CSL-Associated Corepressor and Coactivator Complexes

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Abstract

The highly conserved Notch signal transduction pathway orchestrates fundamental cellular processes including, differentiation, proliferation, and apoptosis during embryonic development and in the adult organism. Dysregulated Notch signaling underlies the etiology of a variety of human diseases, such as certain types of cancers, developmental disorders and cardiovascular disease. Ligand binding induces proteolytic cleavage of the Notch receptor and nuclear translocation of the Notch intracellular domain (NICD), which forms a ternary complex with the transcription factor CSL and the coactivator MAML to upregulate transcription of Notch target genes. The DNA-binding protein CSL is the centrepiece of transcriptional regulation in the Notch pathway, acting as a molecular hub for interactions with either corepressors or coactivators to repress or activate, respectively, transcription. Here we review previous structure-function studies of CSL-associated coregulator complexes and discuss the molecular insights gleaned from this research. We discuss the functional consequences of both activating and repressing binding partners using the same interaction platforms on CSL. We also emphasize that although there has been a significant uptick in structural information over the past decade, it is still under debate how the molecular switch from repression to activation mediated by CSL occurs at Notch target genes and whether it will be possible to manipulate these transcription complexes therapeutically in the future.

Keywords

Notch · CSL · Structure analysis · RAM domain · Coactivator complex · Corepressor complex · DNA-binding · Transcription

Abbreviations

CBF1	C-promoter Binding Factor 1
LAG-1	abnormal cell LINeage-12 (Lin-12) And abnormal Germ line proliferation phenotype-1 (Glp-1)
RBP-J	Recombination Signal-Binding Protein for immunoglobin kappa J region
Su(H)	Suppressor of Hairless

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CBP/CREBBP	C-Adenosine Mono Phosphate Responsive Element (cAMP-RE)-Binding protein (CREB)-Binding Protein; KAT3A
EP300	E1A Binding Protein P300, KAT3B
PCAF	P300/CBP-Associated Factor; KAT2B
GCN5	General Control Of AmiNo Acid Synthesis Protein 5-Like 2; KAT2A
CDK8	Cyclin-Dependent Kinase 8
SCF	S-Phase Kinase Associated Protein1/Cullin/F-Box Protein
SEL10	Suppressor and/or Enhancer of abnormal cell LINeage-12 (Lin-12)-10
FBWX7	F-Box and WD Repeat Domain containing 7
SIRT-1	Sirtuin-1
CARM1	Coactivator-Associated Arginine Methyltransferase1
PRMT4	Protein Arginine N-MethylTransferase 4
CTBP	C-Terminal Binding Protein
CTIP	CTBP Interacting Protein
KYOT2/FHL1	Four and a Half LIM domains 1
NCoR	Nuclear Receptor CoRepressor
SMRT	Silencing Mediator For Retinoid And Thyroid Hormone Receptors
SHARP	SMRT/HDAC1-Associated Repressor Protein
SPEN	SPlit ENds family transcriptional repressor
LID	Little Imaginal Disks
KDM5A	Lysine(K) Demethylase 5A
CIR	Corepressor Interacting with RBPJ
SKIP	Sloan-KetterIng-retroviral oncogene (SKI) -Interacting Protein
L3MBTL3	Lethal(3)Malignant Brain Tumor-Like Protein 3
RITA1	RBPJ Interacting and Tubulin Associated 1
EBNA2	Epstein-Barr Virus Nuclear Antigen 2
NFAT	Nuclear Factor of Activated T-cells
NF-κB1	Nuclear Factor κB1
POFUT1	Protein O-Fucosyltransferase 1
Fringe	Beta-1,3-N-Acetylglucosaminyltransferase

1 Introduction

The Notch signaling pathway is evolutionary conserved in metazoan organisms and represents a short-range cell-to-cell communication mechanism. A fly mutant with *"notches"* in its wing ends served as an eponym for the gene responsible for this particular phenotype (Morgan 1917). In 1985 the *Notch* gene was first cloned in *Drosophila melanogaster* and was found to encode a putative type I transmembrane protein with an extracellular region, a single transmembrane domain, and an intracellular region (Wharton et al. 1985). Further studies in *Drosophila* showed that the NOTCH protein serves as a receptor for two specific ligands, SERRATE and DELTA, which are also type I transmembrane proteins (Struhl and Adachi 1998; Artavanis-Tsakonas et al. 1999). During embryonic development and in the adult organism, Notch signaling affects and regulates stem cell maintenance, cell fate decisions, and cell lineage identity, as well as cell proliferation, differentiation and apoptosis (Borggrefe and Oswald 2009). These different outcomes of Notch signaling seem to be highly dependent on cellular context (Bray 2016). Although Notch signaling has pleiotropic functions, the pathway itself, which is devoid of second messengers and enzyme cascades, is mechanistically very simple.

Five ligands (JAGGED 1 and 2, DELTA-LIKE 1, 3 and 4) and four NOTCH receptors (Notch1–4) are present in mammals (Bray 2006; Kovall et al. 2017). The Notch receptor contains multiple epidermal growth factor (EGF)-like repeats (36 EGF repeats in mammalian NOTCH1), and three LNR (LIN1–2/Notch) repeats, which are located within the so-called Negative Regulatory Region (NRR) in the extracellular domain. The intracellular part of the Notch receptor contains the RAM (RBPJ-associated molecule) domain

and seven ankyrin (ANK) repeats, which are followed by a trans-activation domain (TAD) and a PEST [rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues] domain at its carboxy terminus.

