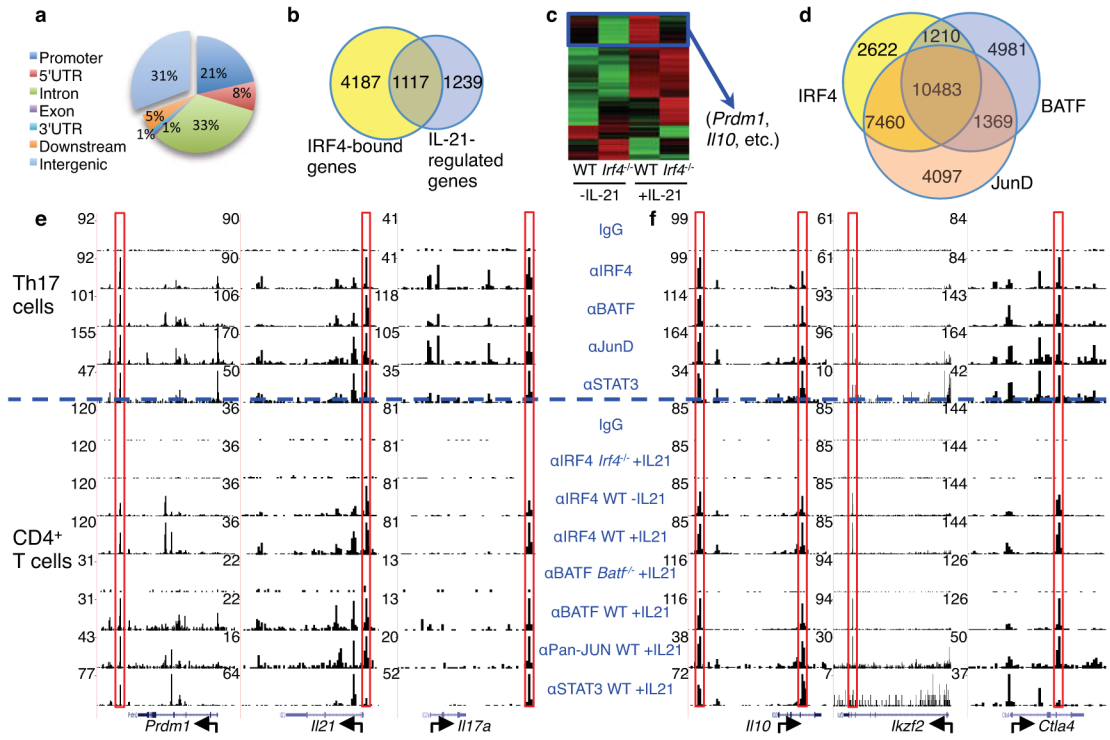

Figure 2. Functionally important IRF4 binding to AP-1 sites

a, Distribution of IRF4 ChIP-Seq peaks. **b,** Venn diagram of genes bound by IRF4 and regulated by IL-21. **c**, Heatmap of 130 genes regulated in pre-activated CD4$^+$ T cells after 1 h stimulation with IL-21. **d,** IRF4, BATF, and JunD binding in Th17 cells. **e,f,** ChIP-Seq data for IRF4, BATF, JunD, and STAT3 in Th17 cells (upper 5 rows) or IRF4, BATF, pan-Jun, and STAT3 in CD4$^+$ T cells (lower 6 rows) treated with IL-21, in *Prdm1*, *Il21*, and *Il17a* (**e**) or *Il10*, *Ikzf2*, and *Ctla4* (**f**) genes. Gene orientations are indicated.

