JunB promotes Th17 cell identity and restrains alternative CD4+ T-cell programs during inflammation

Tiffany M. Carr1, Joshua D. Wheaton1, Geoffrey M. Houtz1 & Maria Ciofani1

T helper 17 (Th17) cell plasticity contributes to both immunity and autoimmunity; however, the factors that control lineage flexibility are mostly unknown. Here we show the activator protein-1 (AP-1) factor JunB is an essential regulator of Th17 cell identity. JunB activates expression of Th17 lineage-specifying genes and coordinately represses genes controlling Th1 and regulatory T-cell fate. JunB supports Th17 cell identity by regulating key AP-1 complex constituents. In particular, JunB limits the expression of the subset repressor IRF8, and impedes access of JunD to regulatory regions of alternative effector loci. Although dispensable for homeostatic Th17 cell development, JunB is required for induction and maintenance of Th17 effector responses in the inflammatory contexts of both acute infection and chronic autoimmunity in mice. Through regulatory network analysis, we show that JunB is a core regulator of global transcriptional programs that promote Th17 cell identity and restrict alternative CD4+ T-cell potential.
Functional plasticity in immune cells enhances the adaptability of responses targeting pathogens, but can also be detrimental to the host. Upon antigenic stimulation, CD4+ T cells adopt one of two opposing fates: a helper T (Th) cell specialized in supporting the clearance of infections, or a regulatory T (Treg) cell that functions to attenuate immune responses. Cytokines and other microenvironmental ligands present during T-cell activation direct diverse effector Th and Treg cell differentiation programs via the induction of function-specifying transcription factors (TF). The resulting subsets include Treg cells defined by Foxp3 expression, Th1 cells defined by T-bet (Tbx21) and IFNγ production, Th2 cells defined by GATA3 and IL-4 secretion, and Th17 cells defined by RORγt and IL-17 production. The identification of CD4+ T cells that co-express TFs and cytokines associated with alternative subsets highlights the vast flexibility inherent to CD4+ T cell diversification.

Among this diversity, Th17 cells stand out as particularly flexible in acquiring features of multiple other CD4+ T-cell subclasses under altered cytokine conditions. At steady state, Th17 cells secreting IL-17 and IL-22 occupy mucosal barriers as the intestinal lamina propria, where they regulate tissue homeostasis by promoting epithelial barrier function. During infection, Th17 cells provide critical support for immunity against extracellular bacteria and fungi, and can readily adopt Th1-like characteristics (e.g., T-bet and IFNγ expression) in such inflammatory settings. Although advantageous during infection, Th17 cell effector flexibility is also associated with deleterious Th17 cell function in autoimmunity. Indeed, pathogenic IFNγ+ IL-17A+ cells have been identified in numerous disease settings, including multiple sclerosis (MS) and inflammatory bowel disease (IBD).

Moreover, genetic fate mapping tools in mice have provided convincing evidence for the context-dependent in vivo conversion of Th17 cells into T follicular helper cells in intestinal Peyer’s patches. IFNγ+ Th1-like cells in an autoimmune model of MS, and T regulatory type 1 cells (T1) during resolution of inflammation. In light of the pervasive role of plasticity in CD4+ T-cell function and dysfunction, an understanding of the underlying mechanisms is of critical importance.

Th17 cell identity is governed by a network of transcriptional regulators that provide dynamic programming downstream of environmental inputs. While the central regulator RORγt is essential for the expression of a limited set of key subset effectors, additional TFs are required to specify the full Th17 cell program. In particular, a core of obligate initiator TFs including STAT3, IRF4, and BATF integrate cytokine (IL-6/IL-21/IL-23) and T-cell receptor (TCR) signals to coordinately activate Th17 cell differentiation. Beyond this, less is appreciated about the molecular mechanisms that sustain subset identity and that facilitate functional plasticity. Early studies suggested the flexibility of Th17 cells is facilitated by a permissive, bivalent histone epigenetic status at polarizing TF loci—such as Tbx21 and Gata3—that may allow for subset reversibility and reprogramming.

Although epigenetic modulators that control effector status in Th17 cells have been identified, the TF regulators that globally program the capacity of CD4+ T cells to dynamically control their functional identity in response to changing contexts are mostly undefined.

TFs of the activation protein-1 (AP-1) family are compelling candidates for mediating CD4+ T-cell identity switches. AP-1 factors are basic leucine-zipper TFs comprised of homodimers or heterodimers of Jun with Fos, or ATF protein classes. These TFs are dynamically regulated by extracellular signals and can either activate or repress transcription. AP-1 consensus motifs predominate in global analyses of Th17 cell cis regulatory regions, which coincides with important functional roles for AP-1 TFs in Th17 cell differentiation. In particular, BATF and its cooperative binding partner IRF4, linking TCR and cytokine signals to epigenetic changes, are essential pioneer factors that establish chromatin accessibility at Th17 regulatory regions downstream of TCR signals. This activity pre-patterns the enhancer landscape for further subset-selective gene regulation. Accordingly, BATF collaborates in high-order regulatory complexes with other Th17 specifying TFs, including STAT3, linking TCR and cytokine signals to epigenetic changes. The identification of Fosl2 as a broad repressor of Th effector genes in Th17 cells adds another layer of complexity in the Th17 AP-1 network. Fosl2 restricts Th1 and Treg cell potential, yet also antagonizes important Th17 program genes (e.g. Il17a/f, Ccl20, Ccru6), suggesting balancing contributions by other AP-1 factors in the regulation of Th17 cell plasticity. In this regard, JunB is a dominant AP-1 dimerization partner for both BATF and Fosl2 in Th17 cells, with an established role in CD4+ T-cell differentiation.

Results

JunB regulates Th17 cell identity. To determine the expression profile of JunB in CD4+ T-cell subsets, we evaluated in vitro differentiated CD4+ T cells at 24, 48, and 72 h for levels of JunB. At each time point, the relative mean fluorescence intensity of JunB was on average 3-fold higher when naive CD4+ T cells were cultured under Th17 cell polarizing conditions (IL-6 and TGFβ), as compared to induced Treg (iTreg, TGFβ) and IL-2, Th1 (IL-12), or Th2 (IL-4) cell polarizing conditions, or to non-polarizing Th0 conditions (TCR stimulation alone) (Fig. 1a). After 5 days, JunB remained most highly expressed in Th17 cells responses with an upregulation of a Th1 cell phenotype. Global analysis of JunB-dependent gene expression and genomic occupancy reveals that JunB controls Th17 cell stability through direct activation of important Th17 effector genes in concert with direct repression of subset-defining regulators of the Th1 and Treg cell lineages (e.g. Tbx21, Foxp3, and Ifng). Beyond these select genes, JunB functions as a core regulatory node in global programs that promote Th17 cell differentiation while constraining alternative effector and regulatory CD4+ T-cell potentials.
To test this hypothesis, we bred mice with a conditional *Junb* allele to the CD4-Cre pan T-cell deleter strain. First, we compared αβ T-cell subsets in naïve *Junb*+/+CD4-Cre (WT CD4cre) and *Junb*fl/flCD4-Cre (KO CD4cre) mice. Total thymocyte numbers and selection, based on TCRβ and CD69 expression, were unaffected in KO CD4cre mice, and mature CD4+ TCRβ+ and CD8α+ TCRβ+ thymocytes developed in the expected frequencies (Supplementary Fig. 1c). In the spleen and mesenteric lymph nodes (LN), total cellularity, as well as the frequencies and numbers of CD4+ TCRβ+ and CD8α+ TCRβ+ cells were similar (Fig. 1b and Supplementary Fig. 1d). Importantly, the frequency of naïve CD4+ TCRβ+ CD44lo CD62L+ cells was also unaltered in the periphery of KO CD4cre mice (Fig. 1b and Supplementary Fig. 1d), permitting further study of effector diversification.

Th17 cells can be differentiated in vitro to mimic distinct functional classes. Exposure to IL-23 elicits pathogenic potential,
such that Th17 cells polarized with IL-6, IL-1β, and IL-23 effectively induce disease compared to non-pathogenic Th17 cells polarized with IL-6 and TGFβ2, 3. Differentiation of naive CD4+ T cells purified from WT CD4cre- and KO CD4cre- mice with IL-6 and TGFβ revealed a striking dysregulation in cytokine and TF expression for mutant cells. JunB-deficient cultures displayed a 2-fold to 4-fold decrease in the proportion of IL-17A-expressing (WT expression (Fig. 1c). IFNγ and IL-17A expression remained nearly mutually exclusive (Supplementary Fig. 1f). Importantly, the reduction in IL-17A+ cells is not due to a defect in cell proliferation, or a selective impairment of IL-17A+ cell proliferation or survival in the absence of JunB (Fig. 1d and Supplementary Fig. 1e). A similar dysregulation occurred using ‘pathogenic’ Th1 cell-promoting conditions (Supplementary Fig. 1g), although, KO CD4cre cultures generated fewer Foxp3+ cells, suggesting that efficient release of iTreg-like potential requires TGFβ-mediated signals. Accordingly, we also observed less atypical Foxp3 expression in KO CD4cre naive CD4+ T cells cultured without exogenous TGFβ in Th0 and Th1 conditions relative to ‘non-pathogenic’ KO CD4Dcre Th17 conditions (Fig. 1c). In addition, the upregulated Th1-like T-bet+ IFNγ+ profile of KO CD4cre Th17 cells also occurred in JunB-deficient Th0, iTreg, and Th1 cultures, suggesting a general role for JunB in limiting Th1 cell differentiation (Fig. 1c). Indeed, similar to studies using Junb-hypomorphic mice2, 3, KO CD4cre Th2 cell cultures produced reduced levels of IL-4 and increased IFNγ, compared to WT CD4cre by day 5 (Supplementary Fig. 1h). Taken together, JunB has a nonredundant role in promoting Th17 cell identity and restraining alternative Th1 and Treg potential during Th17 cell induction.