NOTCH receptors undergo multiple cleavage events and post-translational modifications during their maturation and in response to ligand binding (Fig. 1). The first cleavage event (S1) is ligand independent and occurs in the trans-golgi network by a furin-like convertase (Logeat et al. 1998) (Fig. 1A). S1 results in two protein fragments, the Notch extracellular domain (NECD) and an intracellular fragment that contains the transmembrane domain, which are non-covalently held together and presented as a heterodimer at the cell surface. The extracellular domains of NOTCH receptors are also modified by O-linked glycosylation and fucosylation, which can modulate specific ligand-receptor interactions, thereby affecting signaling outcome (Takeuchi and Haltiwanger 2010, 2014; Rana and Haltiwanger 2011). These modifications within the EGF repeats are catalyzed by protein O-fucosyltransferase 1 (POFUT1) and the fringe glycosyl-transferases RADICAL FRINGE (RFNG), LUNATIC FRINGE (LFNG) and MANIC FRINGE (MFNG) (Okajima et al. 2003; Moloney et al. 2000; Bruckner et al. 2000) (Fig. 1A). After ligand binding, a mechanical pulling force is thought to expose a second cleavage site (S2) in the NRR due to conformational changes that occur within the LNR domain (Fig. 1B) (Gordon et al. 2015). This ligand dependent cleavage step is catalyzed by members of the ADAM (A Disintegrin And Metalloprotease) metalloproteases family, ADAM10 and ADAM17 (Struhl and Greenwald 1999; Brou et al. 2000; Bozkulak and Weinmaster 2009). Subsequently, the remaining transmembrane NOTCH fragment, also called Notch extracellular truncation (NEXT), undergoes a final cleavage step (S3), which occurs within the cellular membrane and is catalyzed by the γ-secretase complex (Mumm et al. 2000). Cleavage at the S3 site releases the Notch intracellular domain (NICD) from the cell membrane (Schroeter et al. 1998) and subsequently NICD translocates to the nucleus to activate transcription of Notch target genes (Fig. 1C) (Struhl and Adachi 1998).

NICD does not bind to DNA itself but rather interacts with the DNA binding transcription factor CSL [for CBF1/RBPJ (C-promoter Binding Factor1/ Recombination Binding Protein Jk), Su(H) (Suppressor of Hairless), and Lag-1] and the transcriptional coactivator MASTERMIND-LIKE (MAML) to form a DNA-bound transactivation complex (Nam et al. 2006; Wilson and Kovall 2006; Kopan and Ilagan 2009; Kovall and Blacklow 2010). The CSL-NICD-MAML transactivation complex recruits histone modifying coactivators, like CREBBP/EP300 (CREB Binding Protein/E1A Binding Protein P300) or PCAF (P300/CBP-associated factor, *aka* KAT2B) and GCN5 (General control of amino acid synthesis protein 5, *aka* KAT2A), together with chromatin remodeling complexes to activate transcription (Fig. 1D) (Kurooka and Honjo 2000; Oswald et al. 2001; Wallberg et al. 2002; Kadam and Emerson 2003). NICD is a short-lived protein, as its PEST domain is phosphorylated by CYCLINC/CDK8 (Fryer et al. 2004), resulting in its ubiquitilation by the SCF/SEL10/FBXW7 E3 ubiquitin ligase complex, leading to its degradation by the proteasome (Fig. 1E). A number of additional post-translational modifications regulate the activity and stability of NICD, *e.g.* deacetylation by SIRT-1 (silent mating type information regulation 2 homolog, *aka* SIRTUIN1) (Guarani et al. 2011) and methylation by CARM1 (Coactivator Associated Arginine Methyltransferase 1)/PRMT4 (Protein Arginine N-Methyltransferase 4) (Hein et al. 2015), which regulate the amplitude and duration of the Notch response (Wu et al. 2001; Tsunematsu et al. 2004).

In the absence of an active Notch signal CSL acts as a transcriptional repressor (Fig. 1F) (Dou et al. 1994). In *Drosophila*, the CSL ortholog Su(H) recruits the HAIRLESS/CtBP (C-terminal Binding Protein)/GROUCHO corepressor complex (Morel et al. 2001; Barolo et al. 2002). In vertebrates, RBPJ directly interacts with corepressor components KYOT2/FHL1 (Taniguchi et al. 1998), SHARP (SMRT/HDAC1-associated