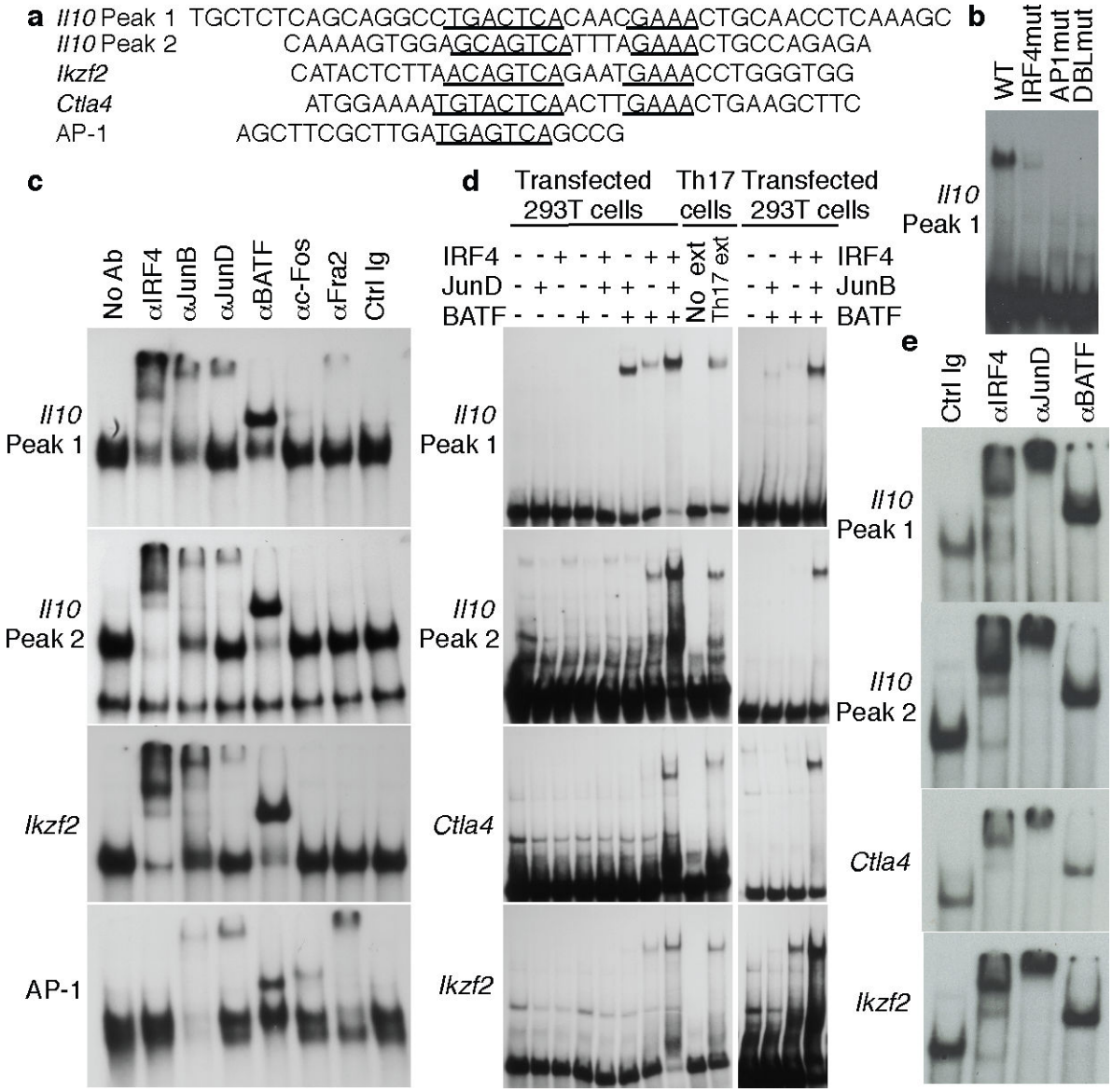

Figure 3. Cooperative IRF4/BATF/Jun binding

a, Probes for IRF4 ChIP-Seq peaks in *Il10*, *Ikzf2*, and *Ctla4*, and an AP1 motif probe; AP-1/IRF motifs are underlined. The AP1 probe is from ref. 15. **b**, EMSA with *Il10* peak1 probe (WT or IRF, AP1, or IRF4/AP1 double mutants) and Th17 nuclear extracts. **c,** EMSAs using *Il10* peak1, *Il10* peak2, *Ikzf2*, or AP1 probes and Th17 nuclear extracts; supershifting was performed as indicated. **d,** EMSAs using nuclear extracts from 293T cells transfected with indicated cDNAs; Th17 extract vs. no extract are also shown (10^{th} vs. 9^{th} lane). **e**, EMSAs with nuclear extracts from 293T cells transfected with IRF4, JunD, and BATF; supershifting was with anti-IRF4, anti-JunD, or anti-BATF. EMSA were performed at least 3 times.

