Structure-function analysis of RBP-J–interacting and tubulin-associated (RITA) reveals regions critical for repression of Notch target genes

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The Notch pathway is a cell-to-cell signaling mechanism that is essential for tissue development and maintenance, and aberrant Notch signaling has been implicated in various cancers, congenital defects, and cardiovascular diseases. Notch signaling activates the expression of target genes, which are regulated by the transcription factor (CBF1/RBP-J, Su(H), Lag-1). CSL interacts with both transcriptional corepressor and coactivator proteins, functioning as both a repressor and activator, respectively. While Notch activation complexes are relatively well understood at the structural level, less is known about how CSL interacts with corepressors. Recently, a new RBP-J (mammalian CSL ortholog) interacting protein termed RITA has been identified and shown to export RBP-J out of the nucleus, thereby leading to the down-regulation of Notch target gene expression. However, the molecular details of RBP-J/RITA interactions are unclear. Here, using a combination of biochemical/cellular, structural, and biophysical techniques, we demonstrate that endogenous RBP-J and RITA proteins interact in cells, map the binding regions necessary for RBP-J/RITA complex formation, and determine the X-ray structure of the RBP-J/RITA complex bound to DNA. To validate the structure and glean more insights into function, we tested structure-based RBP-J and RITA mutants with biochemical/cellular assays and ITC (isothermal titration calorimetry). While our structural and biophysical studies demonstrate RITA binds RBP-J similar to the RAM (RBP-J associated molecule) domain of Notch, our biochemical and cellular assays suggest RITA interacts with additional regions in RBP-J. Taken together, these results provide molecular insights into the mechanism of RITA-mediated regulation of Notch signaling, contributing to our understanding of how CSL functions as a transcriptional repressor of Notch target genes.

Notch signaling is a highly conserved component of metazoan development and tissue homeostasis (1). Genetic ablation of Notch signaling is embryonic lethal (2). Furthermore, mutations leading to dysfunctional Notch signaling have been linked to certain types of cancers, cardiovascular disease, and birth defects, highlighting the importance of the Notch pathway in human disease (3,4). Due to the negative human health outcomes associated with aberrant Notch signaling, there are ongoing efforts towards developing reagents that modulate Notch signaling to be used as potential therapeutics (5).

Notch is a juxtacrine signaling mechanism that is initiated when the extracellular domain of a DSL (Delta-like in mammals, Serrate in flies, LAG-2 in worms) ligand present on one cell binds a single-pass transmembrane Notch receptor on an adjacent cell (6) (Figure 1A). In mammals, there are five DSL ligands (Jagged1,2 and Delta-like1,3,4) and four Notch receptors (Notch1-4) (6). Ligand-receptor interactions induce the Notch receptor to undergo a series of cleavage events, resulting in the release of the NICD (Notch Intracellular Domain) from the cell membrane and its subsequent translocation to the nucleus (6). In the nucleus, NICD binds the transcription factor CSL (CBF1/RBP-J in mammals, Su(H) in flies,
LAG-1 in worms) and the transcriptional coactivator MAM (Mastermind) (Figure 1B) (7).

CSL has three domains that mediate contacts with coactivators and corepressors (Figure 1C-D): the NTD (N-terminal domain), the BTD (β-trefoil domain), and the CTD (C-terminal domain) (7). NICD contains a RAM (RBP-J associated molecule) and an ANK (ankyrin repeats) domain that interacts with the BTD and CTD of CSL, respectively, which allow for MAM binding to the CTD and NTD (8-10). The CSL-NICD-MAM ternary complex binds enhancer and promoter elements of Notch target genes and functions as the switch to activate transcription at these sites (11). In the absence of a Notch signal, CSL functions as a transcriptional repressor by binding to corepressor proteins (11).

When interacting with corepressors, such as KyoT2 (12), MINT (MSX-2 interacting nuclear target)/SHARP (SMRT/HDAC-1-Associated Repressor Protein) (13,14), or Hairless (15), the function of CSL is to anchor the assembly of higher order repression complexes at Notch target gene sites. CSL-corepressor complexes often contain histone-modifying activity responsible for changing the local chromatin into a repressive environment (11). Early models in the field hypothesized that CSL was constitutively bound to DNA and activation was a function of displacing corepressors with coactivators (16). However, more recent studies have demonstrated that CSL-DNA interactions in vivo are more dynamic, showing that CSL occupancy at target genes increases after activation of Notch signaling (17,18). It remains inconclusive whether NICD is competing with corepressors for binding CSL molecules or if preassembled CSL transcriptional complexes are recruited to certain sites on DNA or if both modes are occurring simultaneously.

Structure-function studies of the Notch activator complex (CSL-NICD-MAM) have been seminal to understanding the transcriptional activation of Notch target genes (7); however, parallel structure-function studies of CSL-corepressor complexes and how CSL functions as a transcriptional repressor are lacking. Recently, a new transcriptional coregulator, termed RITA, has been identified from a yeast two-hybrid screen looking for RBP-J-binding partners (19). RITA is a 269 residue protein, which likely has little intrinsic secondary structure based on disorder prediction servers (Figure 2A-B). Proteomic studies have shown that RITA incurs numerous post-translational modifications in cells, including phosphorylation and acetylation (20,21).

Previously, a number of functional domains/regions have been mapped to RITA, including a RBPID (RBP-J interacting domain), a NLS (nuclear localization signal), a NES (nuclear export signal), and a C-terminal tubulin interacting domain (Figure 2A) (19). As such, RITA has been shown to bind RBP-J and facilitate its export out of the nucleus (Figure 1B) (19).

Previous studies have shown that RITA functions as a repressor of Notch mediated transcription in cells, as well as in Xenopus embryos where it is able to reverse the loss of primary neurogenesis caused by Notch overactivation (19). Moreover, RITA overexpression has been shown to suppress growth and promote apoptosis in hepatocellular carcinoma (22). While it is possible that this is due to repression of Notch target gene transcription, this remains to be determined.

The RBPID of RITA contains a conserved hydrophobic tetrapeptide motif (φWφP; where φ is any hydrophobic residue, Figure 2D). This motif is also present in the RAM domain of Notch1-4, the corepressor KyoT2, and the viral proteins EBNA2 (Epstein-Barr virus nuclear antigen 2) and EBNA3C (Figure 2D). The φWφP motif is critical for binding to the BTD of CSL (23,24). The φWφP motif in RITA contains an atypical threonine (T143) in the third position (Figure 2D), and interestingly, T143 has also been shown to be phosphorylated in cells (20). Three additional motifs have been identified in RAM that contribute to the high affinity interaction with the BTD; these motifs consist of an amino terminal basic region, and -HG- and -GF- dipeptide motifs (Figure 2D) (25). However, these motifs are not present in other BTD-binders, including RITA.

In order to deepen our understanding of the RBP-J/RITA corepressor complex, here we undertook a comprehensive structural, biophysical, and cellular characterization of RBP-J/RITA interactions. To this end, we determined the high-resolution X-ray structure of the RBP-J/RITA corepressor complex bound to DNA, defined the biochemical/cellular and thermodynamic interactions between RBP-J and RITA, analyzed how post-translational modifications in RITA...
affect these interactions, and begun to characterize in cells how RITA acts to repress Notch target gene transcription. Taken together, our results provide molecular insights into RBP-J/RITA interaction, expanding our knowledgebase of CSL-corepressor complexes.