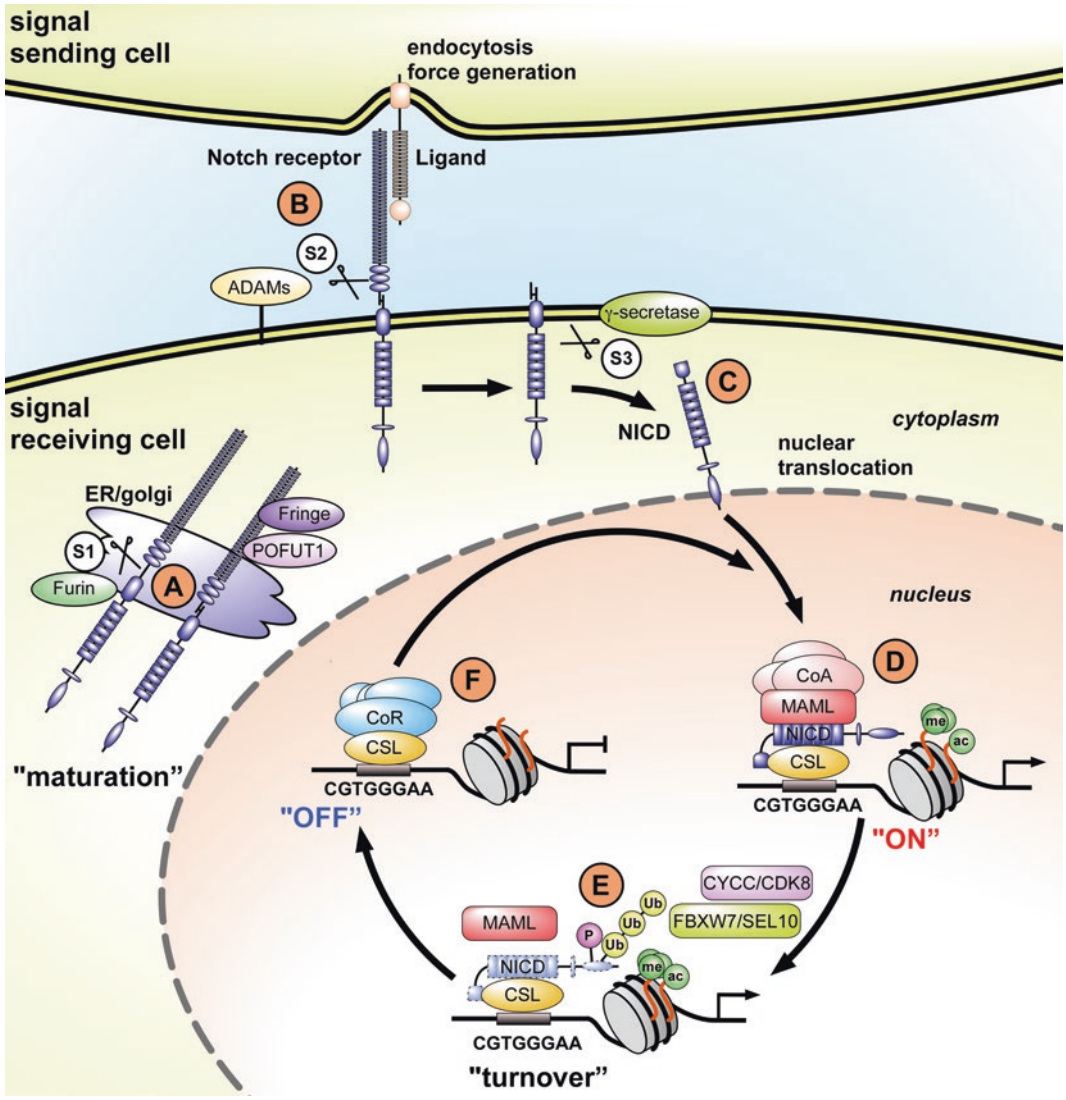

Fig. 1 Schematic representation of the major molecular events during Notch signaling: (**A**) Posttranslational modifications of the Notch receptor during maturation in the trans-golgi network. The Notch receptor precursor protein is cleaved by a furin convertase (S1). Protein fragments are non-covalently linked together as a heterodimer. Additional modifications are catalyzed by fringe glycosyltransferases (FRINGE) and protein O-fucosyltransferase 1 (POFUT1). (**B**) Notch receptors and ligands are single transmembrane spanning proteins. Ligand binding and its endocytosis generate a mechanical pulling force to expose the second cleavage site (S2) and processing by ADAM family metalloproteases. (**C**) A further cleavage step (S3) is catalyzed by a gamma-secretase containing complex, releasing the Notch Intracellular Domain (NICD) that translocates to the nucleus. (**D**) Nuclear NICD interacts with the DNA-binding protein CSL and recruits a coactivator complex composed of Mastermind (MAML) and additional chromatin modifying factors to activate transcription of Notch target genes ("ON"). (**E**) Phosphorylation by the mediator subunit CYCLINC/CDK8 and subsequent ubiquitylation by the FBXW7/SEL10 containing E3 ubiquitin ligase complex lead to rapid degradation of NICD by the proteasome ("turnover"). (**E**) In the absence of activated Notch signaling, CSL recruits various corepressor complexes to downregulate transcription of Notch target genes

repressor protein)/SPEN (Split Ends), also called MINT (Msx2-interacting nuclear target protein) (Oswald et al. 2002; Kuroda et al. 2003), L3MBTL3 [Lethal(3)Malignant Brain Tumor-Like Protein 3] (Xu et al. 2017), the H3K4 demethylase KDM5A [Lysine (K)-Specific Demethylase 5A)/LID (Little imaginal discs), (Moshkin et al. 2009; Liefke et al. 2010) or other cofactors like CIR (CBF1-Associated Corepressor) (Hsieh et al. 1999) and SKIP (Ski-interacting protein) (Zhou et al. 2000). These direct RBPJ binding partners recruit further corepressors, such as CtIP (CtBP interacting protein)/CtBP (Oswald et al. 2005), NCoR(Nuclear receptor corepressor1)/SMRT (silencing mediator for retinoid or thyroid-hormone receptors) (Zhou and Hayward 2001; Oswald et al. 2016), histone modifying enzymes (Xu et al. 2017; Hsieh et al. 1999; Olave et al. 1998) or Polycomb complex components (Qin et al. 2004; Qin et al. 2005) to silence Notch target genes. Therefore, CSL has dual roles within the Notch signaling pathway, acting either as an activator or repressor of transcription, depending on the status of Notch activity. As CSL plays a pivotal role in the regulation of transcription of Notch target genes, here we review the X-ray structures of CSL-mediated transcription complexes and what has been learned from these structural studies.

2 Overall Fold of Transcription Factor CSL

CSL proteins are DNA binding proteins that recognize the consensus sequence –C/tGTGGGAA– (Del Bianco et al. 2010; Meng et al. 2005; Tun et al. 1994) and regulate transcriptional activation and repression of Notch target genes by interacting with coactivators and corepressors, respectively. As originally shown in the X-ray structure of LAG-1 bound to DNA (Fig. 2A) (Kovall and Hendrickson 2004), all CSL proteins contain a conserved structural core that is largely composed of β-strands and consists of three domains: NTD (N-terminal domain), BTD (β-trefoil domain), and CTD (C-terminal domain). Additionally, CSL proteins from different organisms contain poorly conserved N- and C-terminal extensions of the structural core that appear unstructured by secondary-structure/disorder prediction algorithms. In general, the function of these regions is not well understood, but in certain orthologs the N-terminal regions appear to play a role in DNA binding and cooperative interactions with other transcription factors (Prevorovsky et al. 2011; Neves et al. 2007).

The NTD and CTD have immunoglobin type folds, whereas the BTD has a β-trefoil fold, similar to fibroblast growth factors and interleukin-1 (Kovall and Hendrickson 2004). The BTD of CSL has an atypical β-trefoil fold, as it is missing two of the canonical 12 β-strands that compose the classic β-trefoil fold. This results in a large exposed hydrophobic pocket on the surface of CSL, which is the binding site for many of the coregulators that interact with CSL (*see below*), including the RAM domain of NOTCH (Wilson and Kovall 2006; Friedmann et al. 2008), FHL1 (Four and a half LIM domains protein 1) (*aka* KyoT2) (Collins et al. 2014), RITA1 (RBPJ-interacting and tubulin-associated protein 1) (Tabaja et al. 2017), EBNA2 (Epstein–Barr virus nuclear antigen 2) (Johnson et al. 2010), and SPEN (*aka* MINT or SHARP) (VanderWielen et al. 2011). CSL proteins share some structurally similarity to the Rel Homology Domain (RHD) proteins, such as the transcription factors NF-κB1 (Nuclear Factor-κB1) and NFAT (Nuclear factor of activated T-cells) (Kovall and Hendrickson 2004). The NTD and CTD of CSL structurally align with RHD-N and RHD-C domains, respectively. However, the overall fold of CSL is distinct from other RHD members in that the BTD lies between the RHD-N and RHD-C domains of CSL, whereas typical RHD proteins have a RHD-N immediately followed by a RHD-C domain. Moreover, RHD proteins typically bind DNA as homodimers or heterodimers, whereas CSL proteins bind DNA as monomers. The NTD and BTD of CSL form a continuous electropositive surface in which to interact with DNA (Fig. 2) (Kovall and Hendrickson 2004). Much like other RHD proteins, the NTD of CSL inserts a β-hairpin loop within the major groove of DNA to make both specific and nonspecific contacts,