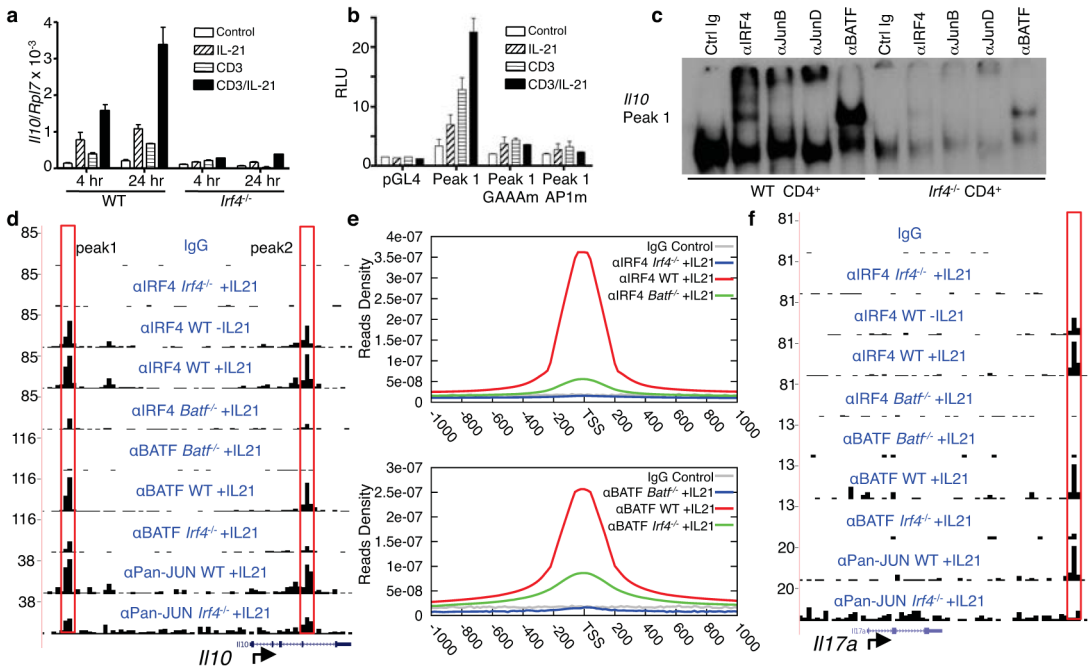

Figure 4. Cooperative action of IRF4, BATF, and Jun

a, *Il10* mRNA expression relative to *Rpl7* in WT or *Irf4*$^{-/-}$ T cells pre-activated and treated as indicated for 4 or 24 h. (n=3; mean ± S.D.). **b**, WT or mutant *Il10* reporter constructs transfected into pre-activated T cells and treated as indicated for 7 h (n=3; mean ± S.D.). **c**, EMSA with *Il10* peak1 probe and nuclear extracts from WT or *Irf4*$^{-/-}$ pre-activated CD4^{+} T cells stimulated with IL-21 for 4 h. Supershifting antibodies are indicated. EMSA was performed twice. **d**, IRF4, BATF, and pan-Jun ChIP-Seq data from WT or *Irf4*$^{-/-}$ T cells pre-activated and stimulated with IL-21 at the *Il10* gene. **e**, IRF4 binding in WT vs. *Batf*$^{-/-}$ cells (upper) and BATF binding in WT vs. *Irf4*$^{-/-}$ cells (lower). **f**, As in **d**, except at *Il17ra*.