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Selective repression of Notch pathway target gene transcription

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ABSTRACT

The Notch signaling pathway regulates metazoan development, in part, by directly controlling the transcription of target genes. For a given cellular context, however, only subsets of the known target genes are transcribed when the pathway is activated. Thus, there are context-dependent mechanisms that selectively maintain repression of target gene transcription when the Notch pathway is activated. This review focuses on molecular mechanisms that have been recently reported to mediate selective repression of Notch pathway target gene transcription. These mechanisms are essential for generating the complex spatial and temporal expression patterns of Notch target genes during development.

Introduction

The Notch signaling pathway is necessary for regulating many cellular processes in metazoan development, including progenitor proliferation, cell-fate specification and cell death (Gazave et al., 2009; Richards and Degnan, 2009). This regulation involves a direct control of gene transcription that is coordinated by the DNA binding transcription factor CSL (CBF-1/RBPJκ/Suppressor of Hairless/LAG-1). A summary of the canonical model by which the Notch signaling pathway regulates gene transcription is provided in Fig. 1. In brief, when the Notch pathway is not active, target gene transcription is blocked by protein co-repressor complexes assembled on CSL (Kopan and Ilagan, 2009; Lai, 2002). Activation of target gene transcription involves the conversion of CSL from a co-repressor to a co-activator, and this conversion requires activation of the Notch pathway. Membrane-bound DSL (Delta/Serrate/LAG-2) proteins expressed by adjacent cells are the canonical ligands that activate the Notch receptors. These ligands bind to the Notch extracellular domain (NECD) and induce a proteolytic cleavage of the Notch receptor that releases the NECD-ligand complex. Endocytic trafficking of the NECD-ligand complex in the ligand-expressing cell is crucial for proper activation of the Notch pathway in the receptor-expressing cell (Le Borgne et al., 2005). Following cleavage of the NECD, the Notch intracellular domain (NICD) is proteolytically released and transported into the nucleus. In the nucleus, NICD forms a complex with CSL and displaces the co-repressor proteins bound to CSL. Mastermind (MAM)/Mastermind-like (MAML)/LAG-3 is the canonical transcription co-activator protein that binds the CSL/NICD complex. CSL/NICD/MAM complexes enhance target gene expression, in part, by recruiting other co-activators and chromatin remodeling enzymes (Kopan and Ilagan, 2009). This ensemble of co-activators bound to CSL/NICD is referred to as the "Notch transcription complex."

Recent proteomic approaches have expanded the number of genes that are direct targets of the Notch transcription complexes (Hamidi et al., 2011; Margolin et al., 2009; Palomero et al., 2006). In any given cellular context, however, only a subset of target genes is transcribed when the pathway is activated. In the *C.elegans* embryo, for example, different members of the *ref-1* family of basic-helix-loop-helix (bHLH) repressor genes are activated in partially overlapping patterns within the AB and EMS cell lineages (Neves and Priess, 2005). Alternatively, in *Drosophila melanogaster*, Notch signaling drives expression of individual genes of the *Enhancer of split Complex* (*E(spl)-C*) in spatially and temporally distinct patterns within the developing embryonic and larval tissues (Cooper et al., 2000; de Celis et al., 1996; Maeder et al., 2009; Nellesen et al., 1999; Wech et al., 1999). Also, murine *Hes5* and *Hes1* show reciprocal expression patterns in the developing midbrain, hindbrain, isthmus and optic vesicles (Hatakeyama et al., 2004). Additional examples of differential expression patterns for Notch target genes in vertebrate development are reported in the kidney (Chen and Al-Awqati, 2005; Leimeister et al., 2003; Piscione et al., 2004), intestine (Schroder and Gossler, 2002), heart (Fischer and Gessler, 2003), as well as embryonic stem cells (Meier-Stiegen et al., 2010).

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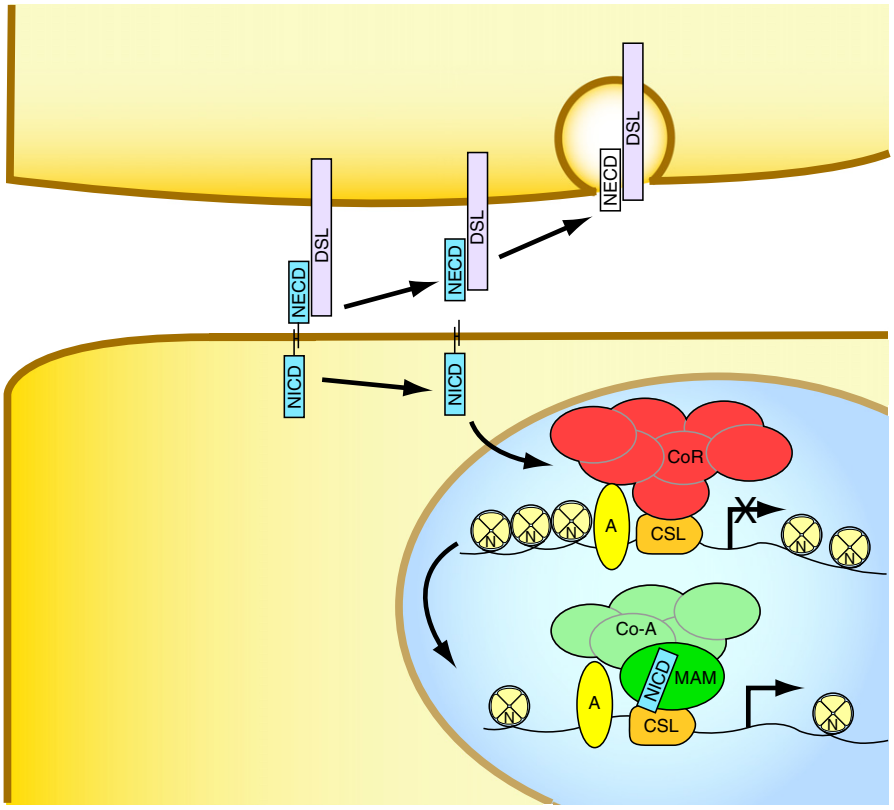