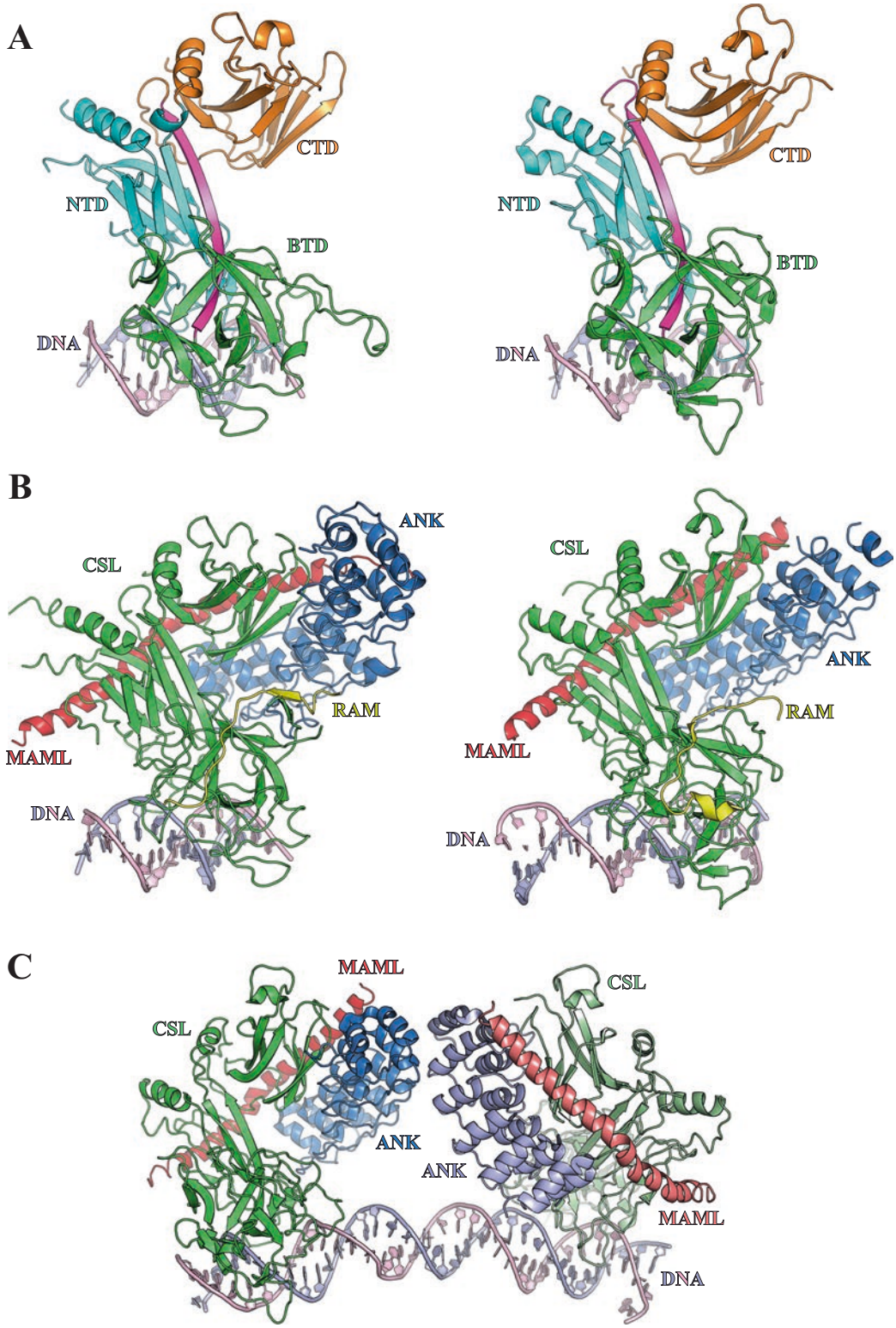

Fig. 2 X-ray structures of unbound CSL and CSL-NICD-MAML ternary complexes bound to DNA: (**A**) *Left,* ribbon diagram of LAG-1 bound to DNA (PDBID: 1TTU) and *right,* ribbon diagram of RBPJ bound to DNA(PDBID: 3IAG). The NTD, BTD, and CTD are colored cyan, green, and orange respectively. A β-strand that

largely recognizing the second half of its consensus binding site (–GGGAA–). The BTD also contributes to DNA binding, in which a β-hairpin loop inserts into the minor groove of DNA, making both specific and nonspecific contacts to the first base steps in the consensus binding site (–CG–) (Fig. 2).

3 The CSL-NICD-MAML Activation Complex

An obligatory step to activate transcription of target genes in response to a Notch signal is the formation of the ternary complex composed of CSL, NICD, and a member of the MAML family of transcriptional coactivators (MAML1–3 in mammals). The activation complex structures of the *Caenorhabditis elegans* and human orthologous proteins have been determined (Fig. 2B) (Nam et al. 2006; Wilson and Kovall 2006), and demonstrate that the RAM domain and ANK repeats of NICD bind the BTD and CTD, respectively, of CSL. MAML, which adopts a short bent α-helical conformation in the complex, forms a tripartite interaction with ANK, and the CTD and NTD of CSL (Fig. 2B). Similar to the RHD-C domains in other proteins, the CTD of CSL functions as a protein-protein interaction domain, binding MAML and NICD in the activation complex, as well as the corepressors SPEN and HAIRLESS detailed below (VanderWielen et al. 2011; Yuan et al. 2016). MAML coactivators are relatively large proteins (~1000 residues) that also interact with CBP/EP300 and the CDK8 module of the Mediator complex to activate transcription (Oswald et al. 2001; Wallberg et al. 2002; Fryer et al. 2004), but only require a small N-terminal domain to form a complex with NICD and CSL (Fig. 2B) (Nam et al. 2006; Wilson and Kovall 2006; Nam et al. 2003). Interestingly, constructs that only correspond to this N-terminal region are termed DN-MAML (dominant-negative MAML), and expressed in cells, these constructs are potent inhibitors of Notch signaling due to the ability of DN-MAML to form ternary complexes with CSL-NICD, but are unable to recruit CBP/EP300 and CDK8 to activate transcription (Weng et al. 2003).