**Th17 cells require continuous expression of JunB.** To interrogate the role of JunB in the maintenance of Th17 cell effector identity we deleted Junb in differentiated Th17 cells and simultaneously employed a fate mapping strategy to track Th17 to Th1 or Treg cell conversion events. Specifically, conditional Junb mutant mice were bred with mice expressing Cre from the Il17a locus (Il17aCre+/+)11, as well as with a reporter strain encoding a floxed-stop ZsGreen conditional allele in the ubiquitous Rosa26 locus32. Consequently, ZsGreen expression permanently marked cells with both a history of Il17a expression and Junb gene deletion in Junbββ cells (Fig. 2a).

Th17 cell plasticity under defined, altered conditions can be modeled in vitro2, 3. We differentiated naive CD4+ T cells from Junb+/+ or Il17aCre+/+Rosa26ZsGreen+ (WT Il17aCreR26+) and JunbββIl17aCre+/+Rosa26ZsGreen+ (KO Il17aCreR26+) mice under Th17 conditions for 4 days to generate Il17a ZsGreen+ fate-mapped cells (Il17aZsGreen-FM+). Purified Il17aZsGreen-FM+ cells were transferred back to Th17 conditions to evaluate the role of JunB in regulating Th17 cell stability, or alternatively, to altered cytokine conditions to establish maximal Th1 and iTreg lineage conversion potential. Compared to wild type, JunB-deficient Il17aZsGreen-FM+ cells preserved in Th17-promoting conditions, displayed a marked reduction in IL-17A and IL-17F expression (Fig. 2b and Supplementary Fig. 2c). Moreover, a prominent fraction of Junb-deficient Il17aZsGreen-FM+ cells underwent effector conversion to Treg- or Th1-like phenotypes (Fig. 2c). The extent of Treg re-direction was similar when Il17aZsGreen-FM+ cells were transferred to a potent iTreg-polarizing condition (Supplementary Fig. 2a), indicating that efficient differentiation occurs when Junb expression is extinguished in Th17 cells. Conversely, the level of T-bet in Junb-deficient Il17aZsGreen-FM+ Th17 cells was higher for cells transferred to Th1 vs. Th17 cell conditions (Supplementary Fig. 2b), suggesting the full acquisition of Th1 status upon conversion is facilitated by altered cytokine inputs. Therefore, continuous Junb expression is required to both maintain Th17 cell effector identity and constrain plasticity towards alternative CD4+ T-cell fates in cells that have initiated the Th17 cell program.
its activity was uniquely JunB-dependent, reduced by 2-fold in JunB-deficient Th17 cells (Fig. 3c). Notably, when test regions were assayed in cis with CNS-5, CNS+10 exclusively synergized with CNS-5 in a JunB-dependent manner (Fig. 3d), suggesting a mechanism for JunB-promoted maintenance of Il17a.

Among JunB-dependent genes, IRF8 is a compelling candidate negative regulator of Th17 cell differentiation and can replace IRF4 in BATF-JunB AP-1 complexes in Th17 cells. IRF8 RNA and protein levels were significantly elevated in KOIl17aCreR26ZS Th17 cells and in KOIl17aZsGreen-FM+ cells (Fig. 3e, f and Supplementary Fig. 3c). Additionally, JunB binds upstream of the Irf8 locus (Fig. 3g), suggesting that direct Irf8 suppression contributes to stabilization of the Th17 cell program.

To assess whether Irf8 upregulation accounts for the decreased differentiation of JunB-deficient Th17 cells, we counteracted Irf8 derepression by conditionally deleting one allele of Irf8 on a KOCD4Cre background (generating Irf8fl/+ Junbfl/fl CD4-Cre mice), thereby restoring Irf8 RNA and protein to near wild-type levels (Supplementary Fig. 3d and Fig. 3f). This coincided with a 50% rescue in IL-17A production (Fig. 3h); and a significant increase in the expression of lineage-relevant Irf8 suppression targets (Il23r, Il21, Ccr6, and Rorc) in Irf8fl/+ KOCD4Cre relative to KOCD4Cre Th17 cells (Supplementary Fig. 3e). Although heterozygous deletion of Irf8 partially restored the Th17 cell program in the absence of Junb, it did not reverse the aberrant elevation of T-bet, IFNγ, and Foxp3 in KOCD4Cre Th17 cells (Supplementary Fig. 3f). Therefore, the release of alternative CD4+ T-cell programs upon loss of JunB is independent of IRF8 upregulation. Taken together, JunB promotes Th17 cell identity by both limiting the expression of Irf8 and directly activating effector loci.

JunB directly represses alternative programs in Th17 cells. The lineage dysregulation observed for JunB-deficient Th17 cells was also apparent by global differential gene expression analysis (Fig. 3a). In particular, transcripts for subset-defining regulators of Treg and Th1 differentiation, including Foxp3, Tbx21, and Ifng, were significantly upregulated in KOCD4Cre Th17 cells (Fig. 3a and Supplementary Fig. 4a). This coincided with significant gene-proximal JunB ChIP occupancy (Fig. 4a), identifying these lineage-defining loci as likely direct JunB repression targets. Gene set enrichment analysis was used to characterize the dysregulated transcriptome of KOCD4Cre relative to WTCD4Cre Th17 cells (Fig. 4b). In the absence of JunB, downregulated targets were significantly enriched in Th17 cell signature genes, whereas upregulated genes were significantly enriched for those associated with Treg and Th1 cell signatures, indicating global shifts in effector programming consistent with the adoption of alternative CD4+ T-cell states.

JunB occupancy defines novel silencer elements. Cell identity is governed by the interaction between environment-sensing TFs, chromatin context, and the DNA elements that regulate lineage-relevant loci. The cis elements that restrict alternative potentials in Th17 cells are undefined. To address this, putative JunB-dependent silencer elements were identified as JunB-occupied CNS proximal to lineage-defining repressed loci (Irf8, Foxp3, Tbx21, and Ifng), which also contained AP-1 elements.
consensus sites (Fig. 3g and Fig. 4a, Supplementary Table 1). Candidate regions were cloned upstream of the JunB-independent Il17a CNS-5 enhancer in a minimal promoter-driven luciferase reporter construct, and assayed in Th17 differentiation cultures for the ability to diminish enhancer activity. Three of seven regions displayed silencer activity, including Ifng CNS+18, Foxp3 CNS+13 (3'UTR-localized), and Irf8 CNS-34. Each significantly attenuated the activity of Il17a CNS-5 (Fig. 4c), and did not display enhancer activity (Supplementary Fig. 4b). Notably, when these 3 silencer CNSs were assayed in KO CD4cre Th17 cells, Il17a CNS-5 activity was significantly restored and, in the case of Irf8 CNS-34, enhanced (Fig. 4c), indicating that their repressive potential is JunB-dependent. Interestingly, none of the Tbx21 CNS demonstrated silencing activity (Supplementary Fig. 4c), potentially reflecting the presence of distal cis elements. Together, these data implicate Ifng CNS+18, Foxp3 CNS+13, and Irf8 CNS-34 as novel JunB-dependent silencer elements, which coordinate to protect Th17 cell status, and through regulation of Ifng and Foxp3, are potential restriction elements that actively repress alternative Th1/Treg cell fates.

JunB deficiency results in enhanced JunD occupancy. JunB is a core component of AP-1 complexes in Th17 cells. As the main BATF heterodimerization partner, JunB binds cooperatively with p300 to Il17a CNS-5 in Th17 cells. As the main BATF heterodimerization partner, JunB binds cooperatively to Il17a CNS-5, enhancing its occupancy in Th17 cells (Fig. 3d, e). This enhanced occupancy is further increased in KO CD4cre Th17 cells, indicating that JunB repression is JunB-dependent. Interestingly, none of the Tbx21 CNSs demonstrated enhanced occupancy in KO CD4cre Th17 cells, suggesting that JunB repression is JunB-dependent. Together, these data suggest that JunB repression is required for the maintenance of Th17 cell identity, and that JunB-dependent silencer elements play a crucial role in regulating Th17 cell function.
AP1-IRF composite motifs with BATF and IRF4, essential pioneer factors for Th17 cell specification. JunB also dimerizes with FosL2, a negative regulator of effector potential in Th17 cells. Consistent with this diversity, ChIP-Seq revealed a high degree of colocalization of JunB with known partners at Th17 cell (II17a, and alternative Th subset (Ifng) loci (Fig. 5a and Supplementary Fig. 5a). To examine global patterns of TF binding in Th17 cells, we merged overlapping binding peaks for AP-1 TFs, IRF4, RORyt, and p300 into putative cis regulatory modules (pCRM), and generated a clustered heatmap of TF distribution at pCRMs. JunB showed a striking, near-exclusive binding overlap with BATF, FosL2, and IRF4, indicating global occupancy of common regulatory regions (Fig. 5b, clusters 1, 2, and 3).