Fig. 1. Summary of the canonical Notch signaling pathway. DSL (Delta/Serrate/LAG2) ligand binding to the Notch extraceullular domain (NECD) induces proteolytic cleavage that releases the NECD-ligand complex, which is subsequently endocytosed by the ligand-expressing cell. The remaining membrane-bound Notch protein is proteolytically cleaved to release the Notch intracellular domain (NICD). In the absence of NICD, the DNA binding transcription factor CSL (CBF-1/RBPJκ/Suppressor of Hairless/LAG1) blocks transcription of pathway target genes by recruiting co-repressor (CoR) protein complexes. When the Notch pathway is activated, NICD translocates to the nucleus and forms a complex with CSL, which displaces the co-repressor proteins bound to CSL. The CSL/NICD complex recruits co-activator protein Mastermind (MAM) and the CSL/NICD/MAM complex serves as a scaffold for other transcription co-activators. Chromatin and nucleosome (N) remodeling enzymes are important components of both co-activator and co-repressor complexes recruited by CSL. Strong activation of gene expression requires synergistic interactions with local transcription factor activator proteins (A) also bound to the target gene cis-regulatory modules.

Selective activation of Notch pathway target gene transcription

Since all canonical Notch pathway target gene transcription is coordinated by a single CSL protein orthologue, a fundamental question is how the Notch pathway differentially activates transcription in a context-dependent manner? Combinatorial interactions between Notch transcription complexes and tissue-specific (or local) transcription factor activators bound to target gene cis-regulatory elements are an effective mechanism to selectively activate transcription (reviewed in Barolo and Posakony, 2002). These combinatorial interactions restrict target gene transcription to those cells that have both the specific local activators expressed and the Notch pathway activated. In the absence of local activators, Notch signaling is either insufficient to activate target gene expression or only induces weak expression levels (Barolo and Posakony, 2002). A well established example of this combinatorial regulation is the synergistic and physical interaction between basic-helix-loop-helix (bHLH) proteins and Notch transcription complexes that activate the expression of specific target genes in *Drosophila* and *Xenopus* neurogenic territories (Castro et al., 2005; Cave et al., 2005, 2009; Cooper et al., 2000; Lamar and Kintner, 2005; Singson et al., 1994). In other cellular contexts, Notch transcription complexes can synergistically and physically interact with other types of local transcription factors to activate transcription of different target genes (Blokzijl et al., 2003; Gustafsson et al., 2005; Hayashi and Kume, 2008; Itoh et al., 2004; Kitamura et al., 2007; Maekawa et al., 2008; Neves et al., 2007; Sakamoto et al., 2008; Sun et al., 2005; Takizawa et al., 2003; Tang et al., 2010).

For organisms that express multiple Notch receptor paralogues, the incorporation of different NICD paralogues into Notch transcription complexes increases the combinatorial complexity with local activators. Mammals, for example, express four Notch receptor paralogues and, in aortic smooth muscle cells, phosphorylated-SMAD2/3 transcription factors physically interact with Notch4-ICD, but not with either Notch1-ICD or and Notch2-ICD (Tang et al., 2010). These differential interactions with local activators are important for establishing target gene preferences for NICD paralogues (Ong et al., 2006).

Although combinatorial interactions between Notch transcription complexes and local activators are likely the predominant mechanism by which Notch target gene transcription is selectively activated, alternative mechanisms have been also reported. Transcription of the mammalian *Hes1* or *Hey2* canonical Notch target genes is activated by Notch-independent mechanisms in some developmental contexts (Curry et al., 2006; Doetzlhofer et al., 2009; Leimeister et al., 2000; Sanalkumar et al., 2010; Stockhausen et al., 2005; Timmerman et al., 2004; Wall et al., 2009). This Notch-independent activation of *Hes1* and *Hey2* transcription requires the activity of other signaling pathways, but how these other pathways abrogate repression mediated by CSL/co-repressor without the assistance of the Notch pathway is unclear. In the developing pancreas and spinal cord, CSL-mediated repression of *Elastase1* and *Neurogenenin2*, respectively, is alleviated by physical interactions between PTF1 protein complex and CSL (Beres et al., 2006; Henke et al., 2009). The PTF1 complex binds the genomic DNA adjacent to CSL and displaces the co-repressors bound to CSL to activate gene transcription. Notch signaling is dispensable for this mechanism since the PTF1 complex excludes NICD from binding to

CSL, which demonstrates that not all CSL-regulated genes are necessarily Notch pathway target genes. By contrast, the expression of genes not typically considered Notch pathway target genes can be activated by non-canonical NICD transcription complexes formed by NICD physically interacting with DNA-binding proteins other than CSL. For example, human Notch1-ICD can co-activate transcription by forming a complex with the LEF-1 transcription factor (Ross and Kadesch, 2001). Also, Notch1-ICD binds to p50 and sustains NF-κB mediated activation of IFN-γ transcription in murine T-cells (Shin et al., 2006).

