DECODING THE NOTCH SIGNAL

Julia Falo-Sanjuan and Sarah J Bray*

Department of Physiology Development and Neuroscience
University of Cambridge
Downing Street
Cambridge CB2 3DY, UK

***sjb32@cam.ac.uk**

Abstract:

Notch signalling controls many key cellular processes which differ according to the context where the pathway is deployed due the transcriptional activation of specific sets of genes. The pathway is unusual in its lack of amplification, also raising the question of how it can efficiently activate transcription with limiting amounts of nuclear activity. Here we focus on mechanisms that enable Notch to produce appropriate transcriptional responses and speculate on models that could explain the current gaps in knowledge.

Background:

The Notch signalling pathway is one of the core pathways controlling key cellular processes, such as proliferation, differentiation and apoptosis, in different cell types and developmental stages across metazoa. A simple transduction mechanism generates different responses through the transcriptional activation of context specific sets of genes, but much of how this complexity arises is still unknown. When Notch, a transmembrane receptor binds its ligand of the DSL (Delta/Serrate/Lag-2) family in adjacent cells, it triggers two successive cleavages of Notch by ADAM and γ- secretase proteases that release the Notch intracellular domain (NICD). NICD enters the nucleus and directly activates transcription of target genes by binding the transcription factor CSL (an acronym for CBF-1/RBPJ-κ in mammals, Suppressor of Hairless in *Drosophila melanogaster*, Lag-1 in *Caenorhabditis elegans*) and the co-activator Mastermind (Mam) (Fig. 1) (Bray, 2006).

While many features deployed by the Notch pathway are common to other signalling pathways, for example involvement of a repression-activation switch and of cooperative activation in target gene regulation (reviewed in (Barolo and Posakony, 2002)), additional measures are required to produce accurate responses to pathway activation. For example, the Notch pathway exhibits linear stoichiometry: activation of one Notch receptor in the membrane will produce one NICD fragment in the nucleus (Bray, 2006; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998), whereas many other pathways are composed of multiple signalling steps, enabling amplification and modulation as seen with MAPK pathways (Ferrell, 1996). Despite the lack of amplification steps and the fact that the levels of NICD in the nucleus are barely detectable by current methods, genes are capable of responding to these extremely low amounts of NICD. How is this achieved?

In this review we focus on the current state of knowledge of the effects at the gene level brought about by NICD to produce appropriate transcriptional responses and speculate on mechanisms that could explain the current gaps in this knowledge. For simplicity we focus on mechanisms that are conserved between species; and will use “Notch” to refer to all Notch receptors, except where we discuss mechanisms specific to one of the 4 mammalian homologues (Notch1-4). Our main focus is on how genes ‘see’ NICD, based on different properties, to enable different modes of response.

How does Notch regulate transcription?

Notch pathway transcription complexes

Upon Notch pathway activation, the released NICD fragment forms a ternary complex with the DNA binding protein CSL and the Mam co-activator. This CSL-NICD-Mam complex in turn recruits other co-activators and histone acetyltransferase (HAT) complexes to promote transcription of Notch target genes (Giaimo et al., 2017; Oswald and Kovall, 2018; Tanigaki and Honjo, 2010). A large-scale profiling of proteins that bind to the ICD from Notch 1 suggests that a diverse array of other co-factors may also be associated with the ternary complex, potentially conferring different characteristics that might even include NICD mediated repression (Yatim et al., 2012).

Structural and biochemical analysis of mammalian and *C. elegans* NICD-CSL complexes predict a step-wise assembly model in which the RAM (RBP-J Associated Molecule) domain of NICD binds with high affinity to the BTD (beta-trefoil domain) of CSL, causing a conformational change and an increase in the local concentration of NICD that allows the otherwise very weak binding of the ANK (ankyrin) repeats in NICD to the CTD (C-terminal domain) of CSL. This creates a binding site for Mam, which stabilizes the ANK-CTD interaction (Kovall and Blacklow, 2010). However, similar studies in *Drosophila* show that NICD binds to Su(H) to some degree in the absence of Mam (Contreras et al., 2015), making it plausible that CSL-

NICD complexes could exist in the absence of Mam. Nevertheless, the CSL-NICD-Mam (Fig. 2A) is the major complex mediating Notch function in all species, as dominant negative Mam largely recapitulates Notch loss of function in most contexts tested (Helms et al., 1999; Maillard et al., 2004; Oyama et al., 2011).