In light of the strong regulatory interaction between AP-1 TFs, we characterized how the loss of JunB influences the chromatin occupancy of JunB binding partners in Th17 cells. Importantly, Batf, Irf4, and Fosl2 transcript levels were not reduced in KO conditions relative to WT conditions. JunB cell proliferation cultures by 48 h (Fig. 5c). ChIP for BATF, IRF4, and Fosl2 from KO conditions cultures demonstrated comparable occupancy to WT conditions at select JunB-bound regions in the II17a, Ifng, Tbx21, Ifng, and Foxp3 loci (Fig. 5d). A notable reduction was observed for IRF4 at the Il17a locus, potentially reflecting replacement by elevated IRF8. Therefore, the DNA binding pattern of cell-relevant AP-1 complex TFs is largely independent of JunB, unlike the collaborative binding of BATF with IRF4, which is greatly reduced in cells lacking the respective partner.

The incomplete block in Th17 cell differentiation in the absence of JunB, compared to either BATF or IRF4, implies that other Jun family members play a compensatory role. JunD is an excellent candidate in this regard. Whereas Jun transcripts were dramatically reduced in KO conditions relative to WT conditions cultures by 48 h (Fig. 5c), JunD expression was elevated in JunB-deficient cells specifically under Th17-promoting conditions (Fig. 5c and Supplementary Fig. 5b). Moreover, 68% of significant genome-wide JunD binding peaks, vs. only 24% of Jun peaks, overlapped with those of JunB (Supplementary Fig. 5c and d). Like JunB, JunD can bind cooperatively to DNA with BATF and IRF4. Accordingly, the global binding pattern of JunD in Th17 cells highly overlapped with that of BATF, and IRF4 as reported, and also with that of Fosl2 and JunB in WT conditions (Fig. 5a, b, cluster 1). The overlap in JunD and JunB occupancy suggests an antagonistic relationship. In addition to shared pCRMs (Fig. 5b, cluster 1), JunB binding in regions devoid of JunD implies that JunD is excluded in some contexts (cluster 2,3). Consistent with TF antagonism, in the absence of JunB, we observed a striking elevation in the binding of JunD at many AP-1 consensus sites typically bound by JunB in lineage-defining regulatory loci (Fig. 5d). Notably, global ChIP-Seq revealed elevated JunD binding in JunB-deficient Th17 cell cultures at numerous sites throughout the genome (Fig. 5e).

Global coordination of JunB with Th17 specification factors. The interplay between AP-1 proteins in Th17 cells is complex as JunB can homodimerize, or form heterodimers with partners such as BATF or Fosl2. To examine global trends in the regulation of shared targets by individual AP-1 factors in Th17 cells, we compared the JunB-dependent differential expression profile with that of BATF and Fosl2 (Fig. 6a, red dots). Notably, the majority of TF-dependent genes (90%) were similarly affected (i.e. both up- and downregulated) in JunB- and BATF-deficient Th17 cells, in contrast to a comparison of JunB- and Fosl2-dependent genes, where a relatively large proportion (32%) demonstrate discordant regulation (Fig. 6a). Highly coordinate regulation of gene expression by JunB and BATF is consistent with the predominant formation of a heterodimer pair, vs. potentially greater JunB-independent diversity in AP-1 and co-factor associations for Fosl2.

To evaluate the regulatory collaboration between JunB and Th17 cell identity factors (BATF, Fosl2, IRF4 and STAT3), we defined direct, functional targets for each TF as those that are significantly differentially expressed in TF knockout vs. wild-type RNA-seq; and for which one or more significant TF ChIP-seq peaks localize within 5 kb of the gene. The high-confidence network of TF-target interactions are visualized in Cytoscape (Supplementary Fig. 6a). JunB target genes display a high degree of co-regulation by other Th17 cell specifying TFs, with 83% regulated by at least one additional TF. Notably, AP-1 TF subnetworks reveal a striking, near-absolutely activation-repression concordance by JunB and BATF, with a lesser degree of coordinate regulation by JunB and Fosl2 (Fig. 6b), consistent with the comparisons of RNA differential expression.
(Fig. 6a). Coherent activation or repression activity by either JunB-BATF or JunB-Fosl2 collaborations are well correlated with actual changes in gene expression in Th17 relative to Th0 cells (i.e., genes activated by both TFs are upregulated in Th17 cells) supporting dominant regulatory action by AP-1 complexes in Th17 cell differentiation (Fig. 6b, c). Conversely, in instances where JunB and Fosl2 show opposing regulation, differential gene expression upon Th17 differentiation is most consistent with JunB activity, implicating JunB as a more influential activator and repressor (Fig. 6c).

Does JunB globally silence alternative CD4+ T-cell programs? Direct repression targets of JunB are significantly enriched for genes that are differentially expressed in Th1 vs. Th17 polarization cultures (Th1 signature in orange; Supplementary Fig. 6b and Fig. 6d), and similarly in iTreg vs. Th17 differentiation (Supplementary Fig. 6c), supporting a role for JunB in broad silencing of Th1- and iTreg-associated loci. This strong correlation is mirrored for repression targets of BATF, IRF4, and STAT3, but not of RORγt (Fig. 6d, Supplementary Fig. 6d). Consistent with a more general role in limiting effector plasticity, Fosl2 represses genes that are upregulated in Th1, iTreg, and Th17 signatures. Focusing our analysis on functional gene classes (e.g., cytokines, receptors, and TFs) reveals a high level of coordinated regulation of global Th17 (colored blue), Th1, and iTreg cell signature genes by JunB, BATF, IRF4, STAT3, and Fosl2 (Fig. 6e, and Supplementary Fig. 6d). The concerted negative regulation is highlighted for key Th1 and Treg loci (Ifng, Tbx21, Il18r1, Tnfsf4, Nrp1, and Foxp3) among others that are upregulated upon Th1 and iTreg differentiation. JunB is also highly integrated into the global Th17 cell activation program coordinated by initiator TFs BATF, IRF4 and STAT314. Indeed, the Th17 cell signature is

---

**Fig. 4** JunB binding defines novel silencer elements in Th17 cells. **a** ChIP-Seq tracks of JunB occupancy at the Foxp3, Tbx21, and Ifng loci in WT Th17 cells from 48 h polarization cultures. Lower track marks the boundaries of significant peaks called by MACS2 with FDR < 0.0005. **b** Gene set enrichment analysis (GSEA; Broad Institute) for KOCD4cre relative to WTCD4cre 48 h Th17 cell polarization cultures demonstrating significant enrichment of gene sets associated with Th1, iTreg and Th17 cell programs in the absence of Junb (p < 1 × 10−5). **c** Luciferase reporter assay of silencer activity for select JunB-bound regions at the Ifng, Foxp3, and Irf8 loci, in WTCD4cre and KOCD4cre naive CD4+ T cells cultured under Th17 polarizing conditions for 48 h. JunB-bound CNS of interest at each locus are indicated in **a.** Error bars represent SEM of three independent experiments. *p<0.05; **p<0.01 (unpaired two-tailed Student’s t-test)
strongly enriched among direct JunB activation targets (Fig. 6d), and JunB co-activates many of these signature genes (Il17a, Il23r, Ccl20, and Rora) in conjunction with Th17 pioneer TFs (Fig. 6e). In addition to influencing ‘master’ regulator loci, JunB directly contributes more broadly to global regulatory programs that promote Th17 cell identity and restrict alternative Th1 and Treg programs.

Steady-state Th17 cells are unaltered in the absence of JunB. Given the dysregulation of JunB-deficient polarization cultures, we examined whether Th17 cell differentiation was also altered in vivo. Notably, there was no difference in the proportion of IL-17A- or IFNγ-expressing steady-state CD4+ T cells localized to the spleen, mesenteric LNs, or small intestine lamina propria (SILP) between WT CD4cre and KO CD4cre mice (Fig. 7a).
frequency of SILP CD4+ T cells expressing ROYt or T-bet was also unaltered in JunB mutants (Fig. 7b, c). However, there was a modest reduction in the frequency and number of Foxp3+ Treg cells in KOCD4cre mice in every tissue evaluated (Fig. 7d and Supplementary Fig. 7a). The decrease in Treg cells in vivo is similar to the defect observed when IL-2 is omitted from mutant iTreg cultures in vitro (Supplementary Fig. 7b). This is in contrast to the unperturbed status of IL-2 supplemented cultures (Fig. 1c), suggesting a potential non cell-intrinsic role for JunB in Treg cell development or maintenance. Accordingly, deletion of junb exclusively in Foxp3+ cells resulted is similar frequencies of Tregs compared to control mice in secondary lymphoid organs (Supplementary Fig. 7c). Overall, steady-state CD4+ Th cell subsets, including homeostatic Th17 cells, were similar in WTCD4cre and KOCD4cre mice.