The RAM domain of NICD binds in an extended conformation across the BTD of CSL in a manner that blankets the exposed hydrophobic surface on the BTD (Figs. 2B and 3A) (Wilson and Kovall 2006; Choi et al. 2012). The RAM domains of all NOTCH receptors (NOTCH1–4 in mammals), as well as a number of other coregulators that bind BTD, have a conserved hydrophobic tetrapeptide motif (φWφP), where φ is any nonpolar amino acid. In addition to the φWφP motif, RAM domains have other conserved motifs that are important for interacting with BTD, including an N-terminal basic region, and –HG– and –GF– dipeptide motifs (Johnson et al. 2010; Lubman et al. 2007). Interestingly, other coregulators that bind BTD similarly to RAM share some, but not all of these other motifs conserved in RAM. Prior to interacting with CSL, RAM is a random coil in solution (Nam et al. 2003; Bertagna et al. 2008). While RAM is ~100 residues in length, only ~20 N-terminal residues are required for interacting with the BTD of CSL (Wilson and Kovall 2006; Friedmann et al. 2008; Choi et al. 2012). The remaining ~80 residues between the RAM domain and ANK repeats of NICD were not resolved in the X-structure of the activation complex (Fig. 2B). However, this intervening region appears to be important for formation of the ternary complex, because (1) statistical models suggest that the length of RAM has been tuned through evolution to optimize the interactions between ANK and CTD (Bertagna et al. 2008), and (2) mutation of

Fig. 2 (continued) makes hydrogen bonding interactions with all three domains is colored magenta. The DNA is colored light pink and light blue. (**B**) Ribbon diagrams of CSL-NICD-MAML ternary complexes bound to DNA for Notch components from *Caenorhabditis elegans* (*left*, PDBID: 2FO1) and humans (*right*, PDBID: 3V79). CSL and MAML are colored green and red, respectively; the ANK and RAM domains of NICD are colored blue and yellow, respectively; and the DNA is colored light pink and light blue. (**C**) Ribbon diagram of dimeric CSL-NICD-MAML complexes bound to SPS element. Coloring is the same as (**B**)

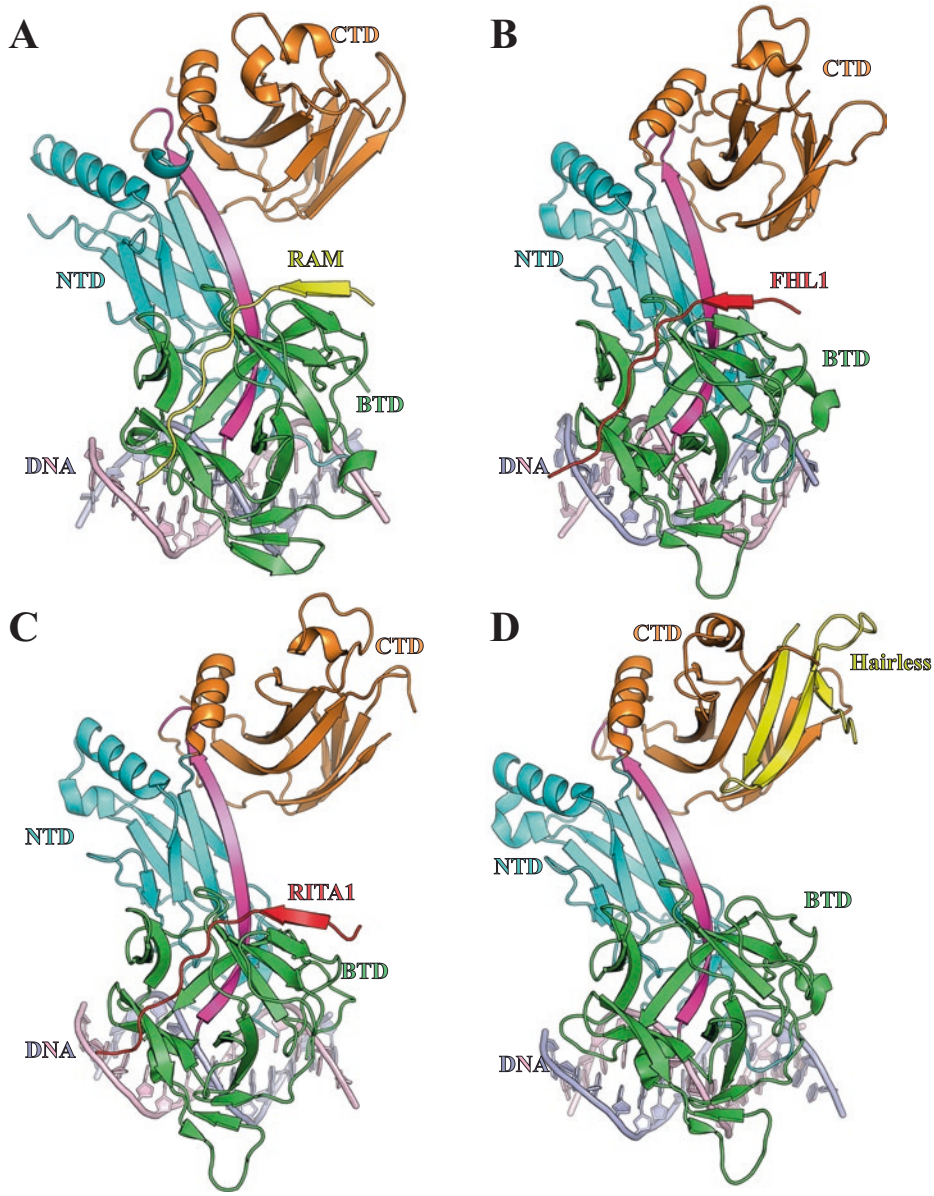

Fig. 3 X-ray structures of CSL-coregulator complexes: (**A**) Ribbon diagram of the RAM domain of NICD bound to LAG-1 and DNA. Coloring is the same as Fig. 2. (**B**) Ribbon diagram of the RBPJ-FHL1 complex bound to DNA. RBPJ-DNA coloring is the same as Fig. 2 and FHL1 is colored red. (**C**) Ribbon diagram of the RBPJ-RITA1-DNA complex. RBPJ-DNA coloring is the same as Fig. 2 and RITA1 is colored red. (**D**) Ribbon diagram of the Su(H)-HAIRLESS-DNA corepressor complex. Su(H)-DNA is colored the same as Fig. 2 and HAIRLESS is colored yellow

sequence specific elements within this intervening region of RAM adversely affect cellular reporter assays, suggesting that this region also contributes to proper transcriptional activation by NICD (Sherry et al. 2015).