Selective repression of target gene transcription

Mechanisms that selectively activate Notch target gene transcription are necessary, but not always sufficient for establishing the distinct expression patterns of individual Notch target genes. In *Drosophila*, for example, combinatorial interactions between Notch transcription complexes and bHLH transcription factors are necessary for the expression of several *E(spl)-C* target genes within proneural clusters of the wing imaginal disc, but these combinatorial interactions are not sufficient to explain why individual *E(spl)-C* target genes are expressed in different subsets of proneural clusters (Cooper et al., 2000). This insufficiency of selective activation mechanisms to fully account for target gene expression patterns indicates that additional mechanisms maintain repression of select target genes when the Notch pathway is activated. Simply repressing the expression of local activators required for Notch target gene transcription, however, is not necessarily an effective mechanism. Derepression of CSL by the Notch pathway in the absence of local activators can still induce weak expression levels of some target genes, and even weak expression levels of some target genes may be detrimental for proper development. Recent studies have reported several novel mechanisms by which target gene transcription can selectively remain repressed when the Notch pathway is activated. These mechanisms, which are discussed in greater detail below, include the differential regulation of local chromatin environments, the assembly of different CSL/co-repressor complexes, combinatorial interactions with local repressors, and binding site architecture in the target gene cis-regulatory regions. These novel repression mechanisms, in combination with selective activation mechanisms, are essential for establishing the distinct expression patterns of individual Notch target genes.

Selective repression by epigenetic mechanisms

In the canonical model of Notch signaling, chromatin remodeling enzymes associated with CSL/co-repressor complexes maintain target gene chromatin environments in a transcriptionally repressive state when the Notch pathway is inactive (Di Stefano et al., 2011; Goodfellow et al., 2007; Hsieh et al., 1999; Kao et al., 1998; Liefke et al., 2010; Moshkin et al., 2009; Mulligan et al., 2011; Oswald et al., 2002, 2005; Pajerowski et al., 2009). The active and repressive transcriptional states of chromatin are distinguished by distinct patterns of post-translational modifications to histones (Jenuwein and Allis, 2001). Modifications associated with active transcription include trimethylation of histone H3 lysine residue 4 (H3K4me3), mono-methylation of histone H3 lysine residue 27 (H3K27m) and acetylation of histone H3 lysine residue 9 (H3K9ac). By contrast, repression is associated with demethylation of H3K4, deacetylation of H3K9, and trimethylation of both lysine 9 and lysine 27 residues on histone H3 (H3K9me3 and H3K27me3, respectively). Repressive chromatin environments prevent activation of target gene transcription by impeding access of local activators, NICD and other co-activators to the target cis-regulatory regions.

Although local chromatin environments of Notch target genes would be expected to lack epigenetic markers of active transcription when the pathway is quiescent, recent studies have shown that is not the case. An analysis of *E(spl)-C* target genes in cultured *Drosophila* cells found that a subset of genes was already enriched for H3K4me3 when Notch pathway activity was absent (Krejci and Bray, 2007). These enriched target genes were also the genes selectively expressed when the Notch pathway was activated. Consistent with these findings, genes transcribed in response to the expression of Notch1-ICD in murine embryonic stem cells were also found to be preferentially enriched for the transcriptionally active H3K4me3 marker when Notch1-ICD was absent (Schwanbeck et al., 2011). About 40% of these murine target genes were enriched with only the H3K4me3 modification and lacked the repressive H3K27me3 marker. By contrast, the other ~60% genes had "bivalent domains" that contained both the H3K4me3 and H3K27me3 markers. Bivalent domains are transcriptionally repressive, but the presence of active epigenetic modifications makes these regions primed to become transcriptionally active when the appropriate cellular context is provided.

Together, these findings indicate that the methylation status of H3K4 in the absence of Notch signaling can identify which target genes will remain repressed when the pathway does become active (Fig. 2). Specifically, those target genes enriched with demethylated H3K4 will remain repressed when the Notch pathway is activated. By contrast, target genes with chromatin environments enriched for H3K4me3, either with or without repressive epigenetic markers such as H3K27me3, are primed to initiate transcription in response to the Notch pathway. However, enrichment of the H3K4me3 marker does not guarantee target gene transcription when the pathway is activated since other cell context-specific factors can repress gene expression.

The importance of target gene-specific epigenetic modifications raises the question of how the local chromatin environments of target genes are differentially regulated within the same cell. Using both biochemical and developmental genetic approaches in *Drosophila*, the Bray lab has shown that CSL can assemble target gene-specific co-repressor complexes that have distinct chromatin remodeling functions. These studies found that CSL, together with the co-repressors SKIP and Hairless, recruits two different transcription silencing complexes (Moshkin et al., 2009). Both complexes have the core LAF complex, which contains the lysine demethylase LID, the histone deacetylase SIN3A and its partner PF1, the transcription repressor EMSY and the chromodomain MRG15 protein. This core LAF complex can associate with either the histone chaperone ASF1 (forming the LAF-A complex) or both the histone deacetylase RPD3 and histone chaperone NAP1 (forming the RLAF-N complex). These interactions between CSL/LAF with either ASF1 or RPD3/NAP1 are mutually exclusive. The LAF-A and RLAF-N complexes both mediate demethylation of H3K4, but the RLAF-N complex also facilitates deacetylation of H3 and increases local histone density. Disruption of the LAF-A complex by RNAi-mediated depletion of ASF1 substantially increased both basal and Notch-induced expression levels for only a select subset of target genes in the *E(spl)-C* (Goodfellow et al., 2007), indicating that different CSL/co-repressor complexes selectively regulate target gene transcription.