JunB promotes Th17 cell subset stability in vivo. To evaluate the role of JunB in restricting alternative CD4+ T-cell programs in an inflammatory setting where Th17 cell plasticity occurs physiologically, we used the EAE mouse model of MS, which is dependent upon the activity of CD4+ Th cells37. In EAE, IFNα- encephalitogenic T cells originate nearly entirely from Th17 cells that have undergone subset conversion11. Notably, JunB levels decrease as IL17a-ZsGreen-FM+ CD4+ T cells transition from IL-17A+ to IL-17A*IFNα+ or to IFNγ* status post EAE induction. Consistent with in vitro differentiation cultures (Fig. 1a), JunB is more highly expressed by IL-17A- vs. IFNγ-producing CD4+ T cells (Fig. 8a). These trends indicate that JunB protein levels are modulated during physiological Th17 cell plasticity, suggesting that JunB is part of the mechanism that regulates effector conversions. Thus, EAE is an ideal model to evaluate the role of JunB in Th17 cell lineage stability.

Following induction of EAE, WTCD4cre mice progressed to near-complete paralysis, whereas KOCD4cre mice were absolutely protected from disease (Fig. 8b). Analysis of cerebellum and spinal columns at peak disease in WTCD4cre mice, uncovered a dramatic reduction in encephalitogenic CD4+CD45+ cells infiltrating the central nervous system (CNS) of KOCD4cre mice, precluding cellular phenotyping (Fig. 8c). This likely accounted for the lack of symptoms, and revealed an unexpected requirement for JunB for pathogenic T-cell CNS infiltration. Accordingly, genes involved in migration are enriched in the altered transcriptome of JunB-deficient Th17 cells (Supplementary Fig. 3a). To assess early CD4+ T-cell responses, draining LNs were examined at 6 days post induction of EAE, where there is no significant difference in baseline effector profiles in naive KOCD4cre mice (Fig. 8d). This revealed a striking dysregulation in CD4+ T-cell cytokine production in KOCD4cre mice similar to that of JunB-deficient Th17 cell cultures (Fig. 8d). In particular, KOCD4cre mice had fewer IL-17A-producing CD4+ T cells compared to WTCD4cre mice, with numbers similar to naive levels (Fig. 8d). Importantly, mutant CD4+ T cells were refractory to activation; rather, KOCD4cre LNs showed a striking predominance of IFNγ- and GM-CSF-producing CD4+ T cells relative to WTCD4cre EAE mice (Fig. 8d). Enhanced GM-CSF was also a feature of KOCD4cre Th17 cell cultures in vitro (Supplementary Fig. 8a). Moreover, taking into account that Treg cells are reduced in steady-state KOCD4cre mice, we observed a selective twofold increase in the frequency of LN Foxp3+CD4+ T cells relative to naive mice at day 6 of EAE, implying that enhanced Treg induction also occurs in vivo in the absence of JunB (Fig. 8e and Supplementary Fig. 8b). Notably, while JunB is essential for inflammatory Th17 cell induction and expression of important effector genes, JunB does not globally regulate the previously defined pathogenic Th17 cell profile10 (Supplementary Fig. 8c). Thus, in an inflammatory setting, JunB is essential for the induction of appropriate Th17 cell responses; for restricting alternative CD4+ Th and Treg programs; and for enabling pathogenic CD4+ T-cell CNS migration during EAE.

To assess the role of JunB in regulating lineage conversion events during inflammation, we induced EAE in WTIl17aCreR26Zs and KOIl17aCreR26Zs mice and tracked fate-mapped Il17a-ZsGreen-FM+ cells in vivo. Similar to KOCD4cre mice, junb deletion in Il17a-expressing cells resulted in a complete rescue from symptoms (Supplementary Fig. 8d). At peak disease, 50% of CD4+ CNS infiltrating cells in WTIl17aCreR26Zs mice were IL17a-ZsGreen-FM+ and enriched for encephalitogenic IL-17A IFNγ+ CD4+ T cells, as reported11 (Supplementary Fig. 8e). In contrast, although CD4+ T cells were identified in the CNS in KOIl17aCreR26Zs mice, these were selectively devoid of the IL17a-ZsGreen-FM+ fraction that marks JunB-deficient cells. Unexpectedly, this specific depletion was mirrored in the draining LN early post-induction of EAE in KOIl17aCreR26Zs vs. WTIl17aCre mice (Supplementary Fig. 8f), and this coincided with a dramatic elevation in the frequency of AnnexinV+ apoptotic cells (Supplementary Fig. 8g). While the loss of JunB-deficient IL17a-ZsGreen-FM+ T cells precluded analysis of fate conversion events, it revealed a distinct requirement for sustained JunB expression for survival of differentiated Th17 cells. Importantly, an unaltered frequency of AnnexinV+ cells among homeostatic SILP Th17 cells suggests that the role of JunB in Th17 cell survival is restricted to an inflammatory setting (Supplementary Fig. 8h).

In addition to the chronic inflammatory context of EAE, we employed an acute cutaneous infection model with Candida albicans to assess the role of JunB in an inflammatory setting where Th17 cells do not typically exhibit plasticity11. IL-17A-producing Th17 cells are important in the defense against C. albicans38, 39. On day 5 post infection, similar frequencies of CD4+ cells were observed in the skin explants from WTCD4cre and KOCD4cre mice, both significantly enhanced relative to mock-treated controls (Fig. 8f). As expected, WTCD4cre mice produced a balanced IL-17A/IFNγ cytokine response to C. albicans11. However, comparable to our findings in the EAE model, infected KOCD4cre mice failed to induce a Th17 cell response, instead

Fig. 5 Enhanced JunD occupancy in the absence of JunB. a ChiP-qseq tracks for JunB, JunD, IRF4, BATF, and FoxI2 at the Il17a and Il17f loci in 48 h polarized wild-type Th17 cells. b Clustered heat map of pCRMs regions of JunB, JunD, BATF, FoxI2, IRF4, p300, and ROYt occupancy in wild-type 48 h Th17 cell polarization cultures. e qPCR analysis for Batf, Irf4, Fox2, clun, and Jun transcripts, relative to Actb expression, in WTCD4cre and KOCD4cre CD4+ naive T cells and naive CD4+ T cells cultured under Th17 cell conditions for 24 and 48 h. d ChiP of WTCD4cre and KOCD4cre 48 h polarized Th17 cells, using α-BATF, α-IRF4, α-FoxI2, or α-Jun followed by qPCR. Primers used encompassed the AP-1 consensus site at select JunB-bound regions or a negative control region, as depicted with boxes in the schematics of the relevant loci. ChiP recovery at negative control loci provides local background for the assay. Error bars represent SEM of two independent experiments. e Differential global occupancy of JunD in KOCD4cre vs. WTCD4cre 48 h Th17 cell polarization cultures displayed as a volcano plot of fold change vs. significance. Differential binding of JunD is considered significant below FDR < 0.05; pCRMs with and without JunB co-occupancy displayed in orange and blue, respectively. The percentage of pCRMs for each subtype with fold change ≥ 2 and p-value < 0.01 are indicated for the regions bounded by gray trendlines. p-value capped at 10−25. f Clustered heat map of pCRMs regions defined by occupancy of JunB and JunD in WT (C57Bl/6), WTCD4cre, or KOCD4cre 48 h Th17 cell polarization cultures, as indicated. *p < 0.05; **p < 0.01 (unpaired two-tailed Student’s t-test)
a

![Graph showing Log2 (fold change Junb KO/WT) vs Log2 (fold change BATF KO/WT) and Log2 (fold change Fosl2 KO/WT) with data points indicating differential expression.]

b

![Network diagram showing differential expression with colors indicating up-regulation (Th17/Th0) and down-regulation (Th17/Th0).]

c

![Box plots for Log2 (fold change JunB:BATF) and Log2 (fold change JunB:Fosl2) with ACT, REP, ACT:REP, Rep:ACT conditions.]

d

![Box plots for Log2 (fold change Junb, Batf, Irf4, Fosl2, Stat3, Rorc) with ACT, REP, ACT:REP, Rep:ACT conditions.]

e

![Network diagram showing cytokines, receptors, and transcription factors (TFs) with differential expression (Th17/Th1) indicated by colors.]
having a proportion of IL-17A+ cells resembling uninfected mice (Fig. 8f). Moreover, T-cell loss of Junb skewed the CD4+ effector profile toward a predominantly Th1 phenotype, characterized by a striking enhancement in IFNγ and GM-CSF production. This dysregulation reflects an inflammation-dependent role for JunB in effector cell generation as steady-state cytokine-producing skin CD4+ cells are unaltered in KO Dörec mice (Fig. 8i). However, as in other tissues, there is a reduction in Foxp3+ Treg cells in the skin of naive KO Dörec mice that persists post infection (Supplementary Figs. 8i and 7d), despite a similar relative expansion in WT Dörec and KO Dörec mice (Fig. 8g). The lack of increased enhancement of Treg cells in mutants relative to controls may reflect the non-supportive environment for Treg cells in KO Dörec mice (Fig. 7d and Supplementary Fig. 7), which is perhaps selectively exacerbated in an acute inflammatory setting of C. albicans infection that is inherently non-permissive to plasticity.