Although CSL binds as a monomer, recognizing a conserved 7-mer motif (Castel et al., 2013; Koromila and Stathopoulos, 2017; Tun et al., 1994), ternary complexes (NTC) containing murine NICD1 can form dimers through contacts between the convex face of the Notch1 ANK domains (Arnett et al., 2010; Nam et al., 2007). The spacing of DNA binding domain in these complexes favors recruitment at CSL motifs in a specific pseudosymmetrical head-to-head configuration that are separated by 15 to 19 bp so-called SPS (Su(H) Paired Sites) (Arnett et al., 2010; Bailey and Posakony, 1995; Cave et al., 2005; Nellesen et al., 1999). Mutations preventing dimerization lead to defects in beta selection during T cell development *in vitro* and prevented T-cell Acute Lymphoblastic Leukemia (T-ALL) in engrafted mice (Liu et al., 2010), indicating the functional relevance. Notably, the highly responsive Notch regulated *HES/HEY* genes have SPS sites in their regulatory regions, as does the mammalian *c-Myc* gene (Liu et al., 2010; Nellesen et al., 1999; Schroeter et al., 1998). Altogether it has been estimated that a third of all Notch targets could rely on SPS sites (Severson et al., 2017). The two foot binding conferred by dimerization could be one factor that favors binding of NICD containing complexes at target enhancers. However, the extent that dimerization is important for the recruitment of CSL-NICD-Mam complexes remains to be established.

CSL also participates in other complexes. For example it interacts directly with several co-repressors, such as Hairless (H), SHARP/MINT or SMRT (silencing mediator for retinoid and thyroid receptor), which have the potential to recruit histone deacetylases (HDAC) and other chromatin remodelling proteins to repress target enhancers (Tanigaki and Honjo, 2010; Yuan et al., 2016; Yuan et al., 2019). Studies on the structure of repressor complexes indicate repressors of different species bind different CSL domains. Hairless binds the CTD of fly Su(H) (Fig. 2A) (Yuan et al., 2016), whereas FHL1 and RITA1 bind the BTD of RBPJ (Collins et al., 2014; Tabaja et al., 2017), similar to the RAM domain of NICD. SHARP interacts with two distinct surfaces on RBPJ, contacting both the CTD and BTD in regions that overlap with those interacting with NICD (Yuan et al., 2019). Interestingly, although there is no sequence similarity between SHARP and Hairless, these two corepressors have evolved to bind the same CTD interface on RBPJ and Su(H) (Yuan et al., 2019). Altogether there is a large repertoire of potential CSL-repressor complexes, that have the potential to contribute to switch-like properties at target enhancers. However, although it is evident that they make a significant contribution in some contexts, by antagonizing NICD and/or by preventing inappropriate activation in Notch off conditions (Barolo et al., 2000; Morel and Schweisguth, 2000; Morel et al., 2001; Ozdemir et al., 2014), their overall significance merits further exploration.

Dynamics of DNA binding and transcription

The classical model for Notch mediated gene activation was based on a repressor-activator switch, in which NICD-Mam displaced the co-repressors on binding CSL. In this model, CSL remained stably bound to DNA while the NICD exchange converted it from repression to activation (Bray and Bernard, 2010). Such a model implied long-term binding of CSL at target sites and higher affinity of NICD for CSL in comparison to co-repressors. Recent studies have challenged these suppositions, leading to a modified dynamic model. Firstly, the evidence indicates a much more dynamic association of CSL with DNA. The CSL-DNA binding affinity appears to be lower than previously measured (Friedmann and Kovall, 2010) and CSL kinetics in Notch of conditions, based on live-imaging experiments, are very dynamic with estimated residence at target sites of circa 1 second (Fig. 2B) (Gomez-Lamarca et al., 2018). Second, *in vitro* measurements revealed that co-repressors and NICD have similar affinities for CSL, or even in some cases repressors have a higher affinity (Collins et al., 2014; Vanderwielen et al., 2011; Yuan et al., 2016), suggesting that a simple subunit exchange would not be thermodynamically favourable, although it remains possible that other factors or protein modifications could modify the protein behaviours. Finally, analysis of CSL occupancy at target

enhancers by chromatin immunoprecipitation (ChIP) and by live imaging revealed that its residence increased upon Notch activation (Fig. 2A) (Castel et al., 2013; Krejcí and Bray, 2007; Wang et al., 2014). The live-imaging suggested a 10-fold increase in residence for CSL in the presence of NICD (Gomez-Lamarca et al., 2018). As structural studies indicate no change in the conformation of CSL itself when bound to NICD, the observed increase in occupancy is likely due to interactions with other factors.

Current evidence therefore invokes a more dynamic model in which CSL-repressive complexes exchange rapidly on and off DNA in the absence of Notch activity and can be replaced by CSL-NICD activating complexes when the pathway is activated. In this model, the CSL-NICD complexes would be favoured because of their longer DNA residence and ability to engage with other factors. Indeed, direct visualization of the dynamics of CSL and the repressor Hairless both in the absence and presence of Notch activity supports this highly dynamic model (Fig. 2A) (Gomez-Lamarca et al., 2018). Based on the change in characteristics, it appears that NICD promotes two independent but complementary behaviours: 1 - through a process referred as "assisted loading" chromatin accessibility increases and enhances the local concentration of both activating and repressing complexes; 2 – the presence of Mam in the complex with CSL-NICD increases the residence time of the activating complex on DNA. Although this model explains many of the recent observations, it still remains uncertain whether it is sufficient to account for the rapid and robust effects of NICD, given that in many cases the levels are below detection.