The local chromatin environments of Notch target genes can also be differentially regulated by Polycomb-Group (PcG) repressor complexes (Fig. 2). Several genetic studies have established that PcG complexes are critical for both the proper function of the Notch pathway and the expression of several Notch target genes (Bejarano and Milan, 2009; Chopra et al., 2009; Dietrich et al., 2005; Ferres-Marco et al., 2006; Janody et al., 2004; Martinez et al., 2009; Miyazaki et al., 2005; Tolhuis et al., 2006). PcG-mediated repression has two basic steps with the initial event being trimethylation of both H3K27 and H3K9 by the Polycomb Repressing Complex 2 (PRC2). The second step is the recruitment of Polycomb Repressing Complex 1 (PRC1), which binds H3K27me3. PRC1 both catalyzes ubiquitination of histone H2A to promote chromatin compaction and inhibits RNA polymerase II transcriptional elongation (Simon and Kingston, 2009). In murine embryonic stem cells, PRC2 is bound to many bivalent

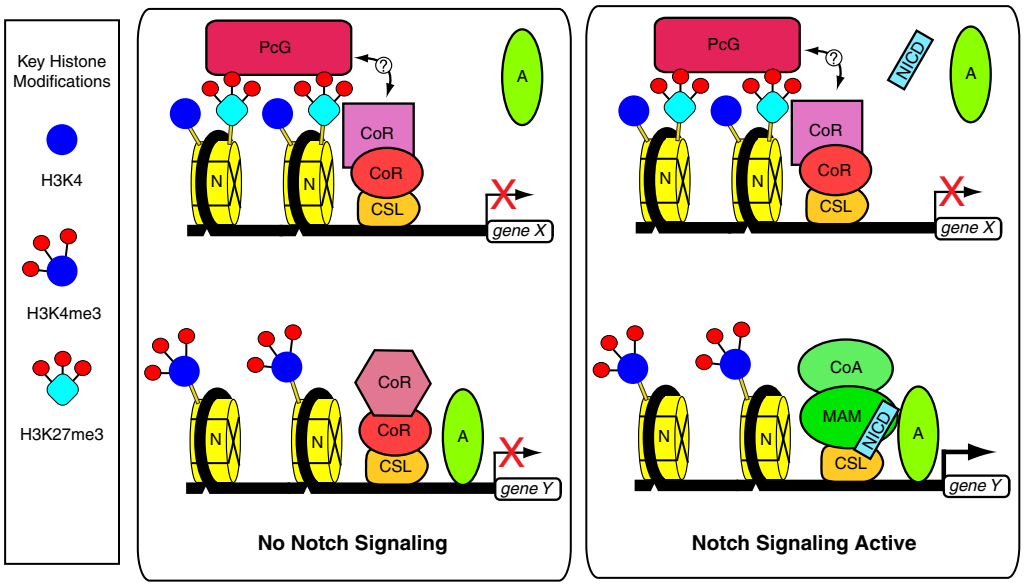

Fig. 2. Epigenetic mechanisms of selective Notch target gene repression. In the absence of Notch signaling, local chromatin environments of target genes are differentially modified. Some genes are enriched with nucleosomes (N) containing unmethylated Histone 3 lysine 4 residues (H3K4) and trimehtylated Histone 3 lysine 27 residues (H3K27me3), which are associated with transcriptional repression. By contrast, some genes have nucleosomes are enriched with trimehtylated Histone 3 lysine 4 residues (H3K4me3), which are associated with transcriptional activation. These different sets of epigenetic modifications can be generated, in part, by target gene-specific CSL/co-repressor (CoR) complexes that have different chromatin remodeling properties (Moshkin et al., 2009). The mechanisms underlying the target gene-specific assembly of CSL/co-repressor complexes remain to be established. Target gene-specific chromatin modifications can also differentially recruit Polycomb Group (PcG) gene silencing complexes (Schwanbeck et al., 2011). PcG complexes bind regions enriched with H3K27me3, but physical interactions between co-repressors that interact both with CSL and subunits of PcG suggest that chromatin remodeling by CSL and PcG co-repression complexes may be coordinated (indicated by double arrow with question mark). When the Notch pathway is activated, target genes (such as *gene X*) enriched with unmethylated H3K4 often remain transcriptionally repressed (Krejci and Bray, 2007; Schwanbeck et al., 2011) due, in part, to the repressive chromatin environments that impede the access of local activators (A) and NICD. By contrast, genes enriched with H3K4me3 (such as *gene Y*) are poised for transcriptional activation provided other repression mechanisms do not interfere.

domains associated with Notch target genes, but only a subset of these PRC2-positive bivalent domains were also co-occupied by PRC1 (Schwanbeck et al., 2011). This differential recruitment of PRC1 indicates that PcG complexes selectively silence subsets of Notch target genes.

Interestingly, the subset of target genes bound by both PRC1 and PRC2 in murine embryonic stem cells was over-represented by those genes encoding transcription factors critical for establishing developmental cell fate lineage. This selective repression by PcG complexes is thought to be necessary for maintaining the pluripotent state of the embryonic stem cells, and the subset of target genes repressed by the PcG complexes is predicted to change as the cell context changes during development and specific differentiation lineages are adopted (Ku et al., 2008; Schwanbeck et al., 2011).

Differential stability of distinct CSL/co-repressor complexes

In addition to modifying local chromatin environments, target gene-specific CSL/co-repressor complexes can also have different susceptibilities to NICD-mediated derepression. For example, the mammalian MTG16 (ETO2) and NCoR/SMRT proteins are non-DNA binding transcription co-repressor proteins that form complexes with CSL and facilitate recruitment of histone deacetylase enzymes (Amann et al., 2001; Kao et al., 1998). In hematopoietic stem cells, MTG16 selectively represses a subset of target genes and NICD can abrogate this repression by physically interacting with MTG16 to disrupt the CSL/MTG16 complex (Engel et al., 2010). By contrast, NICD cannot disrupt the interaction between CSL and NCoR under similar conditions, revealing that distinct CSL/co-repressor complexes have different susceptibilities to NICD-mediated displacement and that some complexes cannot be disrupted by NICD. The inability of NICD to displace some co-repressors from CSL was also reported in thermodynamic studies with the mammalian co-repressor SHARP (MINT) (Vanderwielen et al., 2011). These studies showed that SHARP binds with high affinity to CSL and competes with NICD for binding to CSL. NICD is not likely sufficient to displace SHARP from CSL based on relative affinity since nuclear NICD concentrations are unlikely to be significantly higher than the co-repressors (Vanderwielen et al., 2011). Thus, target genes associated with highly stable CSL/co-repressor complexes can potentially remain repressed when the Notch pathway is activated unless other mechanisms destabilize the CSL/co-repressor complexes.