Taken together, the in vivo findings identify an absolute and selective requirement for JunB in the induction and maintenance of Th17 effector cells during inflammation, in the context of both chronic autoimmunity and acute infection. Coordinate, JunB serves an essential role in constraining Th1 and Treg features, suggesting that the modulation of JunB in an environment-dependent manner can permit controlled adoption of alternative effector programs.

**Discussion**

Previous studies have identified the pioneer factors and transcriptional regulators required to implement the Th17 cell program downstream of TCR and cytokine signals, however, the mechanisms underlying the inherent plasticity of the Th17 cell subset are less characterized.14,17 Here, we define JunB as a novel, nonredundant regulator of global programs that support Th17 cell identity and that restrain alternative Th1 and Treg cell fates in inflammatory contexts. Th17 cells are functionally heterogeneous. Those produced at steady-state promote intestinal barrier homeostasis and defense4, whereas those elicited by IL-23 exposure are pathogenic, express IFNγ and GM-CSF, and exacerbate inflammatory disease.8,41. Although all subsets are RORγt-dependent16, the TFs that distinguish the development of homeostatic vs. inflammatory Th17 cells are less defined. In this regard, JunB is a selective, obligatory regulator of inflammatory Th17 cell induction, but dispensable at steady-state. In contrast to Blimp-1 and RBPI, which confer IL-23R-driven pathogenicity to Th17 cells,42,43 JunB restrains pathogenic IFNγ and GM-CSF production, and supports the underlying Th17 cell program. However, the selective role of JunB during inflammation does not discriminate pathogenic vs. non-pathogenic Th17 cell differentiation. Rather, JunB is necessary in the context of IL-6. JunB is essential for Th17 cell induction in the IL-6-dependent inflammatory contexts of EAE and C. albicans infection14,45, but not for homeostatic IL-6-independent intestinal Th17 cell development46. In addition, JunB expression is upregulated by TCR activation in vitro, suggesting that the priming environment may drive unique inflammatory vs. homeostatic Th17 programs, whereby JunB differentially integrates into AP-1 complexes that program the regulatory landscape.14

JunB is required at distinct stages of inflammatory Th17 cell differentiation. It is necessary during initiation for robust induction of the Th17 cell effector program, and restraint of Th1 and Treg cell potentials. Thereafter, sustained JunB expression is essential to maintain identity and limit plasticity in differentiated Th17 cells. Examination of lineage conversion events in vivo were precluded by apoptosis following JunB withdrawal from IL17a-expressing cells. Therefore, once established, continued JunB activity supports Th17 cell viability in inflammatory environments. Notably, no CD4+ T-cell subset completely lacks JunB expression. Thus, whereas JunB deletion causes apoptosis, reduced levels of JunB, as observed in EAE for non-Th17 or ex-Th17 converted subsets, appear to be compatible with lineage conversion events. A similar modulation of JunB may contribute to Th17 cell transdifferentiation into pathogenic IFNγ+ex-Th17 cells during colitis, or into regulatory cells during resolution of inflammation6,13. Lastly, JunB is obligatory for the infiltration of inflammatory CD4+ T cells into the CNS during EAE, but not the skin in C. albicans infection, implying that JunB regulates a factor that mediates CNS-specific entry. Taken together, JunB plays multiple essential roles in CD4+ T cells.

AP-1 complexes are fundamental to Th17 cell identity. Like JunB, Fosl2 modulates plasticity in Th17 cells.14 Analysis of the collaboration between JunB and Fosl2 revealed both concordant and discordant regulation of shared target genes, highlighting shared and unique roles in Th17 cells. Indeed, both TFs restrain Th1 and Treg potential, however, whereas JunB predominantly activates the Th17 program, Fosl2 represses many Th17-relevant genes.14 Conversely, the highly coincident gene regulation by JunB and BATF identifies BATF as the dominant regulatory partner for JunB in Th17 cells. Further, JunB is integrated into a broad collaboration with pioneer TFs, BATF and IRF4.14 This encompasses both coordinate activation of Th17 lineage genes and repression of alternative Th1/Treg programs. The latter implies an appreciated role for BATF and IRF4 in limiting alternative CD4+ T-cell potentials, which is otherwise masked by their absolute requirement during Th17 cell differentiation.18,19. Thus, it is tempting to speculate that BATF and IRF4 establish a general CD4+ T-cell enhancer.

**Fig. 6** Global regulatory trends for targets of JunB, BATF, and Fosl2. a Comparison of differential expression (DE) targets of JunB in combination with BATF or Fosl2 from RNA-Seq of 48 h Th17 cell polarization cultures with individual TF KO. Genes highlighted in red show DE (FDR<0.05) in both datasets, and represent the subset of genes used for subsequent analyses. Numbers in corners indicate the percentage of all significant genes that fall within that quadrant. Genes with opposing AP-1 mediated regulation fall in quadrants 1 and 3, and select genes in these categories are labeled. b Network representation of shared targets of JunB and BATF or Fosl2. Networks were constructed by combining RNA-Seq and ChIP-seq data to determine direct regulatory targets. Green edges indicate activation, red edges indicate repression based on DE measured by RNA-Seq. Nodes are colored based on log2FC of DE in Th17 vs. Th0 RNA-Seq (only significantly DE nodes are included; FDR < 0.05), and are grouped based on combinatorial interactions between JunB and BATF or Fosl2 (i.e., activation:activation, activation:repression, etc.). c Comparison of the net effect of JunB:BATF and JunB:Fosl2 interactions on Th17-specific gene expression. Common targets were classified based on positive or negative regulatory action (ACT or REP) for each TF, then grouped based on 4 possible modes of cooperative regulation. The log2FC for DE genes (FDR<0.05) from Th17 vs. Th0 RNA-Seq is shown as a boxplot for each group. d Comparison of the Th17 vs. Th1 preference of activation and repression targets of Th17 TFs. Target genes were given regulatory classification as in c. For each category, the log2FC for genes with DE in Th17 vs. Th1 (FDR<0.05) is plotted. e Network representation of Th17 TF targets with functional categorization. Networks were constructed as in b, except with node coloring indicating log2FC of DE in Th17 vs. Th1 (FDR<0.05). Genes were classified and grouped based on molecular function. Only targets with JunB-connected edges are shown. Dashed lines indicated direct targets validated using ChIP-QPCR and luciferase assay that fall outside the 5 kb threshold used for global target classification.
Fig. 7 JunB is not required for homeostatic CD4+ Th cell differentiation. a Flow cytometry of intracellular IL-17A vs. IFNγ in CD4+TCRβ+ cells from the spleen, mesenteric lymph node (mLN), and small intestine lamina propria (SILP), of WTCD4cre and KOCD4cre mice. The frequencies of total IL-17A and IFNγ producing CD4+TCRβ+ cells from each tissue are shown. b Flow cytometry of RORγt and IL-17A expression in CD4+TCRβ+ cells from the SILP of WTCD4cre and KOCD4cre mice. c Frequencies of RORγt+ and T-bet+ CD4+TCRβ+ cells from the SILP of WTCD4cre and KOCD4cre mice are shown. d Flow cytometry of Foxp3 expression by CD4+TCRβ+ cells from the thymus, spleen, and mLN of WTCD4cre and KOCD4cre mice. Frequencies of Foxp3+CD4+TCRβ+ cells from each tissue are shown. **p<0.01; ****p<0.0001 (unpaired two-tailed Student’s t-test)
landscape downstream of the TCR that is poised to respond to inductive cytokine signals, and exploited by various AP-1 complexes to facilitate effector switches.