RESULTS

Endogenous RBP-J and RITA proteins interact in cells—Previous studies overexpressed RBP-J and RITA constructs in cultured cells to demonstrate biochemically that RBP-J interacts with RITA (19). In order to show that endogenous RBP-J and RITA proteins form a complex in cells, we generated a RITA-specific monoclonal antibody (H35-2, Figure 2A). We first tested the H35-2 hybridoma supernatant using lysates from HeLa and HEK-293 cells. The antibody recognizes predominantly a protein of approximately 36 kDa which is consistent with the calculated molecular mass of RITA (Figure 3A). Next we tested lysates from several cell lines of epithelial and hematopoetic origin for RITA expression (Figure 3B). Consistent with RITA mRNA being ubiquitously expressed in vivo (19), we detected RITA in all cell lines used. HEK-293, HeLa, and Jurkat cells exhibited the highest levels of RITA expression. RITA is also detected in A549, HEL-92.1.7, Kasumi-1 and SUP-T1 cells (Figure 3B). While there appears to be a dominant species of RITA in all of the cell lines tested, interestingly, various minor species of RITA appear in different cell lines, suggesting that RITA is post-translationally modified in a cell type specific manner (Figure 3B). To demonstrate that endogenous RBP-J and RITA proteins interact, we performed coimmunoprecipitation experiments using lysates from HeLa cells. As shown in Figure 3C, endogenous RBP-J is coimmunoprecipitated only after RITA is immunoprecipitated with the anti-RITA supernatants H35-2, H35-1 or H35-9, but not with IgG alone.

Next, we sought to better define the regions of RITA that are required for interacting with RBP-J in cells (Figure 4). We designed several deletion constructs of RITA (Figure 4A) and transfected these GFP-RITA fusion constructs into HeLa cells. Fusion protein expression and subcellular location were confirmed using fluorescence microscopy (Figure 4B). Subcellular localization of RITA proteins confirmed the previously identified NLS and Tubulin binding regions (19). As shown in Figure 4C, endogenous RBP-J is co-immunoprecipitated with all GFP-RITA fusion protein constructs that contain the RBPII region, except for the construct that corresponds to RITA residues 120-161. Taken together, these data suggest that the RBPII region is required for the interaction with RBP-J in cells, but not sufficient, as other regions outside of the RBPII, such as RCR2, may also be involved in interactions with RBP-J in vivo.

Thermodynamic analysis of the requirements for RBP-J/RITA complex formation—Subsequently, we used ITC and circular dichroism (CD) with recombinant RBP-J/RITA proteins purified from bacteria to further analyze the binding interactions between RBP-J and RITA in vitro (Figure 2B, Figure 5 and Table 1). As shown in Figure 5A and Table 1, a RITA construct (106-173) that includes the RBPII and corresponds to the smallest construct in cells that interacts with RBP-J binds with a moderate ~1 µM K_d. For perspective, the affinity of RBP-J for RITA is two orders of magnitude weaker than the previously characterized NICD and KyoT2 interactions with RBP-J, but comparable to interactions that the viral coactivator EBNA2 make with RBP-J (25-27). The binding of RITA to RBP-J is enthalpically driven and incurs an entropic penalty (Table 1), which is consistent with RITA being an intrinsically disordered protein prior to interacting with RBP-J. We used far UV CD to confirm that RITA is largely disordered in solution (Figure 2B). The binding affinity of RITA to RBP-J is only marginally (<2fold) increased by the presence of DNA containing a consensus CSL binding site (Table 1). Similar to RBP-J interactions with other coregulators, such as RAM and KyoT2, there is a substantial (>2kcal/mol) favorable increase in the entropy of binding to RITA when RBP-J is prebound to DNA (Table 1) (26,27).

In order to determine the region of RITA necessary and sufficient to bind RBP-J in vitro, we performed a series of ITC experiments with extended and serially truncated RITA constructs (Figure 5 and Table1). In contrast to our cellular/biochemical binding assays, RITA (127-158), which contains only the RBPII, binds RBP-J with similar affinity as the longer RITA construct (106-173) (Figure 5A-B, Table 1). As shown in Table 1 and Figure 5, further shortening
of RITA (133-151, 133-148, 135-148, 137-148, and 137-146) resulted in similar binding to RBP-J as RITA (127-158), except for RITA (139-146), and RITA (133-148) resulted in similar binding to RBP-J (Table 1). These data suggest that RITA (137-148) encompasses the minimal region required to interact with RBP-J in vitro.

To define what domains of RBP-J interact with RITA, we performed ITC experiments with constructs that correspond to the BTD and BTD-CTD domains of RBP-J with RITA (127-158) (Figure 5C-D and Table 1). Binding experiments performed with RITA and either the isolated BTD construct or the BTD-CTD construct showed similar binding as full-length core RBP-J. These data suggest that RITA binds the BTD of RBP-J.

To determine the change in heat capacity ($\Delta C_p$) associated with RITA binding to RBP-J, we performed a series of ITC experiments varying the temperature of the binding reaction. A negative $\Delta C_p$ is indicative of burial of nonpolar surfaces that occur during protein complex formation (28). As shown in Figure 5F and Table 2, the change in free energy, enthalpy, and entropy ($\Delta G^\circ$, $\Delta H^\circ$, $T \Delta S^\circ$) were analyzed as a function of temperature (5°, 15°, 25°, 35°C). While $\Delta G^\circ$ is temperature independent, $\Delta H^\circ$ and $\Delta S^\circ$ change with respect to temperature in order to maintain a constant $\Delta G^\circ$. From this analysis, we determined that the $\Delta C_p$ associated with RBP-J/RITA interactions is -0.51 kcal/mol·K, which is similar to previously characterized CSL complexes, such as CSL-Kyot2 ($\Delta C_p = -0.57$ kcal/mol·K) and CSL-RAM ($\Delta C_p = -0.62$ kcal/mol·K) (26,29), despite RITA’s ~100 fold weaker binding to RBP-J compared to these other coregulators.

Structure of the RBP-J/RITA/DNA Complex—In order to determine the X-ray structure of the RBP-J/RITA complex bound to DNA (PDB ID: 5EG6), we purified recombinant RBP-J (a.a. 53-474) from bacteria, and combined this with a RITA peptide corresponding to residues 133-151 and an oligomeric 15mer DNA duplex, containing a single CSL binding site. Using this complex to screen crystallization conditions resulted in an orthorhombic (P2_12_1) crystal form that diffracted to 2.1 Å resolution. Molecular replacement using published CSL-DNA structures was utilized to solve the RBP-J/RITA/DNA complex structure (27,30). The asymmetric unit contains a single RBP-J/RITA complex bound to DNA (Figure 6A). The final model consists of residues 53-474 of RBP-J, residues 133-148 of RITA, and the 15mer oligomeric DNA duplex, which was refined to an $R_{work}$ and $R_{free}$ of 19.3% and 23.6%, respectively (Table 3).

Overall, the fold of RBP-J in the context of the RBP-J/RITA/DNA complex largely conforms to that observed in previous CSL structures (Figure 6A). However, one notable difference is observed in the CTD, whereby there is a rigid body shift by as much as ~10Å away from the BTD when compared to the structure of RBP-J/DNA (PDB ID: 3IAG or 3BRG), resulting in a more open conformation (Figure 6B). While the significance of this unusual CTD confirmation remains to be determined, it should be mentioned that molecular dynamics simulations of the RBP-J/RITA/DNA complex suggest that the observed CTD conformation is a low energy conformer and not a strained conformation (data not shown).