The molecular basis for the different stabilities of distinct CSL/co-repressor complexes remains to be established, but biophysical studies investigating the formation of the CSL/NICD and CSL/NICD/MAM co-activation complexes have provided some insight into structural differences between co-activation and co-repression complexes that are induced by NICD. These studies (reviewed in Gordon et al., 2008; Kovall and Blacklow, 2010) have shown that the NICD RAM and ankyrin repeat domains bind to the β-trefoil and C-terminal rel-homology domains of CSL, respectively (Friedmann et al., 2008; Nam et al., 2006; Wilson and Kovall, 2006). The interaction between the NICD RAM and CSL β-trefoil domains induces the repositioning of a loop region within the CSL N-terminal rel-homology domain that facilitates recruitment of MAM (Friedmann et al., 2008). The interaction between NICD and CSL also induces formation of an anti-parallel β-sheet with residues from within both the NICD RAM domain and a loop region in the CSL β-trefoil domain. The amino acids within the CSL β-trefoil domain that form this β-sheet are also critical mediating repression by SMRT/NCoR and CIR, suggesting that conformational changes in the β-trefoil domain induced by NICD disrupt interactions between CSL and co-repressors (Wilson and Kovall, 2006).

The allosteric mechanism in the β-trefoil domain was elucidated with Notch pathway proteins from mammals and nematodes, but recent studies suggest that *Drosophila* NICD may mediate a different allosteric mechanism to disrupt CSL/co-repressor complexes. In *Drosophila*, the ubiquitously expressed Hairless protein is a co-repressor of CSL that is critical for repressing Notch pathway target genes in many developmental contexts (Maier, 2006). Unlike mammalian co-repressors

which bind the CSL β-trefoil domain, Hairless binds with high affinity to the CSL C-terminal domain (Maier et al., 2011). Mutations to the CSL β-trefoil domain that disrupt the binding of mammalian co-repressors have no effect on the binding of Hairless to CSL. The affinity of Hairless for the CSL C-terminal domain is approximately 10,000 times greater than the NICD ankyrin repeats, which also bind this region of CSL. Despite this lower binding affinity, NICD can displace Hairless form CSL. A potential explanation for this finding is that NICD induces a conformational change within CSL that decreases the affinity for Hairless and enables the NICD ankyrin repeats to more effectively compete for binding to CSL (Maier et al., 2011). Thus, the use of NICD-mediated allosteric mechanisms to destabilize CSL/co-repressor complexes appears evolutionarily conserved, but the specific structural changes within CSL that mediate derepression may be species-dependent. These findings also imply that mechanisms that enhance the stability of NICD-induced conformational changes in CSL or the overall stability of CSL/NICD complexes will facilitate both displacement of co-repressor complexes and activation of specific target gene transcription. Conversely, target gene repression will be favored by CSL conformations that stabilize co-repressor binding.

The avidity of CSL binding to DNA may be another important determinant of individual target gene transcription responses to the Notch pathway. In the absence of Notch signaling, CSL occupancy on individual *Drosophila* target gene cis-regulatory regions can be dramatically different and directly correlated with the presence of RNA polymerase II and histone markers of transcriptional active chromatin (Krejci and Bray, 2007). The genes enriched with these markers were found to have the highest levels of transcription induced when the Notch pathway was activated. Unexpectedly, transcription of these genes was also accompanied by an increase in CSL occupancy on the target gene promoter, suggesting that CSL/co-repressors have lower resident life-times when compared to Notch transcription complexes (Krejci and Bray, 2007). This dynamic equilibrium between free and DNA-bound CSL may reflect the modest DNA binding affinity of CSL (Friedmann and Kovall, 2010) and the increased binding site occupancy by CSL may be generated by stabilizing interactions between Notch transcription complexes and local activators (Krejci and Bray, 2007).

An interesting possibility presented by the significant dissociation rate of CSL/co-repressor complexes is that activation of target gene transcription may not actually require the physical displacement of co-repressors from CSL by NICD in order for Notch transcription complexes to assemble. The dissociation of the CSL/co-repressor complexes from DNA could enable new CSL molecules that lack co-repressors to bind the DNA. In the absence of Notch pathway activity, co-repressor proteins can re-assemble on CSL to maintain target gene repression. If Notch signaling is active, however, the new CSL protein bound to the DNA can recruit NICD and assemble Notch transcription complexes. This potential mechanism would enable derepression and activation of target genes that are repressed by highly stable CSL/co-repressor complexes that cannot be displaced by NICD. Also, any mechanism that reduces the dissociation rate of CSL/co-repressor complexes on specific target genes would increase the probability that those genes selectively remain repressed when the Notch pathway is activated.

Combinatorial interactions with local repressors

In addition to the role of CSL/co-repressor complexes, local repressors are also a key determinant of whether a target gene remains repressed when the Notch pathway is activated. The cell-specific expression of local repressors in combination with their sequence specificity of DNA binding enables these proteins to repress select target genes in a specific cell context. For example, the zinc-finger FEZF1/2 proteins are transcription repressor proteins expressed in forebrain neural stem cells where they function redundantly to initiate differentiation of cortical neurons and prevent the rostral forebrain from adopting a caudal diencephalic fate. The control of neural stem cell differentiation by these proteins is exerted by selectively repressing Notch-mediated transcription of *Hes5* without affecting *Hes1* expression (Shimizu et al., 2010). The selectivity of this target gene repression requires the sequence specificity of the FEZF1/2 DNA binding domain, which directly binds the *Hes5* promoter.