Alterations in AP-1 complex composition coincide with CD4\(^+\) T-cell effector flexibility. In the absence of JunB, the binding of BATF, IRF4, and Fosl2 are largely unaltered at effector loci, whereas JunD binding is significantly elevated, suggesting that JunD replaces JunB in AP-1 complexes and alters regulatory dynamics. Accordingly, this coincides with a repression-to-activation switch at Th1- and Treg-promoting loci,
suggesting antagonistic roles for JunB and JunD, consistent with their regulation of Il4 expression in Th2 cells\(^{8,17}\). Moreover, Tf\(s\) such as BACH2, the global repressor of CD4\(^+\) T-cell effector programs\(^{8,18}\), may collaborate with JunB in restricting alternative fates. Indeed, in CD8\(^+\) T cells, BACH2 impedes access of JunD to enhancers of terminal differentiation effector loci and prevents their aberrant activation\(^8\). A similar mechanism may regulate JunD function in Th17 cells, where extensive colocalization with p300 implies a dominant activating role for JunD. However, at Th17–relevant loci (e.g., Il17a), which are downregulated in JunB deficiency, JunD activity may be countered by the concomitant derepression of the Th17 cell negative regulator, IRF8\(^{15}\). The capacity of IRF8 to replace IRF4 in AP-1 complexes suggests a mechanism for the downregulation of Il17a and other Th17 cell-defining loci repressed by IRF8. Altogether, this supports the model that dynamic changes in AP-1 complex and co-regulator composition coordinates the loss of Th17 cell effector function with the acquisition of alternative fates during lineage conversion. Silencers are conserved elements that permit context-dependent expression of target loci. Capitalizing on the role of JunB as a plasticity regulator, we identified novel silencers at Ifng, Foxp3, and Ifng where negative regulation by JunB is central to maintenance of Th17 cell effector status. It is tempting to speculate that the coordinated loss of JunB activity at a network of such silencers facilitates transdifferentiation by simultaneously reversing the restriction of alternative potentials (e.g., via Foxp3, and Ifng) and releasing antagonists of Th17 cell identity (e.g., Ifng). Although there are examples of silencers that enforce long-term lineage identity (e.g., Cds\(^{20}\), and Zbtb7b\(^31,52\), some of these elements underlie plasticity permitting flexible changes in expression between CD4\(^+\) T-cell states are undefined. An interesting candidate is Ifng CNS-18, implicated as an enhancer in Th1 cells\(^3\), and here, as a potent silencer in Th17 cells. Such dual-activity elements have been described\(^34,55\). Encompassing activating and repressive functions in single cis elements proposes a mechanism for efficient toggling between effector states in changing environments.

The mechanisms governing Th17 cell plasticity in immunity and autoimmunity are unclear. Here, we identify JunB as an obligatory regulator of Th17 cell stability that promotes the development of inflammatory Th17 cells and restricts flexibility towards alternative effector and regulatory programs. Targeted modulation of JunB to attenuate conversion to pathogenic Th17 cell subtypes, or rather, to promote transdifferentiation to anti-inflammatory regulatory cells presents possible avenues for therapeutic intervention in disease.

Methods

Mice. Mice encoding a conditional Junb floxed allele were obtained from Erwin Wagner (CNIO, Spain), and backcrossed at least five generations onto the C57BL/6 background. Junb\(^{fl/}\) mice were bred with Cd4cre\(^{29}\) and Il17a\(^{loxP-STOP-ZsGreen}\) conditional allele in the ubiquitous Rosa26 locus\(^{32}\) (Jackson Laboratory). We bred to Junb\(^{fl/}\) mice in order to obtain Junb\(^{fl/}\)Il17a\(^{loxP-STOP-ZsGreen}\) mice. Junb\(^{fl/}\)CD4cre mice were also mated with mice harboring a conditional Ifng floxed allele, to generate Junb\(^{fl/}\)Ifng\(^{floxed}\)CD4cre mice (Jackson Laboratory).

For detection in Treg cells, Junb\(^{fl/}\) mice were bred with Foxp3\(^{ymC expressing}\) mice (Jackson Laboratory). C57BL6 wild-type mice were obtained from Taconic Biosciences. Experimental mice were between 7 and 12 weeks of age, with no preference to gender. Sample size was chosen using Mead’s equation to ensure adequate power. Blinding and randomization was used in specific-pathogen-free conditions at Duke University and all experiments were performed with permission from and in accordance with the guidelines of the Duke Institutional Animal Care and Use Committee.

Cell culture. Naïve CD4\(^+\) T cells were sort-purified from pooled spleen and lymph nodes, using the MoFlo XDP (Beckman Coulter). In short, red blood cells were removed by incubation with ACK lysis buffer (Lonza), and the resultant single cell suspensions enriched for CD4\(^+\) T cells using depletion-based magnetic separation (MagneSil Mouse CD4 T Cell Kit; eBioscience; 8804-6821-74), in accordance with the manufacturer’s protocol. Thereafter, cells were surface stained with antibodies specific for CD4, CD25, CD44, and CD262L, and sorted to isolate CD4\(^+\)CD25\(^{low}\)CD44\(^{low}\)CD262L\(^{low}\) naïve T cells. The sort-purified naïve CD4\(^+\) T cells were retitrated into cultures at 12, 24, or 48-well plates and activated with anti-CD3e (145-2C11; 0.25 µg/ml) and anti-CD28 (37.51; 1 µg/ml). Cultures were also supplemented with anti-IL-4 (1B1; 2 µg/ml) and anti-IFNγ (XMG12; 2 µg/ml), in addition to IL-2 (50 U/ml) for Th0 cell conditions; IL-6 (1.25 ng/ml) and TGFβ1 (0.3 ng/ml) for Th17 cell conditions; IL-6 (1.25 ng/ml), IL-1β (20 ng/ml), and IL-23 (25 ng/ml) for pathogenic Th17 cell conditions; or TGFβ1 (5 ng/ml) and IL-2 (50 U/ml) for iTreg conditions. In Th1 cell polarizing conditions, cultures were supplemented with anti-IL-4 (1B1; 2 µg/ml) and anti-IFNγ (XMG12; 2 µg/ml), while Th2 cell cultures included anti-IFNγ (2 µg/ml) and IL-4 (2 ng/ml). After 72 h, Th1 and Th2 cultures were removed from TCR stimulation and supplemented with IL-2 (50 U/ml), while Th0, Th17, and iTreg cell cultures continued to receive TCR stimulation. If maintained past 72 h, blocking antibodies were removed from the culture conditions at varying times.

Isolation of cells from tissues. Single cell suspensions of the thymus, spleen, and lymph nodes were obtained by mechanical disruption of the tissue with a 40 µm cell strainer (Falcon). To isolate leucocytes from the small intestine lamina propria (SLIP), the small intestine was harvested, fat removed, Peyer’s patches excised, and luminal contents expelled. The intestine was cut into 1–2 cm pieces and shaken in PBS containing 1 mM DTT to remove mucus. The intestinal pieces were then transferred to PBS supplemented with 5 mM EDTA and 10 mM Heps, and incubated twice at 37 °C for 10 min with rotation, to remove the epithelial cell layer. After the second incubation, intestinal pieces were collected and washed with HBSS. Pieces were transferred to HBSS supplemented with collagenase D (1 mg/ml; Roche), DNaseI (0.1 mg/ml; Sigma), and dispase (0.1 U/ml; Worthington), and subsequently, minced with scissors and incubated in the digestion cocktail at 37 °C for 25 min with rotation. The intestinal suspension was passed through a 100 µm cell strainer and a discontinuous 40:80 Percoll gradient used to further remove contaminating immune cells.

**Fig. 8** JunB promotes inflammatory Th17 cells and limits alternative CD4\(^+\) Th cell programs in vivo. a Flow cytometry of JunB expression by Il17a\(^{Cre-GFP}\) CD4\(^+\) TCR\(β\) cells producing IL-17A, IFNγ, or CD4\(^+\)IFNγ in the draining lymph node of WT Il17a\(^{Cre}\)R26ZS mice. At day 6 post induction of EAE, with KOCd4cre as a negative staining control (left). Flow cytometry of JunB expression in CD4\(^+\)TCR\(β\) IL-17A or IFNγ producing cells from the inguinal lymph node of WT Cd4cre mice, with KOCd4cre cells as a negative staining control (right). b Flow cytometry of JunB expression by Il17a\(^{Cre-GFP}\) CD4\(^+\) TCR\(β\) cells producing IL-17A, IFNγ, or CD4\(^+\)IFNγ in the draining lymph node of WT Cd4cre mice, with KOCd4cre cells as a negative staining control (right). Data are representative of two independent experiments. b WT Cd4cre and KO Cd4cre mice were injected with an emulsification of Complete Freund’s Adjuvant and MOG35-55 peptide and monitored for disease severity over time. Error bars represent SEM of six mice in each cohort, from three independent experiments. c Flow cytometry of IL-17A, IFNγ, and Foxp3 expression in CD4\(^+\)CD45\(^+\) cells isolated from the cerbellum at day 18 post induction of EAE and restimulated ex vivo. Plots are representative of all mice analyzed in a. Total cell numbers of CD4\(^+\)CD45\(^+\) cells in the cerebellum and spinal column of WT Cd4cre and KOCd4cre mice, as well as naïve control, when WT Cd4cre mice were at max score are shown. d Flow cytometry of cytokine production in WT Cd4cre and KOCd4cre mice, as well as naïve control, when WT Cd4cre mice were at max score are shown. e Relative frequency of Foxp3\(^+\)CD4\(^+\)TCR\(β\) cells in the draining lymph node of WT Cd4cre and KOCd4cre mice at day 6 post-induction of EAE relative to naïve mice of the same genotype. f C. albicans cutaneous infection model in WT Cd4cre and KOCd4cre mice. Flow cytometry of cytokine production by ex vivo restimulated CD4\(^+\)CD45\(^+\) cells harvested from the skin 5 days post infection, as well as naïve control. Data are compiled from three independent experiments. g Relative frequencies of Foxp3\(^+\)CD4\(^+\)CD45\(^+\) by ex vivo restimulated WT Cd4cre and KO Cd4cre cells harvested from the skin 5 days post infection, compared to their respective naïve controls. \(p<0.05; \star<0.01; \star<0.001; \star<0.0001\) (unpaired two tailed Student’s t-test).
epithelial cells and debris. Percoll gradient separation was performed by centrifugation at 2200 rpm for 22 min at room temperature, with no brake. SLP lymph node cells were washed and resuspended in complete IMDM, followed by stimulation and flow cytometric analysis as detailed above.