Consistent with our ITC binding studies, RITA binds exclusively to the BTD of RBP-J in an extended conformation (Figure 6A,C). A $\beta$-hairpin loop within the BTD forms a short antiparallel $\beta$-strand with N-terminal residues of RITA (a.a.135-148) (Figure 6A,C). The $\varphi$WφP motif of RITA (-LWTP-; a.a.141-144) binds a nonpolar pocket on the surface of the BTD, and overall, the RBP-J/RITA interaction buries an estimated 987Å$^2$ of surface area, similar to other BTD-binders such as the RAM domain of Notch (950Å$^2$) and the corepressor Kyot2 (874Å$^2$). Residues 133-148 of RITA had appreciable electron density while the remaining three C-terminal residues were not well resolved (Figure 6C), which is consistent with our ITC binding studies that suggest these C-terminal residues contribute little to complex formation (Table 1).

Next, we compared the structure of RITA bound to RBP-J with the structures of RAM (PDB ID: 3V79 and 3BRD) and Kyot2 (PDB ID: 42JX) bound to the BTD of RBP-J (Figure 6E) (26,27,31). Overall, there is a large degree of structural correspondence between all of the structures, in particular in the region containing the $\varphi$WφP motif, with more structural differences located at the N- and C-terminal residues of RITA, RAM, and Kyot2 (Figure 6E). This is consistent with less sequence similarity for these
proteins in this region (Figure 2D). Interestingly, this structural comparison revealed that both RITA and KyoT2 form salt bridges with the BTD of RBP-J. In this case, R138 of RITA and K187 of KyoT2 form ionic interactions with the well conserved residues E259 and E260 of RBP-J (Figure 6D), providing a structural explanation for the conserved role of these residues in RBP-J. Consistent with the importance of this interaction in RBP-J/RITA complex formation, and likely other BTD-binders as well, disruption of the salt bridge by mutation results in a ~30 fold ($\Delta \Delta G^o = 2 \text{kcal/mol}$) loss of binding in ITC binding studies (Table 4).

**Binding Analysis of Structure Based Mutants**—Given the similar binding mode of RITA and RAM for RBP-J, we analyzed the binding of RITA to RBP-J using a number of BTD mutants (Figure 6F) that we previously showed to affect RBP-J/RAM interactions (26,32). All of these mutants had a dramatic effect on RAM binding to RBP-J, ranging from ~10-500 fold reductions in affinity (32). As shown in Table 4, BTD mutations at positions F261 and A284, which are near where the $\phi W\phi P$ motif binds, have the largest effect on the affinity of RITA for RBP-J, ranging from ~10-fold reduction in binding (A284R) to no detectable binding by ITC (F261R). The BTD mutations at V263 and Q333, which interact with residues near the N- and C-termini of RITA, have a more modest effect on binding, ranging from a ~2-4 fold reduction in affinity. We also mutated the WTP motif of RITA (WTP/AAA), and as expected, no binding with RBP-J was detected (Table 4).

Proteomic studies have revealed that RITA is post-translationally modified in cells, and interestingly, a number of these modifications are contained within the RBPID of RITA (Figure 2C) (20,21). Therefore, we characterized the effect of these modifications on RBP-J/RITA binding, as these post-translational modifications may have a role in regulating this interaction. Previous studies have shown that RITA is phosphorylated on residues T143 and T147, and is acetylated on residues K131 and K136 (Figure 2C) (20,21). As shown in Table 5, phosphorylation of T143, which resides within the $\phi W\phi P$ motif of RITA, results in >10 fold weaker binding ($\Delta \Delta G^o = 1.5 \text{kcal/mol}$). In contrast, phosphorylation of T147 of RITA, which in the structure is at the C-terminus and is characterized by higher temperature factors, results in only a modest effect on binding (~2fold, Table 5). Acetylation of K131/K136 of RITA results in ~5 fold weaker binding to RBP-J (Table 5), consistent with our structural data that shows that these lysine residues are not making critical interactions with RBP-J (Figure 6C).

**Cellular characterization and validation of the RBP-J/RITA corepressor complex**—We used transcriptional reporter assays to validate our RBP-J/RITA/DNA structure and further characterize RBP-J/RITA interactions in cells. To perform these studies, we used mouse embryonic fibroblasts (MEFs) derived from RBP-J null embryos and transduced these MEFs with retroviruses expressing either wild-type or mutant RBP-J proteins. A luciferase reporter that contains four RBP-J binding sites (4xCBS) and a constitutively active form of the Notch1 receptor (NICD1) are co-transfected into the cells to monitor and activate Notch signaling (26,32).

To demonstrate that RITA was functioning in our cultured MEFs similar to previous studies (19), we transfected increasing amounts of RITA and analyzed the effect it had on the Notch activated reporter. As shown in Figure 7A, RITA represses transcription from the reporter in a dose dependent manner. Next, we analyzed the effects the BTD mutants of RBP-J (F261A, V263, A284V, Q333A) had on the ability of RITA to repress activity from the transcriptional reporter in cells. It should be mentioned that these BTD mutants are inherently reduced for reporter activation due to the mutations affecting NICD binding to RBP-J (Figure 7B) (26,32). Thus, “relative activity” plotted on the y-axis represents relative to the maximal activity of that particular mutant and not the native protein (Figure 7C-7F). Consistent with our binding studies and RBP-J/RITA structure, all four BTD mutants showed significantly less repression by RITA. These data suggest that the BTD mutations weaken the interactions between RBP-J and RITA in cells when compared to native RBP-J, thereby requiring higher levels of RITA expression to repress transcription from the reporter. However, somewhat contrary to our ITC binding studies, the F261A mutant only modestly affected RITA mediated repression of the reporter, whereas Q333A had a stronger effect. These data suggest
that regions outside the RBPID of RITA may be affecting interactions with RBP-J.

Finally, we tested several different RITA constructs in our cellular assays to address the importance of the RBPID and NLS/NES regions in mediating repression from the reporter in cells (Figure 8). Consistent with previous studies (19), removal of either its nuclear export or import sequences results in a significant loss in the ability of RITA to repress transcription from the reporter (Figure 8A-B). Next, we transfected a RITA construct that corresponds to residues 106-173, which contains the RBPID, but not the NES. In this case, similar repression was observed for the truncated construct compared to native full-length RITA, but not the NES. In this similar repression was observed for the truncated construct compared to native full-length RITA, but statistically significant differences were observed at some amounts of transfected RITA (Figure 8C). These data provide additional support that the NES of RITA and nuclear export of RBP-J are part of the RITA mediated repression mechanism in cells. We also tested a full-length RITA construct, in which its WTP motif had been mutated to alanine (WTP/AAA, Figure 8D). Interestingly, the WTP/AAA mutation severely affected the ability of RITA to repress activity from the reporter, but did not completely abrogate it, supporting the hypothesis that regions outside the RBPID contribute to RBP-J/RITA interactions in vivo.