Many cell context-dependent factors control the expression of local repressors and, in some cases, Notch pathway effector proteins can function as local repressors that selectively repression other Notch target genes. In hippocampal neural progenitors, for example, Notch signaling activates transcription of *Nfia*, which encodes a CCAAT-box binding transcription factor. Notch-induced expression of NFIA promotes glial differentiation by simultaneously repressing Notch-mediated *Hes1* transcription and activating expression of glial-specific genes, such as *Gfap* (Piper et al., 2010). The selectivity of NFIA repressing *Hes1* is mediated by the sequence specificity of the NFIA DNA binding domain, which directly binds the *Hes1* promoter. An interesting feature of this cross-regulation of between Notch target genes is that *Hes1* is expressed in neural progenitors as early as embryonic day 7.5 (Kageyama et al., 2008), but *Nfia* is not expressed until embryonic day 13 (Plachez et al., 2008). This temporal difference in the initial expression of these genes reveals that *Nfia* transcription is itself selectively repressed in neural progenitors between E7.5 and E13. The mechanisms responsible for this selective repression *Nfia* are unclear, but there is clearly a change in the cellular context of neural progenitors at E13 that enables Notch signaling to activate *Nfia* transcription. This temporal control in the selective repression of the *Nfia* enables Notch signaling to exert developmental stage-specific control over a subset of its target genes, including *Hes1*, during neurogenesis.

The molecular mechanisms by which FEZF1/2 and NFIA repress Notch target gene transcription are not established, but at least two different mechanisms are possible. One mechanism is that local repressors assist in recruiting co-repressors on to CSL and/or stabilize the CSL/co-repressor complexes (Fig. 3A). Many of the co-repressor proteins that associate with CSL, such as SMRT/NCoR, Groucho/TLE and CtBP, can also interact with other DNA binding repressor proteins (Chinnadurai, 2007; Cinnamon and Paroush, 2008; Jepsen and Rosenfeld, 2002). Local repressor-dependent recruitment and stabilization of CSL/co-repressor complexes provides a solution to the paradox of differential recruitment of co-repressors by CSL. The co-repressors by themselves do not bind DNA and lack any inherent target gene specificity. Furthermore, recognition and binding by CSL to consensus DNA binding sites also lacks any target gene specificity. Local repressors, however, do have target gene specificity due to the sequence specificity of their DNA binding domains. Thus, combinatorial interactions between CSL and local repressors can provide for the assembly of target gene-specific CSL/co-repressor complexes. As discussed above, these target gene-specific complexes can selectively repress transcription by either differentially modifying local chromatin environments or by having differential susceptibilities to NICD-mediated displacement of co-repressors.

An alternative repression mechanism is that local repressors can selectively block co-activation of CSL/NICD complexes (Fig. 3B). In this mechanism, local repressors are not necessary to stabilize CSL/co-repressors, rather they essential for recruiting co-repressors that block co-activation of CSL/NICD complexes. An example of this selective repression was recently demonstrated in the establishment of left-right asymmetry in the developing *Xenopus* embryo (Sakano et al., 2010). *Pitx2* is expressed specifically in the left plate mesoderm and the DNA binding transcription repressor BCL6 promotes *Pitx2* expression in the left plate mesoderm, in part, by selectively repressing transcription of the Notch target gene *Esr1*. This repression is mediated by BCL6 binding to the *Esr1* promoter and recruiting the BCOR co-repressor. The BCL6/BCOR co-repressor complex physically interacts

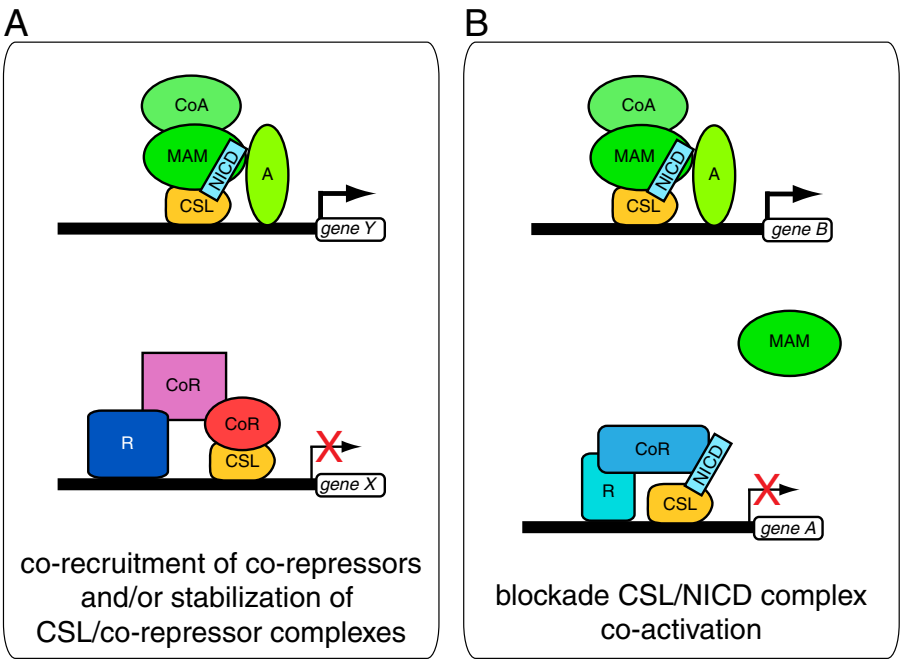

Fig. 3. Selective repression of Notch target gene transcription by combinatorial interactions between local repressors and CSL/co-repressor complexes. A, local repressors (R) bind to the cis-regulatory regions of select target genes (such as *gene X*) based the sequence specificity of their DNA binding domains. Local repressors can facilitate recruitment of co-repressors (CoR) on to CSL and/or stabilize CSL/co-repressor complexes. Transcription of target genes (such as *gene Y*) not bound by local repressors can be activated by synergistic interactions between local activators (A) and Notch transcription complexes. B, alternatively, local repressors can selectively repress transcription of target genes (such as *gene A*) by recruiting co-repressors that block co-activation of CSL/NICD complexes.