There are seven ankyrin repeats within the ANK domain of NICD, as well as an N-terminal capping repeat (Fig. 2B) (Wilson and Kovall 2006). The folding of the terminal repeats is coupled to forming a complex with CSL and MAML

(Choi et al. 2012). There are several structures of the isolated ANK repeats of NICD (Nam et al. 2006; Zweifel et al. 2003), which overlay with a high degree of correspondence with the ANK repeats from the ternary complex structures, suggesting that formation of the CSL-NICD-MAML ternary complex does not induce any large conformational changes within ANK. There was a large rigid body shift observed in the domains of CSL when comparing the unbound structure with the activation complex, such that CSL assumed a more compact conformation with its BTD and CTD moving closer together (Wilson and Kovall 2006). However, these domain movements were only observed in the ternary complex structure with the *Caenorhabditis elegans* orthologous proteins. Whether this conformational change is organism specific or a general property of the activation complex remains to be determined.

In vitro studies using purified recombinant proteins have analyzed the interactions that consititute the CSL-NICD-MAML ternary complex and suggest that its assembly occurs in a stepwise manner (Kovall and Blacklow 2010). The RAM domain of NOTCH was originally identified in a yeast two-hybrid screen for RBPJ binding partners (Tamura et al. 1995) and subsequently shown to form a high affinity (K_d ~10 nM) interaction with the BTD of CSL (Friedmann et al. 2008; Lubman et al. 2007; Del Bianco et al. 2008). In the absence of MAML, the ANK repeats of NOTCH bind weakly to the CTD of CSL (Friedmann et al. 2008; Lubman et al. 2007; Del Bianco et al. 2008). Interestingly, the affinity of ANK for CTD seems to vary in different organisms – in mammals and nematodes the ANK-CTD interaction is very weak and technically difficult to detect (Friedmann et al. 2008; Lubman et al. 2007; Del Bianco et al. 2008), whereas the affinity of ANK for CTD in flies is stronger and binds with ~0.5 μM affinity (Contreras et al. 2015). Why the strength of ANK-CTD interactions varies in different organisms is unclear, but in the case for *Drosophila*, perhaps this is due to competition with the corepressor HAIRLESS, which also binds the CTD with high affinity (K_d ~1 nM) (Maier et al. 2011). MAML does not interact with CSL or NICD individually, but binds to the preformed CSL-NICD binary complex, rigidifying and stabilizing the ternary complex (Nam et al. 2003; Choi et al. 2012). To date, there are no studies that have quantitated the affinity of MAML for CSL-NICD. Taken together, these studies suggest that the high affinity RAM interaction for BTD targets NICD to CSL in the nucleus. The intrisically disordered region of RAM ideally positions ANK to interact with the CTD, and subsequently, MAML binds a groove formed by the CTD and ANK (Kovall and Blacklow 2010).

4 CSL-DNA Binding

CSL proteins bind the consensus sequence –C/tGTGGGAA– with a modest affinity of ~100 nM (Friedmann and Kovall 2010), although some known *in vivo* sites that deviate from the consensus bind considerably weaker (K_d ~1 μM) (Torella et al. 2014). The residues in CSL that contact DNA are absolutely conserved and comparative binding/structural studies of the mouse, worm, and fly orthologs suggest that all CSL proteins bind DNA in a similar manner with similar affinities (Kovall and Hendrickson 2004; Friedmann and Kovall 2010). This is in contrast to the protein-protein interactions that CSL makes with coregulators, e.g. the RAM domain of NICD, in which the affinities for complex formation can vary significantly (>10 fold) (Friedmann et al. 2008; Contreras et al. 2015). As mentioned above, the NTD of CSL interacts specifically with the major groove of DNA, whereas the BTD makes specific contacts in the minor groove (Kovall and Blacklow 2010). All CSL structures to date show very similar major groove contacts made by the NTD; however, in some CSL structures there is variability in how the BTD contacts the minor groove of DNA (Friedmann et al. 2008; Yuan et al. 2016; Friedmann and Kovall 2010). Specifically, a β-hairpin loop in BTD can assume several different conformations to make seemingly equivalent specific and nonspecific interactions with DNA. This may suggest that the BTD can assume different conformations to interact with DNA depending on the nearby base pairs it

contacts, which is consistent with the variability observed in the consensus sequence for CSL (Del Bianco et al. 2010; Meng et al. 2005; Tun et al. 1994).

In addition to binding monomeric DNA binding sites, in some metazoans CSL can also bind dimeric sites, which are known as SPS [Su(H) Paired Sites or Sequence Paired Sites] (Bailey and Posakony 1995). SPS are composed of two CSL binding sites arranged in a head-to-head arrangement with 15–19 base pairs separating the two sites (Nam et al. 2007). A typical SPS contains two CSL consensus-binding sites; however, cryptic paired sites have also been identified, in which one of the DNA binding sites significantly deviates from the consensus and is unable to support binding of monomeric CSL complexes (Arnett et al. 2010). When the Notch pathway is activated, two CSL-NICD-MAML can bind an SPS in a cooperative manner, whereby modest interactions between the ANK repeats of the two NICD molecules mediate the cooperativity (Fig. 2C). Interestingly, mutations that abrogate the cooperative interactions between ANK molecules affect transcription from Notch target genes that contain an SPS, but have no effect on targets that only contain monomeric sites (Arnett et al. 2010).