**DISCUSSION**

Activation of the Notch signaling pathway results in transcription of target genes, mediated by the transcription factor CSL (1). This mechanism involves NICD and MAM binding CSL, and recruiting additional coactivators and general transcription factors (11). CSL can also function as a repressor by binding to corepressors, such as MINT/SHARP, KyoT2, and RITA in mammals, and Hairless in flies (13-15,19). Understanding the molecular details of how coactivators and corepressors interact with CSL will be critical to building a detailed mechanistic model of the Notch pathway. Furthermore, knowledge gleaned from structure-function studies of CSL-mediated transcription complexes will inform and guide the discovery and design of reagents capable of modulating Notch signaling for therapeutic benefit.

Building upon prior structure-function studies of CSL-coregulator complexes, our work here comprehensively characterizes the interactions between RBP-J and the corepressor RITA both in vitro and in cells. We show that RITA is expressed in a variety of commonly used cell lines (Figure 3B), and importantly, that endogenous RBP-J and RITA proteins interact (Figure 3C). Consistent with previous studies (19), we also show that RITA is a potent repressor of reporter activity in our transduced MEFs (Figure 7A), which is dependent on both the NES and NLS of RITA (Figure 8A-B).

These studies prompted us to better define the regions in RITA that are required to interact with RBP-J in cells. We show that a region that includes the RBPID of RITA, i.e. the φWφP motif, which is essential for a number of coregulators to bind CSL, is necessary for interactions with RBP-J, but interestingly, not sufficient (Figure 4C). Consistent with this result, a RITA construct, in which its φWφP motif has been mutated (WTP/AAA), still retains some activity in our transcriptional reporter assays (Figure 8D). Conversely, our ITC studies of RBP-J/RITA complexes showed no differences in binding for RITA constructs that only contained the RBPID when compared to longer RITA constructs (Figure 5 and Table 1). These data suggest that sequences outside of the RBPID contribute to RITA binding interactions with RBP-J in vivo. However, the nature of these upstream regions is unclear, but may involve unidentified post-translational modifications of RITA and/or an additional binding partner. Conceivably, these additional interactions may involve the CTD of RBP-J, as previous studies have implicated the CTD in RBP-J/RITA interactions (33).

Nonetheless, we determined the 2.1Å X-ray structure of the RBPID of RITA in complex with RBP-J and bound to DNA (Figure 6 and Table 3). RITA binds the BTD of RBP-J in much the same way as the RAM domain of NICD and the corepressor KyoT2. However, RITA responds differently to BTD mutants that were designed to affect RBP-J/RAM interactions. On one hand, RBP-J/RITA interactions are drastically affected by mutations at F261 and A284, which interact with more central residues of the RBPID including the φWφP motif; but on the other hand, RBP-J/RITA interactions are significantly less affected when compared to RAM by substitutions at V263 and Q333, RBP-J side chains that interact with...
RITA residues located more peripherally. Similar results with respect to the RBP-J mutants were observed in binding studies with KyoT2 (26); however, there are sequences differences in RITA and KyoT2 that discriminate their binding interactions with RBP-J as well. For example, the isolated KyoT2 φWφP motif (–VWWP–) weakly binds RBP-J with ~10 µM affinity (26), whereas the RITA φWφP motif (–LWTP–) does not (Figure 2D and Table 1); and interestingly, the RITA 8mer peptide (–ALLWTPPP–) binds ~20 fold more weakly than the corresponding KyoT2 peptide (–APVWWPMK–). Taken together, these data illustrate that different regions within coregulators that interact with BTD contribute different amounts of binding energy to achieve high affinity interactions with RBP-J. This may allow for more flexibility in the types of sequences that can interact with the BTD of RBP-J.

Our structural studies also uncovered an unappreciated interaction between conserved residues in the BTD of RBP-J and RITA, and which likely extends to all coregulators that bind the BTD of CSL. In this case, R138 of RITA is flanked by E259 and E260 of RBP-J, making simultaneous ionic interactions with both glutamates. A similar interaction in the corepressor KyoT2, which involves a lysine instead of an arginine, is also observed in the context of the RBP-J/KyoT2/DNA complex structure (26). Alanine substitution at these sites (EE259AA) results in an unexpected 30 fold reduction in binding (ΔΔG°=2kcal/mol), emphasizing the importance of this interaction. Previously, Johnson et al. demonstrated the contribution of the conserved –HG– dipeptide motif from the RAM domain of Notch in interactions with RBP-J (25); however, the structural basis for this energetic contribution to binding was not understood. The histidine in the –HG– dipeptide motif of RAM corresponds to R138 of RITA (Figure 2D). It is interesting to speculate that a similar ionic interaction between this histidine in RAM and BTD residues E259/E260 of RBP-J occurs, which would provide a structural rationale for the importance of this interaction. Albeit, the relatively low resolution structure of the human RBP-J/NICD/MAM/DNA complex that contains RAM does not support this interaction (31). Nonetheless, if this interaction occurs in solution, then the pKa of the histidine may be perturbed to maintain its positive charge and ionic interactions with E259/E260. Future RBP-J/RAM studies that analyze binding as a function of pH may lend support to this hypothesis.

Finally, similar to other transcriptional coregulators, such as NICD, RITA incurs numerous post-translational modifications, including phosphorylation, acetylation, and methylation, and interestingly RITA is acetylated and phosphorylated within its RBPID (20,21). Moreover, the threonine (T143) within RITA’s –WTP– motif is phosphorylated, a region that makes substantial contributions to binding RBP-J. Phosphorylation of T143 (pT143) results in a >10 fold loss in binding (Table 5). Given RITA’s other functions in the cell, e.g. tubulin binding, perhaps pT143 is a mechanism to target RITA binding to RBP-J or tubulin within the cell, but not both simultaneously. Intriguingly, the viral transcriptional coregulator EBNA3C, which also interacts with RBP-J, contains a –WTP– motif and is involved in a myriad of virus associated functions. Possibly phosphorylation of the threonine in the –WTP– motif of EBNA3C is also involved in regulating RBP-J/EBNA3C interactions.

RITA is acetylated on lysine residues within its RBPID, but this modification only modestly affects binding to RBP-J. Interestingly, the RAM domain of Notch is also acetylated, albeit on different lysines. In this case, RAM acetylation leads to stabilization and increased half-life of NICD in the nucleus, whereas SIRT1 deacetylates NICD, leading to downregulation of signaling. In future studies, it will be interesting to test whether RITA acetylation is also coupled to its half-life in vivo.

EXPERIMENTAL PROCEDURES

Cloning, expression, and protein purification—The Mus musculus CSL ortholog (RBP-J), residues 53-474 (CSL core domain), residues 203-393 (BTD), and residues 203-474 (BTD-CTD) were each cloned into the pGEX-6P-1 vector. Expression and purification was performed as previously described in (30). Briefly, transformed bacteria were grown at 37 °C in LB medium, cooled to 20 °C, induced with 0.1 mM isopropyl β-d-thiogalactopyranoside (IPTG), and grown overnight at 20 °C. The bacteria were
harvested by centrifugation and resuspended in PBS. The resuspended cells were lysed by sonication, cleared by centrifugation and filtration, and subsequently loaded onto a glutathione-Sepharose column. The column was washed with PBS, and the GST fusion protein was eluted using buffer containing reduced glutathione. The elutants were dialyzed and the GST tag cleaved with Precision Protease (GE Healthcare) per the manufacturer’s protocol. All of the protein constructs were further purified to homogeneity using ion exchange and size exclusion chromatography.