with the ankyrin repeats of NICD and blocks recruitment of the MAM co-activator. The repression is selective since the expression of other Notch target genes in the left lateral plate mesoderm, such as *Hairy2*, are unaffected by knock-down BCL6 and BCL6 binding to *Hairy2* promoter is not detected in Chromatin immunoprecipitation (ChIP) assays (Sakano et al., 2010).

In some cases, local repressors can do more than combinatorially interact with CSL/co-repressors, they can supplant the role of CSL/co-repressor complexes. Mutating CSL binding sites or eliminating CSL (by either knock-down or gene mutation) typically results in target gene derepression and a "broadening and weakening" of the expression pattern (Barolo and Posakony, 2002). However, in T-cell development, the loss of CSL does not de-repress Notch target gene expression (Chari et al., 2010; Tanigaki et al., 2004). The maintained repression of these genes in the absence of CSL is mediated by Ikaros (Chari and Winandy, 2008), a DNA-binding transcription factor that recruits chromatin remodeling complexes, such as NURD (John and Ward, 2011). Whether Ikaros binds Notch target gene cis-regulatory regions in cooperation (Kathrein et al., 2008) or in competition (Beverly and Capobianco, 2003; Kleinmann et al., 2008) with CSL/co-repressor complexes remains to be clarified, but Ikaros-mediated repression in these cells is crucial since the disruption of this repression is strongly associated with T-cell acute lymphoblastic leukemia (Demarest et al., 2008). Thymocytic cultures lacking both CSL and Ikaros do not expand as aggressively as those lacking only Ikaros (Chari et al., 2010), suggesting that a subset of CSL-regulated genes is not targeted by Ikaros. Thus, an important question for future studies to address is whether local repressors, such Ikaros, repress only select Notch target genes and, if so, how is this selectivity is achieved.

Role of binding site architecture

For most target genes, binding sites in genomic DNA cis-regulatory regions are necessary to recruit both CSL and local transcription factors. The architectural arrangement of these binding sites within the genomic DNA can be critical for mediating context-dependent transcriptional regulation. Critical binding site architectural features include number, order, spacing and orientation. Recent studies in the developing *Drosophila* eye elegantly demonstrated the importance of binding site architecture (Swanson et al., 2010). The *dPax2* gene is specifically expressed in cone cells under the control of the *sparkling* enhancer region, which integrates input from the Notch signaling pathway (via a CSL binding site), the EGFR signaling pathway (via an ETS-domain binding site) and the local transcription factor LOZENGE (Flores et al., 2000). Disrupting the function of individual cis-regulatory regions by either deleting them, which alters the spacing between the remaining regions, or mutating them, which maintains the native spacing, had very different effects on reporter gene expression levels in cone cells. Furthermore, changing the order of the cis-regulatory regions within the enhancer region was sufficient to alter the cell-specificity of reporter gene expression from cone cells to photoreceptors (Swanson et al., 2010). Together, these findings clearly demonstrate that binding site architecture can be critical for direct proper expression patterns of Notch target genes.

For many Notch target genes, a specific CSL binding site architecture that assembles dimers of Notch transcription complexes is essential for activating transcription. This distinct CSL binding site architectural motif is called the SPS element (CSL Paired Site) and is defined by an inverted repeat of CSL binding sites that are separated by 15–17 nucleotides (Bailey and Posakony, 1995; Nellesen et al., 1999). CSL/NICD complexes cooperatively assemble on the SPS element and these dimeric complexes are stabilized, in part, by homotypic interactions between NICD proteins (Arnett et al., 2010; Nam et al., 2007). By expressing NICD mutants that disrupt transcriptional activation mediated by CSL/NICD dimers without interrupting regulation mediated by monomeric CSL/NICD complexes, the Blacklow laboratory showed that the activation of transcription for all Notch target genes can be divided into at least three distinct subsets: 1, those that require CSL/NICD dimers; 2, those that are completely independent of CSL/NICD dimers; and 3, those that utilize both monomeric and dimeric CSL/NICD complexes (Liu et al., 2010).

The architecture of CSL binding sites can also be critical for maintaining transcriptional repression when the Notch pathway is activated. In

Drosophila proneural clusters that generate mechanosensory bristles, the expression of *achaete*, *m8* and *mα* is regulated by both CSL and bHLH binding sites in each gene promoter. This combination of binding sites activates expression of *m8* and *mα* specifically in the non-neural precursor cells of the proneural cluster. By contrast, *achaete* is strongly expressed only in the neural precursor cells independently of Notch signaling (Cave et al., 2011). Thus, the combinational code of CSL and bHLH binding sites is not sufficient to either describe the transcriptional response of these genes to Notch signaling or predict their cell-specific expression in proneural clusters. In the case of *achaete* and *m8*, the differential transcriptional response to the Notch pathway was mediated, in part, by differences in the number and orientation of CSL bindings sites. The *achaete* promoter contains a single CSL binding site that recruits NICD when the pathway is activated, but the formation of the CSL/NICD complex is not sufficient to initiate transcription (Cave et al., 2011). By contrast, the *m8* promoter contains an SPS element that is necessary for physical interactions and transcriptional synergy between Notch transcription complexes and local bHLH proteins (Cave et al., 2005, 2009). The creation of an SPS element in the *achaete* promoter by adding a second CSL binding site was sufficient to switch the gene response to Notch signaling so that *achaete* transcription was synergistically activated when the pathway was activated. The example with *m8* and *achaete* clearly shows that CSL binding site architecture can be an important component of differential responses to the Notch pathway, but a major challenge moving forward is to elucidate the molecular mechanisms by which binding site architecture integrates the cell context-specific cues to influence whether a target gene is either expressed or remains repressed when the Notch pathway is activated.