Classical models of Notch transcriptional regulation posit that CSL is constitutively bound to DNA, and corepressors and coactivators are exchanged on the DNA (Kao et al. 1998; Hsieh and Hayward 1995). However, more recent studies cast serious doubt on this model and suggest that the exchange of CSL-mediated corepressor and coactivator complexes is a much more dynamic process, and likely occurs in the nucleoplasm rather than while CSL is bound to DNA (Castel et al. 2013; Krejci and Bray 2007). Previous genome wide studies have shown that when the Notch pathway is activated CSL binds more sites at target genes than when the pathway is inactive (Castel et al. 2013; Krejci and Bray 2007; Hass et al. 2016). Although the molecular basis of this observation is unknown, generally speaking, two possibilities exist: (1) the affinity of CSL for DNA increases when bound to NICD and MAML and/or other general transcription factors; and (2) increased Notch activity or the activity of other transcription factors, *e.g.* pioneer factors, change the local chromatin environment, making it more accessible for CSL to bind. *In vitro* studies have shown that neither the affinity of CSL for DNA changes when it is bound to coregulators, such as NICD, FHL1, HAIRLESS, SPEN or RITA1, nor does the specificity of CSL change when bound to NICD and MAML (Del Bianco et al. 2010; Friedmann et al. 2008; Collins et al. 2014; Tabaja et al. 2017; VanderWielen et al. 2011; Maier et al. 2011). Albeit these *in vitro* studies have used only constructs that correspond to the structural cores of CSL, NICD, and MAML, and have not been performed with full-length proteins. Thus, it is an open question in the field as to what is the molecular basis that underlies the observed increase in CSL binding genome wide when Notch is active in cells.

5 CSL as a Repressor

Without a doubt CSL, in conjunction with NOTCH and MAML, plays an essential role in the upregulation of transcription from all Notch target genes in all organisms; however, its role as a transcriptional repressor is a bit more enigmatic and may have different roles in different organisms. In the model organism *D. melanogaster*, there is overwhelming genetic, cellular/biochemical, and structural evidence that Su(H) (the fly CSL ortholog), when in complex with the corepressor HAIRLESS, functions as a transcriptional repressor (Brockmann et al. 2014; Maier 2006). In other organisms, such as mammals and nematodes, the function of CSL as a repressor is not as clear. There is compelling biochemical, cellular, and structural data that RBPJ (the mammalian CSL ortholog) interacts with the corepressors FHL1, RITA1, SPEN, and L3MBTL3 (Taniguchi et al. 1998; Oswald et al. 2002; Kuroda et al. 2003; Xu et al. 2017; Tabaja et al. 2017; VanderWielen et al. 2011; Wacker et al. 2011). However, there is not a preponderance of genetic data supporting the function of RBPJ as a repressor. Nonetheless, there are several cellular and genetic studies that suggest loss

of RBPJ results in upregulation of transcription at some Notch target genes (Castel et al. 2013; Hu et al. 2012; Surendran et al. 2010). Interestingly, loss of RBPJ has been shown *in vivo* to promote tumorigenesis (Kulic et al. 2015), suggesting its role as a repressor may be important for tumor suppressor functions. While its role as a transcriptional repressor in the Notch pathway remains to be completely elucidated, the emerging picture seems to suggest that CSL is required for activation of all target genes, but its role as a repressor is important for a subset of target genes.

HAIRLESS is the major antagonist of Notch signaling in *Drosophila* and binds Su(H) with high affinity via a relative short peptide-like sequence (Yuan et al. 2016; Maier et al. 2011). HAIRLESS also interacts with the corepressors CtBP (C-terminal Binding Protein) and GROUCHO in order to function as a transcriptional repressor (Morel et al. 2001; Barolo et al. 2002; Nagel et al. 2005). Consistent with previous studies, HAIRLESS binds the CTD of Su(H) (Fig. 3D) (Yuan et al. 2016; Maier et al. 2011). Unexpectedly, HAIRLESS binding induces a large conformational change in the CTD, whereby HAIRLESS wedges itself between the two β-sheets that compose the Ig fold of the CTD (Yuan et al. 2016). This results in HAIRLESS primarily interacting with residues that form the hydrophobic core of the CTD rather than surface exposed residues (Fig. 3D). This large structural change is incompatible with NICD and MAML binding (Yuan et al. 2016). In future studies, it will be interesting to see whether other coregulators, such as SPEN, interact with this conserved binding pocket on the CTD.

Two other corepressors, FHL1 and RITA1, interact with RBPJ via a peptide-like sequence that resembles the RAM of NICD (Fig. 3B, C) (Taniguchi et al. 1998; Collins et al. 2014; Tabaja et al. 2017; Wacker et al. 2011). FHL1 proteins are characterized by N-terminal LIM (**L**IN11, **I**SL-1 & **M**EC-3) domains, which are protein-protein interaction motifs thought to interact with PRC (Polycomb Repressive Complex), and a C-terminal sequence that binds the BTD of RBPJ (Fig. 3B) (Qin et al. 2004; Qin et al. 2005). FHL1 binds RBPJ with high affinity and has a hydrophobic tetrapeptide sequence similar to RAM (Collins et al. 2014). However, FHL1 does not contain the other motifs in RAM, e.g. N-terminal basic residues, and –HG– and –GF–, required for high affinity binding of RBPJ. RITA1 also contains a hydrophobic tetrapeptide motif that is essential for its interaction with RBPJ and is also missing the other motifs in RAM that are required for high affinity interactions with RBPJ (Fig. 3C) (Tabaja et al. 2017; Wacker et al. 2011). In contrast to FHL1, RITA1 only binds RBPJ with moderate affinity (~1uM K_d) (Tabaja et al. 2017). Additionally, RITA1 has other functional domains, such as nuclear import and export sequences, and a C-terminal domain that interacts with tubulin, and interestingly, RITA1 appears to have Notch independent functions outside the nucleus (Wacker et al. 2011; Steinhauser et al. 2016).