The human RITA ortholog, residues 106-173 or residues 127-158, was cloned into a modified pET 28b(+) vector. This vector encodes a fragment of SMT3 (suppressor of Mif2 temperature-sensitive mutant 3) for increased protein stability and expression, producing a His-SMT3-RITA fusion protein. The fusion protein was overexpressed as described above. The cleared lysate was incubated with Ni-NTA resin (Qiagen) and loaded into a gravity column. The column was washed, and fusion protein was eluted with imidazole-containing buffer. The fusion protein was then cleaved to remove His-SMT3 from the RITA moiety using the Ulp1 protease, which leaves only an N-terminal serine residue following cleavage. The RITA constructs were further purified to homogeneity using ion exchange and size exclusion chromatography. Constructs smaller than RITA 127-158 were purchased as HPLC purified synthetic peptides from Peptide 2.0 and received as lyophilized powder.

Fluorescence Microscopy—HeLa cells were plated at a concentration of 1x10^5 cells/cm^2 on chamber coverslips (Nunc). After 16 h, cells were transfected with 300 ng of GFP or GFP-RITA expression plasmids using the Nanofectin transfection reagent (PAA). 24 h after transfection pictures were taken from living cells using a fluorescence microscope (IX71, Olympus) equipped with a digital camera (C4742, Hamamatsu) and a 100-W mercury lamp (HBO 103W/2, Osram). The following filter set was used for GFP detection, ex: HQ470/40, em: HQ525/50.

Isothermal titration calorimetry—ITC experiments were performed using a Microcal VP-ITC micocalorimeter. For all binding reactions, syringe concentrations varied between approximately 200-250 µM RITA and cell concentrations varied between approximately 20-25 µM RBP-J. Titrations consisted of an initial 1 µL injection followed by 39 7 µL injections. ITC binding experiments were performed in 50 mM sodium phosphate pH 6.5, 150 mM NaCl at 5°C, 15°C, 25°C, or 35°C. Samples were buffer matched using size-exclusion chromatography. The collected data were analyzed using the ORIGIN software and fit to a one site binding model.

Crystallization and data collection—A 15-mer DNA duplex (TTACTGTGGGAAAGA, AATCTTTCCCACAGT) with single-stranded TT/AA overhangs and containing a single CSL binding site from the HES-1 gene was co-crystallized with mouse CSL and human RITA. RBP-J/RITA/DNA complexes were set up in a 1:1.1:1.1 molar ratio and screened for crystallization conditions using the Hampton Research Index Screen and an Art Robbins Phoenix Crystallization Robot. The final optimized crystallization conditions were in a mother liquor containing 0.1M Bis-Tris pH5.5,
Structure-function of the RBP-J/RITA complex

0.2M Ammonium Acetate, 10% 1,4-butanediol, and 16% polyethylene glycol 3350. Crystals were grown at 4°C using microbatch under paraffin oil methods, were cryoprotected in mother liquor solutions containing 20% 1,4-butanediol, and flash frozen in liquid nitrogen. The diffraction data were collected at the Advanced Photon Source (LS-CAT). The crystals diffracted to 2.1 Å and belong to the orthorhombic space group P2₁2₁2₁, with unit cell dimensions of 76.78Å, 96.41Å, and 96.71Å.

Structure determination, model building, and refinement—Collected data was processed and scaled using HKL-2000 (34). Phaser was used to generate a molecular replacement solution (35), using the structure of mouse CSL bound to DNA (PDB ID: 3IAG) as a search model (27). Coot was used to manually rebuild missing parts of the model (36). Translation/libration/screw (TLS) parameters were calculated and used for refinement in Phenix Refine (37). Structural validation was performed using Molprobity (38). Our final model of the RBP-J/RITA complex bound to DNA consists of amino acids 53-474 of RBP-J, amino acids 133-148 of RITA, and the entire DNA duplex. The structure has been refined to a Rₜₜw = 19.3% and R_free = 23.6% and deposited in the Protein Data Bank (PDB) (www.rcsb.org) with PDB ID: 5EG6 (39). We used PyMOL Molecular Graphics System, Version 1.3 for structural visualization and alignments (pymol.sourceforge.net) (40). The PDBePISA server (http://www.ebi.ac.uk/pdbe) was used to analyze protein-protein interfaces (41).

Cellular Reporter Assays—Mouse embryonic fibroblasts (MEFs) originating from RBP-J knockout embryos (OT11) were transduced with retroviruses that express either wild-type or mutant RBP-J proteins as described in (32). Transduced MEFs were grown to 50% confluence in six-well plates and transiently transected with a constitutively active NICD1 construct, a 4xCBS luciferase reporter containing four CSL-binding sites, and Renilla luciferase construct (phRL). Wild-type or mutant RITA constructs were cotransfected in increasing concentrations in order to measure the repressive effects of RITA on Notch-mediated transcriptional activation of the luciferase reporter. TransIT®-2020 transfection reagent (Mirus) was used for all transfections along with pBlueScript (Stratagene) in order to normalize the amount of DNA transfected in each experimental group. 48 hours post transfection, cells were harvested and prepared for measurement of firefly luciferase and Renilla luciferase activity. The Dual Luciferase Kit (Promega) was used to measure luciferase activity. For each experiment, firefly luciferase activity from the 4xCBS reporter was first normalized to Renilla luciferase activity. Normalized data was reported as either fold activation or relative activity. Average values, errors, and SD were determined from at least three independent experiments performed in duplicate.

Circular Dichroism—RITA (127-158) was characterized in a buffer containing 10mM Tris-phosphoric acid pH 7.4 and 50mM NaF at a concentration of 384µM (1.36 mg/ml). CD data were analyzed on DICHROWEB using the CDSSTR analysis program with reference set 7 (42,43). CD measurements were taken in triplicate using an Aviv Circular Dichroism Spectrometer Model 215 at 25 °C in a 0.01 cm cuvette. Wavelength scans were performed between 185nm and 290nm using 1nm increments.

ACKNOWLEDGEMENTS
We thank the Kovall and Oswald labs for their technical assistance. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Atomic coordinates and structure factors (PDB ID: 5EG6) were deposited in the Protein Data Bank (www.rcsb.org).

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
RAK and FO conceived of the study. RAK, NT, ZY, and FO coordinated the research and contributed to data interpretation. NT and ZY expressed and purified proteins, and NT solved the crystal structure of the RBP-J/RITA complex bound to DNA. NT, ZY, and FO designed DNA constructs and conducted experiments. All authors wrote, edited, and approved the final draft of the manuscript.

REFERENCES


**FOOTNOTES**

Project support was provided by the National Institutes of Health Grants 5R01CA178974 to RAK and 5T32CA117846 to NT. This work was further supported by the German Research Foundation (DFG, SFB1074/A3) and by the BMBF (Federal Ministry of Education and Research, research nucleus SyStAR) to FO.