Conclusions

Several inter-connected molecular mechanisms have evolved to selectively repress Notch target gene transcription. These mechanisms can differentially modify target gene chromatin environments so that access of local activators and NICD to target genes is selectively blocked. Specific sets of epigenetic markers within the local chromatin can distinguish which genes are capable of being transcribed when the pathway becomes activated. These distinct epigenetic modifications are generated by the differential recruitment of PcG gene silencing complexes and the assembly of target gene-specific CSL/co-repressor complexes that have distinct chromatin remodeling functions. Target gene-specific CSL/co-repressor complexes may also have different stabilities that influence the sensitivity of target gene transcriptional responses to Notch pathway activation. Highly stable CSL/co-repressor complexes are likely to have a lower susceptibility to NICD-mediated displacement and maintain repression when the pathway is activated. Local repressors that selectively bind target gene cis-regulatory regions may also be important for recruiting different sets of co-repressors on to CSL as well as stabilizing the CSL/co-repressor complexes. Alternatively, local repressors can maintain repression of the select target genes by blocking co-activation of CSL/NICD complexes. The ability of local repressors and CSL/co-repressor complexes to integrate cell context is mediated, in part, by binding site architecture for these proteins within target gene cis-regulatory regions.

In any given context, several of the mechanisms described above can contribute to the repression of a specific target gene. As development proceeds, however, the specific mechanisms that repress target genes are likely to change as the cell context also change. Important changes in the cell context that influence repression mechanisms include modifications to the local chromatin environments of target genes. A key question regarding the regulation of chromatin environments is whether CSL and PcG chromatin remodeling complexes coordinate their activities to repress specific target genes. A recent RNA interference screen in *Drosophila* revealed a complex interaction network between PcG and the Notch pathway genes (Saj et al., 2010). Also, previously reported physical interactions between Notch pathway and PcG proteins raise the possibility that CSL/co-repressor complexes can recruit PcG complexes (Qin et al., 2004, 2005). A second key question for future studies to address is whether CSL co-repressor proteins have a role similar to YAF2, which serves as a molecular bridge between PcG complexes and sequence-specific DNA-binding transcription factors in *Drosophila* and mammals (Wilkinson et al., 2010). Elucidating the interactions between the Notch pathway and PcG complexes, however, will be challenged by fact that the subunit composition of PcG complexes varies in different cellular and developmental contexts (reviewed in Saj et al., 2010; Simon and Kingston, 2009).

Cell context-dependent changes in the composition of the proteome can also influence the repression mechanisms acting on a target gene. In particular, changes in the expression of local repressor proteins can have a significant effect on the transcriptional response of a target gene to activation of the Notch pathway. An important question for future studies will be to establish whether combinatorial interactions between local repressors and CSL are necessary for the target gene-specific recruitment of different co-repressors. Also needing to be addressed is whether all target genes assemble a single "core CSL repression complex" that is analogous to the canonical CSL/NICD/MAM co-activation complex. In mammalian cells, the co-repressor SHARP has been suggested to function as a "hub" that recruits different co-repressors onto a CSL/SHARP core complex (Borggrefe and Oswald, 2009). The "hub" role in *Drosophila* may be played by Hairless, which has been suggested to be the functional analog of the mammalian SHARP co-repressor (Borggrefe and Oswald, 2009), but the molecular and structural details of how Hairless and SHARP function may be substantially different (Maier et al., 2011). Future studies will also need to establish the factors regulate the stability and turnover of CSL/co-repressor complexes.

Other changes in cell context that can influence repression mechanisms include the activity of non-Notch signaling pathways and miRNA post-transcriptional mechanisms. Several signaling pathways interact with the Notch pathway during development, including Wnt/wingless (Hayward et al., 2008), TGF-β/BMP (Kluppel and Wrana, 2005), EGFR (Doroquez and Rebay, 2006), TNFα/NF-κB (Osipo et al., 2008), and cytokine/JAK/STAT (Bigas et al., 1998; Josten et al., 2004). In some cases, these pathway interactions can enhance Notch target gene repression by post-translationally modifying local repressors and co-repressors. The Pak1 kinase, for example, is a down stream effector of the Rho family of GTPases that phosphorylates the C-terminal repression domain of SHARP. This post-translational modification of SHARP may stabilize the physical interaction between the SHARP and SMRT co-repressors (Vadlamudi et al., 2005). Alternatively, post-transcriptional regulatory mechanisms mediated by miRNA can influence target gene repression mechanisms. Recent studies in *C.elegans* found that Notch pathway activity is de-repressed during vulval development when the local transcription repressor LIN-14 is knocked-down by the developmental stage-specific expression of *lin-4* miRNA (Li and Greenwald, 2010). The extent to which either miRNA or signaling pathway cross-talk contribute to the selective repression of Notch pathway target gene transcription remains to addressed, but these mechanisms are likely to be important determinants of how cell context determines the transcriptional response of target genes when the Notch pathway is activated.

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