6 Coregulator Competition

An open question in the field is whether corepressors and coactivators compete for binding to CSL in the nucleus, or alternatively, are there different pools of CSL-mediated transcription complexes in the nucleus that are then recruited to different Notch target genes. As mentioned previously, the classical model of Notch signaling proposes that CSL is constitutively bound to DNA, and in the absence of a Notch signal, DNA bound CSL-corepressor complexes actively repress transcription from Notch target genes; when Notch becomes activated in the cell, NICD translocates to the nucleus directly binding CSL, recruiting MAML and simultaneously displacing corepressors, thereby activating transcription at these sites. Numerous *in vitro* studies have shown that corepressors and coactivators can compete for binding to CSL. In pulldown assays from cellular extracts it has been shown that overexpression of one coregulator can displace the binding of another coregulator to CSL (Xu et al. 2017). For example, overexpression of NICD in cells can outcompete SHARP/SPEN for binding to CSL (Oswald et al. 2002; Kuroda et al. 2003).

Similarly, with purified recombinant proteins it has been shown that coregulators can compete for binding to CSL (Johnson et al. 2010; VanderWielen et al. 2011). Another example is the competitive binding of NICD and HAIRLESS for Su(H) (Maier et al. 2011). In this case, NICD is very effective at competing off HAIRLESS bound to Su(H) even in the absence of MAM. Similar experiments performed with the mammalian proteins RBPJ, MAML, NICD, and SPEN demonstrate that MAML is required for NICD to effectively compete off SPEN binding to RBPJ (VanderWielen et al. 2011). While it has been shown *in vitro* that corepressors and coactivators can compete for binding to CSL, it is not clear whether this actually occurs in cells under normal physiological conditions. Put another way, does every NICD molecule have to compete with a CSL bound corepressor in order to activate transcription or are their free molecules of CSL in the nucleus that NICD can easily access, and therefore corepressor displacement is an *in vitro* artifact? At the present time it is unclear whether one or both of these mechanisms are functioning in cells. Certainly, future studies that quantitate the number of CSL, corepressor, and NICD molecules within the cell, coupled with the known *in vitro* affinities of these complexes, will then begin to allow for a clearer picture of whether coregulators compete for CSL binding or not.

7 Modulation of CSL-Mediated Transcription Complexes

Given that numerous corepressors and coactivators bind to the BTD of CSL raises the question as to whether small molecules or biologic reagents can be identified that inhibit the binding of one, or some, coregulators, but not inhibit interactions with all coregulators. On the face of it this seems to be an arduous task because of the structurally similar manner, in which many coregulators bind to the nonpolar surface on the BTD of CSL. However, there is some experimental data that suggests it may be possible to identify reagents that selectively inhibit one coregulator, sparing the binding of others. A number of years ago, the Kempkes laboratory, using a yeast two-hybrid screen, identified mutations in RBPJ that selectively inhibited binding to the RAM domain of NICD or the viral coactivator EBNA2, but not to both (Fuchs et al. 2001). Interestingly, these subtle mutations lie right in the middle of the RAM binding site on the BTD. More recently, these binding results were confirmed by the Barrick laboratory using purified recombinant proteins and isothermal titration calorimetry (Johnson et al. 2010). Moreover, RBPJ binding data from the Kovall laboratory is consistent with the Kempkes results, i.e. in some cases mutations in the BTD can have drastically different impacts on the binding of different coregulators (Xu et al. 2017; Collins et al. 2014; Tabaja et al. 2017; Yuan et al. 2012). Taken together, these results raise the exciting prospect that it may be possible to identify selective reagents that affect either the repression or activation function of CSL, but not both, which could have biomedical applications for human diseases that are characterized by either insufficient or overactive Notch signaling.

8 Summary, Concluding Remarks and Open Questions

Progress made over the past decade has provided amazing insights into the molecular structures of the transcriptional components of the Notch signaling pathway. Available structures that contain CSL transcription complexes are summarized in Table 1. Structural studies of CSL-associated coactivator and corepressor complexes from different species have revealed the intriguing evolutionary conservation of these molecular interactions and mechanism, albeit with some species-specific differences. We now know that many corepressors interact with CSL by "mimicking" the RAM domain of NICD and its interactions with the BTD of CSL; however, there appear to be significant differences associated with their affinities and specificities for CSL. This has led to an understanding as to why there is competitive binding of NICD and KyoT2 or

Table 1 Available CSL complex structure data (**P**rotein **D**ata **B**ank, PDB)

PDB-ID	Complex	Species	Reference
1TTU	CSL bound to DNA	*C. elegans*	Kovall et al. (2004)
2FO1	Activator complex bound to DNA[a]	*C. elegans*	Wilson et al. (2006)
2F8X	Activator complex bound to DNA[b]	*H. sapiens*	Nam et al. (2006)
3BRD	CSL-RAM bound to DNA	*C. elegans*	Friedmann et al. (2008)
3BRF	CSL-RAM bound to DNA	*C. elegans*	Friedmann et al. (2008)
3BRG	CSL bound to DNA	*M. musculus*	Friedmann et al. (2008)
3NBN	Activator complex dimer bound to DNA	*H. sapiens*	Arnett et al. (2010)
3V79	Activator complex bound to DNA[a]	*H. sapiens*	Choi et al. (2012)
3IAG	CSL bound to DNA	*M. musculus*	Friedmann et al. (2010)
4J2X	Repressor complex bound to DNA[c]	*M. musculus*	Collins et al. (2014)
5E24	Repressor complex bound to DNA[d]	*D. melanogaster*	Yuan et al. (2016)
5EG6	Repressor complex bound to DNA[e]	*M. musculus, H. sapiens*	Tabaja et al. (2017)

[a](CSL/ANK/RAM/MAML)
[b](CSL/ANK/MAML)
[c](CSL/KYOT2)
[d](Su[H]/HAIRLESS)
[e](CSL/RITA1)

RITA1 for CSL. Future studies that seek to elucidate the structures of CSL complexes like CSL-SHARP and CSL-L3MBTL3 will provide additional molecular insights into how CSL functions as a repressor and will further refine our knowledge of these transcription factor-switching mechanisms. Despite this progress, there are still a lot of open questions in the field, for example: (I) Do the CSL-associated coactivator and corepressor complexes exchange on DNA or are there pre-existing complexes in the nucleoplasm or is it some combination of both mechanisms? (II) Are CSL-corepressor complexes gene-, binding site- and cell type-specific, and if so, how are these specificities regulated? (III) Does CSL DNA-binding affinity change when complexed with NICD or corepressors? And finally, (IV) will it be possible to manipulate CSL specific cofactor binding with small molecules or biologics in order to modulate the Notch response for clinical applications in the future?

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