**Antibodies and flow cytometry.** To analyze cytokine production, cultured or primary cells were incubated in complete IMDM with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma) and ionomycin (50 ng/ml; Sigma), in the presence of GolgiStop and 4 µM monensin. In some experiments, cell suspensions were surface stained with fluorochrome-conjugated antibodies in PBS containing 0.5% BSA and 2 mM EDTA (staining buffer) for 30 min at 4 °C. Primary cells were blocked with anti-FcR (93; Biolegend), prior to staining surface markers. To allow for the exclusion of dead cells in all analyses, a fixable viability dye (eFluor 780; eBioscience, 00-5523-00), with subsequent fixation and permeabilization, was used following antibody staining. For intracellular staining, cells were washed twice and resuspended in Fixation-Permeabilization solution (Foxp3/Transcription Factor Staining Buffer Set; eBioscience; 00-5323-00), with subsequent intracellular staining performed as detailed in the protocol provided with the kit. For surface staining, antibodies were purchased from eBioscience against the following antigens: CD4 (RM4-5; 1 : 500), CD8α (53-6.7; 1 : 500), TCRβ (H57-597; 1 : 500), CD69 (H1.2E3; 1 : 200), and CD45 (30-F11; 1 : 500). Antibodies were also purchased from eBioscience for intracellular staining of the following antigens: IL-17A (eBioscience; 80-1781-88), Foxp3 (eBioscience; 05-5820-85), RORγt (M1-14D12; 1 : 600). AnnexinV staining (Annexin V Apoptosis Detection Kit; eBioscience; 88-8006-74) was performed prior to fixation and in accordance with the manufacturer’s protocol provided. For analysis of cell proliferation, cells were labeled with CFSE (CellTrace CFSE Cell Polyrlation Kit; Life Technologies; C34554) according to the protocol provided by the manufacturer and cultured in Th17 cell polarizing conditions for 72 h. Antibodies were purchased from eBioscience unless otherwise indicated. Cells were acquired on a BD FACSCantoII flow cytometer. Brieﬂy, the infected skin was washed once with PBS and resuspended in complete IMDM, followed by stimulation and flow cytometric analysis as detailed above.

**Western blot.** For immunoblot of JunB, WT/Cd4Cre naive CD4+ T cells were cultured for 5 days in Th17 iTreg, Th1, or Th2 polarizing conditions. KC/Cd4Cre naive CD4+ T cells were also cultured in Th17 cells conditions as a negative control. On day 5, cells were harvested, resuspended in RIPA lysis buffer (25 mM Tris, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), and incubated at 4 °C for 30 min with rotation. Lysates were sonicated and subsequently centrifuged to remove debris. Equivalent masses of protein were mixed with LDS sample buffer (Invitrogen), separated on a 10% acrylamide gel, and transferred to PVDF membrane. Membranes were blocked with 5% BSA containing 0.5% nonfat dry milk, and subsequently, incubated with anti-JunB (N-17; sc-46x; 1 : 2000) or anti-Actin Ab-5 (C4/actin; BD Transduction laboratories; 1 : 2000) as a loading control. ECL western blotting substrate (Thermo Fisher Scientific) was used in accordance with the manufacturer’s instructions, for detection of horseradish peroxidase-labeled secondary antibodies.

**Luciferase assay.** Select JunB-bound regions within the Il17a, Ifnb, Thbs2, Ifng, and Foxp3 loci were assessed by dual luciferase reporter assays for enhancer and silencer activity by cloning the sequences upstream of a minimal promoter driving a luciferase gene (pGL4.21[luc2/minP]) or a characterized enhancer from the Il17a locus (CNS-5) cloned into pGL4minP, respectively. Primers used for cloning are available in Supplementary Table 3. Sort-purified naive CD4+ T cells were co-cultured for 48 h in a 2 : 1 cell ratio in culture incubated in complete IMDM. Cell suspensions were surface stained with fluorochrome-conjugated antibodies in PBS containing 0.5% BSA and 2 mM EDTA (staining buffer) for 30 min at 4 °C. Primary cells were blocked with anti-FcR (93; Biolegend), prior to staining surface markers. To allow for the exclusion of dead cells in all analyses, a fixable viability dye (eFluor 780; eBioscience, 00-5523-00), with subsequent fixation and permeabilization, was used following antibody staining. For intracellular staining, cells were washed twice and resuspended in Fixation-Permeabilization solution (Foxp3/Transcription Factor Staining Buffer Set; eBioscience; 00-5323-00), with subsequent intracellular staining performed as detailed in the protocol provided with the kit. For surface staining, antibodies were purchased from eBioscience against the following antigens: CD4 (RM4-5; 1 : 500), CD8α (53-6.7; 1 : 500), TCRβ (H57-597; 1 : 500), CD69 (H1.2E3; 1 : 200), and CD45 (30-F11; 1 : 500). Antibodies were also purchased from eBioscience for intracellular staining of the following antigens: IL-17A (eBioscience; 80-1781-88), Foxp3 (eBioscience; 05-5820-85), RORγt (M1-14D12; 1 : 600). AnnexinV staining (Annexin V Apoptosis Detection Kit; eBioscience; 88-8006-74) was performed prior to fixation and in accordance with the manufacturer’s protocol provided. For analysis of cell proliferation, cells were labeled with CFSE (Cell Trace CFSE Cell Polyrlation Kit; Life Technologies; C34554) according to the protocol provided by the manufacturer and cultured in Th17 cell polarizing conditions for 72 h. Antibodies were purchased from eBioscience unless otherwise indicated. Cells were acquired on a BD FACSCantoII and then analyzed with FlowJo software (Tree Star).

**Cutaneous infection with Candida albicans.** C. albicans strain SC5314 was kindly provided by the laboratory of Dr. Joseph Heitman. They were grown in YPD medium at 30 °C overnight for yeast morphology, and the next day diluted to OD600 of 0.1 and cultured under anaerobic conditions. The cDNA was washed once with PBS, and resuspended to infect each mouse with 2 × 10⁸ yeast in 50 µl PBS. An ~2 cm² region of the ventral skin was plucked and scotch tape used to abrade the area. The 50 µl C. albicans suspension was subsequently applied to the skin. On day 5 post infection, mice were euthanized and leukocytes isolated from infected skin for phenotyping by flow cytometry. Brieﬂy, the infected skin was excised and minced in complete IMDM. The tissue was digested for 1 h at 37 °C in complete IMDM supplemented with Collagenase D (1 mg/ml), dispase (5 U/ ml), and DNase I (0.1 mg/ml). The digests were subsequently filtered through a 100 µm cell strainer and the cells washed once with PBS. The cell pellet was washed once with PFH and resuspended in complete IMDM, followed by stimulation and flow cytometric analysis as detailed above.

**Statistical analysis.** A standard unpaired two tailed Student’s t test was used, unless otherwise stated, to determine the statistical probability of the difference observed between two populations of cells in our phenotyping analyses (GraphPad Software): *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Parametric tests were used when variance between groups was similar, as determined by F-tests; however, when variance was significantly different non-parametric tests were performed.