Abbreviations used: RITA, RBP-J interacting and tubulin associated; CSL, CBF1/RBP-J, Su(H), Lag-1; ITC, isothermal titration calorimetry; DSL, Delta-like/Serrate/LAG-2; NICD, Notch Intracellular Domain; CSL, CBF1/Su(H)/LAG-1; MAM, Mastermind; NTD, N-terminal domain; BTD, β-trefoil domain; CTD, C-terminal domain; RAM, RBP-J associated molecule; ANK, ankyrin repeats; MINT, MSX-2 interacting nuclear target; SHARP, SMRT/HDAC-1-Associated Repressor Protein; RBPID, RBP-J interacting domain; NLS, nuclear localization signal; NES, nuclear export signal; EBNA2, Epstein-Barr virus nuclear antigen 2;

**TABLES**

**TABLE 1: Calorimetric data for RITA binding to RBP-J**

<table>
<thead>
<tr>
<th>RBP-J</th>
<th>RITA</th>
<th>$K$ ($M^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP-J 106-173</td>
<td>1.1 ± 0.2 x 10^6</td>
<td>0.98</td>
<td>-8.2 ± 0.1</td>
<td>-6.4 ± 0.7</td>
<td>-1.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>RBP-J 127-158</td>
<td>9.9 ± 0.5 x 10^6</td>
<td>1.01</td>
<td>-8.2 ± 0.1</td>
<td>-6.1 ± 0.3</td>
<td>-2.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>RBP-J+DNA 127-158</td>
<td>1.7 ± 0.2 x 10^6</td>
<td>0.59</td>
<td>-8.5 ± 0.1</td>
<td>-4.5 ± 0.1</td>
<td>-4.0 ± 0.2</td>
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<tr>
<td>BTD 127-158</td>
<td>5.2 ± 0.5 x 10^5</td>
<td>1.94</td>
<td>-7.8 ± 0.1</td>
<td>-4.0 ± 0.2</td>
<td>-3.8 ± 0.3</td>
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<tr>
<td>BTD-CTD 127-158</td>
<td>5.8 ± 0.2 x 10^5</td>
<td>1.71</td>
<td>-7.8 ± 0.1</td>
<td>-5.5 ± 0.1</td>
<td>-2.3 ± 0.1</td>
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</tr>
<tr>
<td>RBP-J 133-151</td>
<td>1.9 ± 0.3 x 10^6</td>
<td>0.53</td>
<td>-8.5 ± 0.1</td>
<td>-7.6 ± 0.3</td>
<td>-0.9 ± 0.2</td>
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</tr>
<tr>
<td>RBP-J 133-148</td>
<td>2.1 ± 0.3 x 10^6</td>
<td>0.49</td>
<td>-8.7 ± 0.1</td>
<td>-7.7 ± 0.3</td>
<td>-1.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>RBP-J 135-148</td>
<td>5.0 ± 1.0 x 10^6</td>
<td>0.21</td>
<td>-9.1 ± 0.1</td>
<td>-6.7 ± 0.1</td>
<td>-2.4 ± 0.3</td>
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</tr>
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<td>RBP-J 137-148</td>
<td>2.0 ± 0.1 x 10^6</td>
<td>0.50</td>
<td>-8.6 ± 0.1</td>
<td>-7.9 ± 0.1</td>
<td>-0.7 ± 0.1</td>
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<tr>
<td>RBP-J 137-146</td>
<td>2.4 ± 0.5 x 10^6</td>
<td>0.42</td>
<td>-8.7 ± 0.01</td>
<td>-8.5 ± 0.1</td>
<td>-0.1 ± 0.1</td>
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<tr>
<td>RBP-J 139-146</td>
<td>2.4 ± 0.3 x 10^4</td>
<td>43.2</td>
<td>-6.0 ± 0.1</td>
<td>-9.5 ± 0.8</td>
<td>3.5 ± 0.9</td>
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</tr>
<tr>
<td>RBP-J –LWTP–</td>
<td>NBD</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>

All experiments were performed at 25°C. Values are the mean of at least three independent experiments and errors represent the standard deviation (S.D.) of multiple experiments. NBD represents no binding detected.
### Table 2: Temperature dependence of RITA binding to RBP-J

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>$K (M^{-1})$</th>
<th>$K_d (\mu M)$</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP-J + RITA^{(133-148)}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>$4.0 \pm 0.3 \times 10^6$</td>
<td>0.43</td>
<td>$-7.8 \pm 0.2$</td>
<td>$3.3 \pm 0.3$</td>
<td>$-11.1 \pm 0.1$</td>
</tr>
<tr>
<td>15</td>
<td>$1.1 \pm 0.3 \times 10^6$</td>
<td>1.0</td>
<td>$-7.9 \pm 0.2$</td>
<td>$-2.9 \pm 0.1$</td>
<td>$-5.0 \pm 0.3$</td>
</tr>
<tr>
<td>25</td>
<td>$2.1 \pm 0.3 \times 10^6$</td>
<td>0.49</td>
<td>$-8.7 \pm 0.1$</td>
<td>$-7.7 \pm 0.3$</td>
<td>$-1.0 \pm 0.4$</td>
</tr>
<tr>
<td>35</td>
<td>$1.0 \pm 0.4 \times 10^6$</td>
<td>1.0</td>
<td>$-8.5 \pm 0.1$</td>
<td>$-11.9 \pm 0.1$</td>
<td>$3.4 \pm 0.1$</td>
</tr>
</tbody>
</table>

Values are the mean of three independent experiments and the errors represent the S.D. of multiple experiments.

### Table 3. Data collection and refinement statistics.

#### Data Collection Statistics

<table>
<thead>
<tr>
<th>Beam Line</th>
<th>APS LS-CAT 21-ID-F</th>
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<tr>
<td>Resolution (Å)</td>
<td>40.83 - 2.09 (2.15 - 2.09)</td>
</tr>
<tr>
<td>Space Group</td>
<td>P2_1 22_1</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97872</td>
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<td>Unit Cell a, b, c (Å)</td>
<td>76.78, 96.41, 96.71</td>
</tr>
<tr>
<td>Unit Cell α, β, γ (°)</td>
<td>90.00, 90.00, 90.00</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>0.07 (0.54)</td>
</tr>
<tr>
<td>$I/\sigma I$</td>
<td>22.8 (4.79)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>89.6 (48.3)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>7.1 (5.5)</td>
</tr>
<tr>
<td>Average mosaicity</td>
<td>0.46</td>
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</table>

#### Refinement Statistics

<table>
<thead>
<tr>
<th>$R_{work}/R_{free}$ (%)</th>
<th>19.3 / 23.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of reflections</td>
<td>38,486</td>
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<tr>
<td>Number of atoms</td>
<td>4285</td>
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<tr>
<td>Complexes/asymmetric unit</td>
<td>1</td>
</tr>
<tr>
<td>Wilson B/Mean B value (Å²)</td>
<td>25.8 / 30.9</td>
</tr>
<tr>
<td>RMSD Bond Lengths (Å)</td>
<td>0.007</td>
</tr>
</tbody>
</table>
### Table 4: Calorimetric binding data for RITA and RBP-J mutants