**Library preparation and sequencing.** RNA was purified from Th cultures for RNA-Seq using the Trizol method and used directly for library preparation. For ChIP-Seq, the same general ChIP protocol (see above) was used with some modifications. Brieﬂy, 4 × 10⁶ Th17 cells were cross-linked and the resulting sonicate was subjected to immunoprecipitation using 10 µg of anti-JunB (N-17; sc-46x), anti-JunB (C-11; sc-8051x), anti-BATF (WW8; sc-100974), anti-IRF4 (M-17; sc-6059x), anti-Fra2 (Q-20; sc-604x), or anti-JunD (329; sc-74x). The labeled beads were extensively washed, followed by resuspension in 200 µl of lysis buffer (25 mM Tris, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS), and incubated at 4 °C for 30 min with rotation. Lysates were sonicated and subsequently centrifuged to remove debris. Equivalent masses of protein were mixed with LDS sample buffer (Invitrogen), separated on a 10% acrylamide gel, and transferred to PVDF membrane. Membranes were blocked with 5% BSA containing 0.5% nonfat dry milk, and subsequently, incubated with anti-Fra2 (Q-20; sc-604x), or anti-JunD (329; sc-74x). The labeled beads were subsequently fragmented with a Vibra-Cell VCX130PB (Sonics & Materials). For immunoprecipitation, anti-mouse IgG1 or protein G Dynabeads were incubated overnight at 4 °C with 10 µg of anti-JunB (N-17; sc-46x), anti-JunB (C-11; sc-8051x), anti-BATF (WW8; sc-100974), anti-IRF4 (M-17; sc-6059x), anti-Fra2 (Q-20; sc-604x), or anti-JunD (329; sc-74x). Following reverse-crosslinking, ChIP and input samples were processed to obtain purified DNA as described.
integrity was first verified for quality using an Agilent Bioanalyzer. Libraries were then prepared for RNA-Seq using a Kapa Stranded mRNA-Seq Kit (Kapa Biosystems; Cat # KK8420), or for ChIP-Seq using a Kapa Hyper Prep Kit (Kapa Biosystems; Cat # KK8504). Libraries were then run on an Illumina HiSeq 2500 to generate 50 bp single-end reads. Sequence read quality was subsequently verified using FastQC, and only high-quality samples were used for downstream analysis.

Sequence read alignment. Raw sequence data was either generated in house (Junb KO RNA-Seq and Junb ChIP-Seq) or downloaded from the NCBI SRA database (see Supplementary Data 1 for full listing of datasets used). For RNA-Seq experiments, raw sequence reads were aligned to the Mus musculus mm10 genome using the gapped-read alignment software TopHat2 (ver. 2.0.13), with the following parameters: "--q 10 --g 20--novel-juncs".23 UscSc mm10 genomes as a reference. For ChIP-Seq experiments, raw sequence reads were sequenced to the Mus musculus mm10 genome using Bowtie2 (ver. 2.2.5), with the setting "–k 1".28

Differential expression analysis. Reads were assigned to features found in the UCSC mm10 genome annotation and counted using the “count” function from the HTSeq package (ver. 0.6.1), with options "–s no –m union".29 Raw feature counts were then analyzed for differential expression using the edgeR (ver. 3.16) software package.30 Briefly, within each comparison, raw counts were filtered to remove genes with less than 1 count per million reads in at least two of the samples analyzed. Filtered counts for biological replicates were then used to sequentially estimate library size and dispersion by calling the estimateSizeFactors(), estimateDisp() functions, and estimate the Log2FoldChange() function. Finally, differential expression testing was performed on normalized counts using the exactTest() function to generate a differentiation expression table, including log2 fold-change and FDR-adjusted p-values (Benjamini-Hochberg method) used in subsequent analyses. Genes were considered significantly different if having an FDR-adjusted p-value < 0.05. Two independent biological samples were used for analysis.

Pathway analysis. Ingenuity canonical pathway analysis (IPA, Qiagen), was performed on significantly differentially regulated genes with an FDR<0.01 based on DE analysis of RNA-Seq data comparing Junbfl/fl Cd4cre vs. Junbfl/fl Cd4cre Th17 cell differentiation cultures. This list included a total of 480 genes. Enriched pathways with a significance threshold of p<0.05 were reported.

Network creation, analysis, and visualization. Custom scripts were used to integrate RNA-Seq and ChIP-Seq outputs to compile a network of direct targets of transcription factors. Briefly, for each transcription factor-knockout RNA-Seq experiment (Junb, Batf, Stat3, IfNa, Rorc, and Foxl2), all differentially expressed genes (FDR<0.05) were added to the network and classified as “DIRECT” targets if at least one ChIP-Seq peak was found within 5 kb of the gene body, or “INDIRECT” if no proximal ChIP-Seq peaks were found. Targets were also classified as either “activation” targets (if gene expression was increased in TF-KO), or “repression” targets (if gene expression was increased). The resulting network was visualized using Cytoscape (ver. 3.4), where various differential expression datasets comparing in vitro differentiated T helper subsets were layered into the analysis for node coloring as indicated.25 For certain validated targets with ChIP-Seq peaks falling outside the 5 kb threshold (Ifng, Ifrb, and Tbx21) “DIRECT” network edges were added manually. In addition, the resulting network table was used to analyze the net effect of transcription factor regulatory pairs on subset-preferential gene expression by comparing differences in log2 fold-change of significant targets using custom scripts in R.

Code availability. All code is available upon request from the corresponding author.

Data availability. Sequence data that support the findings of this study have been deposited in GEO with the primary accession code GSE98414. Other sequence data referenced in this study are listed in Supplementary Data 1. All other data that support the findings of this study are available from the corresponding author upon request.

Received: 25 January 2017 Accepted: 12 June 2017 Published online: 21 August 2017

References

1. Nakayama, S., Takahashi, H., Kanno, Y. & O’Shea, J. J. Helper T cell diversity and plasticity. Curr. Opin. Immunol. 24, 297–302 (2012).
2. Lee, Y. K. et al. Late developmental plasticity in the T helper 17 lineage. Immunology 30, 92–107 (2009).
3. Mukasa, R. et al. Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. Immunity 32, 616–627 (2010).
4. Gugliani, L. & Khader, S. A. Th17 cytokines in mucosal immunity and inflammation. Curr Opin HIV AIDS 5, 120–127 (2010).
5. Zielinski, C. E. et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature 484, 514–518 (2012).
6. Morrison, P. J. et al. Th17-cell plasticity in Helicobacter hepaticus-induced intestinal inflammation. Mucosal Immunol. 6, 1143–1156 (2013).
7. Annunziato, F. et al. Phenotypic and functional features of human Th17 cells. J. Exp. Med. 204, 1849–1861 (2007).
8. Kehri, H. et al. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. Ann. Neurol. 66, 390–402 (2009).
9. Feng, T. et al. Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production. J. Immunol. 186, 6313–6318 (2011).
10. Harbour, S. N., Maynard, C. L., Zindl, C. L., Schoeb, T. R. & Weaver, C. T. Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. Proc. Natl Acad. Sci. USA 112, 7061–7066 (2015).
11. Hirota, K. et al. Fate mapping of IL-17-producing T’ cells in inflammatory responses. Nat. Immunol. 12, 235–243 (2011).
12. Hirota, K. et al. Plasticity of Th17 cells in Peyer’s patches is responsible for the induction of T cell-dependent IgA responses. Nat. Immunol. 14, 372–379 (2013).
13. Gagliani, N. et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. Nature 523, 221–225 (2015).
14. Ciofani, D. et al. A defined regulatory network for th17 cell specification. Cell 151, 289–303 (2012).
15. Yosef, N. et al. Dynamic regulatory network controlling Th17 cell differentiation. Nature 496, 461–468 (2013).
16. Ivanov, I. I. et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126, 1121–1133 (2006).

NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-00380-3 ARTICLE
25. Li, B., Tournier, C., Davis, R. J. & Flavell, R. A. Regulation of IL-4 expression by T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 8, 958–966 (2007).

26. Blonska, M., Joo, D., Zweidler-McKay, P. A., Zhao, Q. & Lin, X. CARMA1 is critical for IRF4-mediated transcription in T cells. *Nature* 490, 543–546 (2012).

27. Oliver, P. M. et al. NATFiP1 protein promotes the function of T(+) helper cell differentiation. *EMBO J.* 18, 420–432 (1999).

28. Blonska, M., Joo, D., Zweidler-McKay, P. A., Zhao, Q. & Lin, X. CARMA1 controls Th2 cell-specific cytokine expression through regulating JunB and GATA3 transcription factors. *J. Immunol.* 188, 3160–3168 (2012).

29. Oliver, P. M. et al. Ndt1p protein functions in the promotion of ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation. *Immunity* 25, 929–940 (2006).

30. Kenner, L. et al. Mice lacking JunB are osteopetrotic due to cell-autonomous osteoblast and osteoclast defects. *J. Cell Biol.* 164, 613–623 (2004).

31. Vahedi, G. et al. STATs shape the active enhancer landscape of T cell differentiation. *Nature* 506, 950–951 (1997).

32. Visel, A. et al. ChIP-seq accurately predicts tissue-specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Nature* 490, 543–546 (2012).

33. Visel, A. et al. ChIP-seq accurately predicts tissue-specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Nature* 490, 543–546 (2012).

34. Wang, X. et al. Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Nat. Immunol.* 13, 991–999 (2012).

35. Ghoreschi, K. et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 467, 967–971 (2010).

36. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

37. Wang, X. et al. Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Immunity* 36, 23–31 (2012).

38. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

39. Wang, X. et al. Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Immunity* 36, 23–31 (2012).

40. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

41. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

42. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

43. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

44. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

45. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

46. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

47. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

48. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

49. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

50. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

51. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

52. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

53. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

54. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

55. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

56. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

57. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

58. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

59. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).

60. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858 (2009).