<table>
<thead>
<tr>
<th>RBP-J</th>
<th>RITA</th>
<th>$K$ (M$^{-1}$)</th>
<th>$K_d$ (uM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
<th>$\Delta AG^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE259AA</td>
<td>WT$^{135-148}$</td>
<td>1.7 ± 1.0 x 10$^5$</td>
<td>6.21</td>
<td>-7.1 ± 0.1</td>
<td>-4.9 ± 0.6</td>
<td>-2.1 ± 0.7</td>
<td>2.0</td>
</tr>
<tr>
<td>F261R</td>
<td>WT$^{133-151}$</td>
<td>NBD</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>F261A</td>
<td>WT$^{133-151}$</td>
<td>6.9 ± 1.0 x 10$^5$</td>
<td>147.2</td>
<td>-5.2 ± 0.1</td>
<td>-8.1 ± 0.8</td>
<td>2.9 ± 0.9</td>
<td>3.3</td>
</tr>
<tr>
<td>V263R</td>
<td>WT$^{133-151}$</td>
<td>0.9 ± 0.2 x 10$^4$</td>
<td>1.29</td>
<td>-8.0 ± 0.2</td>
<td>-6.9 ± 0.6</td>
<td>-1.1 ± 0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>V263A</td>
<td>WT$^{133-151}$</td>
<td>1.1 ± 0.9 x 10$^4$</td>
<td>0.92</td>
<td>-8.1 ± 0.7</td>
<td>-5.9 ± 0.3</td>
<td>-3.0 ± 1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>A284R</td>
<td>WT$^{133-151}$</td>
<td>1.8 ± 0.4 x 10$^5$</td>
<td>5.88</td>
<td>-7.2 ± 0.1</td>
<td>-12.6 ± 1.1</td>
<td>5.5 ± 1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>A284V</td>
<td>WT$^{133-151}$</td>
<td>4.6 ± 0.8 x 10$^5$</td>
<td>21.9</td>
<td>-6.3 ± 0.1</td>
<td>-4.0 ± 0.9</td>
<td>-2.3 ± 1.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Q333R</td>
<td>WT$^{133-151}$</td>
<td>4.8 ± 0.3 x 10$^5$</td>
<td>2.1</td>
<td>-7.8 ± 0.1</td>
<td>-7.0 ± 0.3</td>
<td>-0.8 ± 0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Q333A</td>
<td>WT$^{133-151}$</td>
<td>1.2 ± 0.3 x 10$^4$</td>
<td>0.90</td>
<td>-8.3 ± 0.1</td>
<td>-5.7 ± 0.2</td>
<td>-2.5 ± 0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>WT</td>
<td>WTP/AAA</td>
<td>NBD</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

All experiments were performed at 25°C. Values are the mean of at least three independent experiments and errors represent the S.D. of multiple experiments. NBD represents no binding detected.

### Table 5: Calorimetric data for acetylated and phosphorylated RITA binding to RBP-J

<table>
<thead>
<tr>
<th>RBP-J</th>
<th>RITA</th>
<th>$K$ (M$^{-1}$)</th>
<th>$K_d$ (uM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$-T\Delta S^\circ$ (kcal/mol)</th>
<th>$\Delta AG^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP-J</td>
<td>130-148</td>
<td>1.1 ± 0.2 x 10$^6$</td>
<td>0.90</td>
<td>-8.2 ± 0.1</td>
<td>-6.1 ± 0.4</td>
<td>-2.1 ± 0.3</td>
<td>---</td>
</tr>
<tr>
<td>RBP-J</td>
<td>130-148 PT143</td>
<td>8.6 ± 0.1 x 10$^4$</td>
<td>12.0</td>
<td>-6.7 ± 0.1</td>
<td>-3.9 ± 0.4</td>
<td>-2.8 ± 0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>RBP-J</td>
<td>130-148 PT147</td>
<td>5.4 ± 0.7 x 10$^5$</td>
<td>1.9</td>
<td>-7.8 ± 0.1</td>
<td>-5.3 ± 0.1</td>
<td>-2.5 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>RBP-J</td>
<td>130-148 acK131/acK136</td>
<td>1.9 ± 0.4 x 10$^5$</td>
<td>5.0</td>
<td>-7.2 ± 0.1</td>
<td>-6.8 ± 0.2</td>
<td>-0.4 ± 0.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

All experiments were performed at 25°C. Values are the mean of at least three independent experiments and errors represent the S.D. of multiple experiments.
FIGURE LEGENDS

FIGURE 1: Overview of Notch signaling. (A) Notch signaling occurs between neighboring cells, in which interactions on the cell surface between a Notch receptor and a DSL ligand, results in cleavage of Notch and release of its intracellular domain (NICD) that subsequently transits to the nucleus. (B) In the absence of a Notch signal, CSL can bind corepressors, such as RITA, to repress transcription from Notch target genes. RITA binding to CSL can also cause CSL to be exported out of the nucleus. Upon activation of the Notch signaling pathway, NICD and MAM (Mastermind) form a ternary complex with CSL that activates transcription from Notch target genes. (C) Structure of the CSL-NICD-MAM ternary complex bound to DNA (PDB ID: 2FO1). The structural core of CSL is composed of three domains: NTD (N-terminal domain), BTD (β-trefoil domain), and CTD (C-terminal domain), which are colored cyan, green, and orange, respectively. A β-strand that makes hydrogen-bonding interactions with all three domains is colored magenta. The RAM and ANK domains of NICD are colored red and yellow, respectively. MAM and DNA are colored gray. Domain schematics are colored similarly to the structure.

FIGURE 2: RITA domain schematic, secondary structure analysis, and sequence alignment with other CSL binding partners. (A) RITA is a multidomain protein containing an N-terminal nuclear export signal (NES; red), RITA conserved repeats (RCR1/RCR2; yellow), nuclear localization sequence (NLS; grey), the RBP-J interacting domain (RBPID; blue), and a C-terminal tubulin interacting domain colored green. As revealed by epitope mapping, the monoclonal antibody H35-2 colored red recognizes amino acids 40-55 of human RITA. (B) Far-UV Circular Dichroism (CD) spectra (wavelengths 185-200nm) for the RBPID of RITA (amino acids 127-158). The RBPID consists of mostly random coil as indicated by the minimum at 200 nm. Secondary-structure was determined using Dichroweb and CDSSTR with reference set 7. The normalized root mean square deviation parameter value for the RITA CD data is 0.038. (C) Schematic representation of the RITA constructs used in this study with the φωφ motif colored green and the arginine implicated in salt-bridge formation with E260 of RBP-J colored red. Previously identified post-translational modifications within the RBPID are shown: lysine acetylation sites are colored green and threonine phosphorylation sites are colored red. (D) Sequence alignment of coregulators that bind the BTD of CSL, including the RAM domains of human Notch1-4, the RAM domains of fly (dNotch) and worm (LIN-12) Notch receptors, the viral coactivator EBNA2, and the corepressor KyoT2. Boxed in blue, is the RAM basic motif; boxed in yellow, the -HG- dipeptide motif; boxed in green, the φωφ motif; boxed in magenta, the -GF- dipeptide motif; and boxed in red, are the basic residues of KyoT2 and RITA implicated in salt-bridge formation with RBP-J.

FIGURE 3: Endogenous RITA and RBP-J interact in cells. (A) Detection of endogenous RITA protein in HeLa cells (left panel) and HEK-293 cells (right panel). The indicated amounts of whole cell lysates were used for western blotting. Membranes were incubated with the anti-RITA hybridoma supernatant H35-2. (B) RITA expression levels in different human cell lines. Short exposure (upper) and long exposure (middle) is shown. Expression of tubulin served as a loading control (lower panel). Membranes were incubated with the anti-RITA hybridoma supernatant H35-2 (upper and middle) or an anti-tubulin antibody (lower). (C) Coimmunoprecipitations of endogenous RBP-J with RITA in HeLa cells. RBP-J was coimmunoprecipitated with RITA specific hybridoma supernatants H35-1 (lane 1), H35-2 (lane 2), and H35-9 (lane 3), but not with an IgG control (lane 4). Purified RBP-J protein served as a positive control for western blotting (lane 6). The asterisk denotes the heavy chain of the antibodies.

FIGURE 4: Mapping the minimal RBP-J binding region of RITA within cells. (A) Schematic representation of RITA deletion constructs used for analysis of subcellular localization and interactions with endogenous RBP-J. Abbreviations, NES, nuclear export signal; RCR1 and RCR2, RITA conserved repeat 1 and 2; NLS, nuclear localization signal; RBPID, RBP-J interaction domain; Tubulin, tubulin binding region. (B) Subcellular localization of GFP-RITA fusion proteins used for coimmunoprecipitation experiments. RITA(wt) and RITA(Δ128-156) show predominant tubulin association due to its tubulin binding region and rapid nucleo-cytoplasmic shuttling (19). RITA(156-296)
is also located at tubulin fibers confirming the tubulin binding region at the carboxy-terminus of RITA. RITA(83-173) and RITA(66-173) show predominantly nuclear localization confirming the NLS within RITA. RITA(120-161) and RITA(106-173) show equal distribution within the cell. GFP localization served as a control. HeLa cells were transfected with the indicated GFP fusion constructs. 24h after transfection the living cells were imaged by fluorescence microscopy. Scale bar = 10 µm. (C) Coimmunoprecipitations of RBP-J with RITA deletion constructions. (Upper panel): RBP-J interacts with RITA(wt) (lane 1), RITA(83-173) (lane 3), RITA(106-173) (lane 4), and RITA(66-173) (lane 5), but not with RITA(156-269) (lane 2), RITA(Δ128-165) (lane 6), and RITA(120-161) (lane 7). Expression of RITA proteins (middle) and endogenous RBP-J (lower) was verified by western blotting. Coimmunoprecipitations were performed 24h after transfection of the indicated GFP-RITA fusions. The asterisk denotes the heavy chain of anti-GFP antibody used for immunoprecipitation of RITA proteins.  

FIGURE 5: Binding analysis of RBP-J/RITA interactions by ITC. Figure shows representative thermograms from individual ITC experiments with various constructs of RBP-J and RITA. All ITC experiments were conducted with CSL in the cell at approximately 20-25 µM and RITA in the syringe at approximately 200-250 µM. Experimental temperature was set at 25°C and experiments were performed in triplicate (n=3). (A) A RITA construct (106-173) that corresponds to the region necessary to interact with RBP-J binds with ~1 µM affinity. (B) A RITA construct (127-158) that corresponds to the RBPID also binds with ~1 µM affinity. (C-D) The BTD and BTD-CTD constructs of RBP-J bind RITA (127-158) with ~2 µM affinity. (E) Further truncation of the RBPID (139-146) results in a significant loss of binding to RBP-J. (F) ΔCp analysis of RBP-J/RITA interactions. ITC experiments were performed at 5°C, 15°C, 25°C, and 35°C. The average change in Gibbs free energy (ΔG°), enthalpy (ΔH°), and entropy (−TΔS°) were plotted as a function of temperature.  

FIGURE 6: High resolution structure of the RBP-J/RITA corepressor complex bound to DNA. (A) The x-ray structure of RBP-J/RITA/DNA (PDB ID: 5EG6) was determined to 2.1 Å resolution. Figure shows ribbon and surface representation of the complex structure with the NTD, BTD, and CTD colored cyan, green, and orange, respectively; RITA is colored yellow. (B) Structural overlay of CTD domains from the RBP-J/RITA/DNA structure determined here with the CTD from the previously published RBP-J/DNA structure (PDB ID: 3IAG), highlighting the 10.5 Å rigid body shift of the CTD of RBP-J when bound to RITA. (C) Zoomed view of RITA binding the BTD of RBP-J, emphasizing the φWφP motif. RITA is shown in a stick representation with corresponding 2Fo-Fc electron density contoured at 1σ. (D) Figure shows the salt bridge formed between R138 of RITA (yellow) and E259 and E260 of RBP-J (green). (E) Structural alignment of BTD-binding proteins, including RITA, in yellow (PDB ID: 5EG6); KyoT2, in blue (PDB ID: 42JX), worm RAM, in pink (PDB ID: 3BRD), and human RAM, in red (PDB ID: 3V79). Cα traces are shown with the side chains of the φWφP motifs depicted as sticks. (F) Figure shows location of the mutation sites (F261, V263, A284, Q333) within the BTD that affect RAM binding and used in this study.  

FIGURE 7: Cellular reporter assays of RITA-mediated repression in the context of CSL mutants. RBP-J null MEFs were transduced with a retrovirus encoding either wild-type or mutant RBP-J constructs. To activate and readout Notch signaling, cells were transfected with a construct that expresses an activated form of the Notch1 receptor (NICD1) and the 4xCBS reporter, which has four CSL binding sites upstream of the firefly luciferase gene. To assay for RITA-mediated repression, cells were cotransfected with increasing amounts a construct that expresses RITA: 0ng (--), 50ng (+), 100ng (++), 200ng (+++), 400ng (++++). Experiments were performed in triplicate and the error bars represent the S.E.M. (A) RITA represses Notch reporter activity in a dose dependent manner. Fold activation is relative to luciferase activity from control cells not transfected with NICD1. (B) Plot shows reduced reporter activity for RBP-J mutants (F261A, V263A, A284V, Q333A). Fold activation is relative to luciferase activity from control cells not transfected with NICD1. (C-F) Plots show RITA-mediated repression for the RBP-J mutants compared to wild-type. Data are normalized to cells with NICD1, but without RITA, and shown
as relative activity. Statistical significance was determined by unpaired t test with *, $p \leq 0.05$; **, $p \leq 0.01$; and ns, not significant.

**FIGURE 8:** Cellular reporter assays of RITA-mediated repression in the context of RITA mutants. Cellular reporter assays in retrovirally transduced MEFs were performed similarly as described for Figure 5. (A) Plot shows relative repression activity of RITA (106-173), which contains the RBPIID, compared to full-length RITA. (B) Plot shows reduced, but not completely abolished, RITA mediated repression of the reporter for the RITA mutant WTP/AAA, which mutates the $\varphi W\varphi P$ motif. (C) Plot shows reduced reporter activity, compared to wild-type, for the construct RITA$\Delta$NES, which deletes the nuclear export sequence of RITA. (D) Plot shows reduced reporter activity, compared to wild-type, for the construct RITA$\Delta$NLS, containing a non-functional NLS. Error bars represent the S.E.M and statistical significance was determined by unpaired t test with *, $p \leq 0.05$; **, $p \leq 0.01$; and ns, not significant.
Figure 1
Figure 2
Figure 4

A

B

C

GFP

1-269 (wt)

Δ128-156

156-269

120-161

66-173

83-173

106-173

GFP-hsRITA

kDa

95

72

*  

55

36

72

55

IP: α-GFP
WB: α-RBP-J

RITA

WB: α-GFP

RBP-J

WB: α-RBP-J

1-269 (wt)

Δ128-156

156-269

120-161

66-173

83-173

106-173

not transfected

control

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Figure 5
Figure 6
Figure 8
Structure-function analysis of RBPJ-interacting and tubulin-associated (RITA) reveals regions critical for repression of Notch target genes
Nassif Tabaja, Zhenyu Yuan, Franz Oswald and Rhett A. Kovall

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