The observed dynamic behaviour of CSL nuclear complexes is similar to that of many other transcription factors. As a result of techniques to image or quantify behaviours of single molecules (Liu and Tjian, 2018), there has been a shift from the classic view of static TF binding to DNA to one where proteins bind their target enhancers in a dynamic manner. This raises the question how these more dynamic binding can be converted into efficient transcription initiation. It is suggested that one or more essentially irreversible steps could drive this transition (Coulon et al., 2013), but this remains an open question. Furthermore, the transcription field has also seen a shift in paradigm. With the development of techniques to measure transcription quantitatively and in real time, it has emerged that most eukaryotic genes are transcribed in bursts (Bothma et al., 2014; Chubb et al., 2006; Golding et al., 2005). This 'bursty' transcription has been formalized as a 2 state model of a promoter's activity, which cycles between ON and OFF states with stochastic rate constants (Larson et al., 2009; Peccoud and Ycart, 1995). In agreement with most other examples of eukaryotic transcription, Notch target genes are transcribed in bursts rather than in a poissonian manner. This has been simultaneously reported in *C. elegans* (Lee et al., 2019) and *Drosophila* (Falo-Sanjuan et al., 2019), with observed bursts ranging from approximately 5 min to 1h in both organisms depending on the context (Fig. 2B). Oscillatory behaviour of Notch target genes has also been observed in neural progenitors (Manning et al., 2019), neural and muscle stem cells (Lahmann et al., 2019; Sueda et al., 2019) and during somitogenesis in mouse, chick and zebrafish embryos (Dequéant et al., 2006; Holley et al., 2002; Oates and Ho, 2002; Palmeirim et al., 1997). However, this is thought to be consequence of genetic feedback rather than the bursting behaviour of a single gene. Bursts of Notch target gene transcription in the order of hours have also been observed in cultured cell models (Nandagopal et al., 2018), but whether this represents bursts at the promoter level or it is also the result of feedback regulation remains unclear.

Regardless of whether the time scale of transcriptional bursting is minutes or hours, binding of CSL-NICD complexes to target enhancers appears to occur over a range that is at least an order of magnitude faster. Furthermore, the system needs to operate effectively with low amounts of NICD released. How can the current model, with fast dynamics of CSL-NICD recruitment and transcriptional bursting, explain how NICD effectively programs transcription?

Bridging the timescale and concentration gap: hubs?

The paradox of dynamic binding properties is not unique to CSL complexes. Most TF which have been studied in real-time by direct protein visualization, *e.g.* by using FRAP (Fluorescence Recovery After Photobleaching), SMT (Single Molecule Tracking) or FCS (Fluorescence Correlation Spectroscopy), exhibit very fast kinetics, with an estimated DNA dwell time in the order of hundreds of milliseconds to seconds (Cheutin et al., 2003; Mir et al., 2017; Mir et al., 2018; Swinstead et al., 2016). Structural proteins, such as Histones or CTCF, are the exception with dwell times in the order of minutes to hours (Agarwal et al., 2017; Hansen et al., 2017; Hansen et al., 2018; Kimura and Cook, 2001). The discrepancy in timescales between TF binding and transcriptional bursting is therefore a general one and not specific to Notch regulation. A further challenge to understanding the mechanisms involved is that the basis for transcriptional bursting is not fully understood. Some studies have correlated bursting frequency with enhancer-promoter interactions via induced looping (Bartman et al., 2016). Others find no correlation between enhancer-promoter proximity and transcriptional activity (Alexander et al., 2019; Chen et al., 2018), suggesting other mechanisms are involved. However, the outcome, in terms of total number of mRNA molecules produced in a single bursting event (bursting size), has been correlated with the lifetime of PolII clusters (Fig. 2B) (Cho et al., 2016), suggesting that the formation of these clusters is an important step and hence a likely consequence from NICD recruitment.

The formation of PolII clusters or aggregates is thought to be driven by weak interactions between intrinsically disordered regions (IDRs) in PolII and other proteins (Boehning et al., 2018; Cho et al., 2018; Lu et al., 2018). Mediator complex and other transcription complexes are also present in these heterotypic clusters (Cho et al., 2018), from which constituents can exchange dynamically in and out (Fig. 2B). Similarly, many TFs (Oct4, p53, Myc, nanog, Sox2, RARa, GATA-2 and ER) are seen to colocalize with Mediator in aggregates or droplets *in vitro* and in cultured cells (Boija et al., 2018). The number and size of Mediator clusters and their presence at regulated enhancers is affected by the presence or absence of specific TFs, suggesting that the capability of TFs to drive cluster formation, of Mediator and/or PolII, could be a key factor for promoting transcription.

In agreement with this model, several TFs (Bicoid (Mir et al., 2017; Mir et al., 2018), Dorsal (Yamada et al., 2019), Zelda (Dufourt et al., 2018; Mir et al., 2018) and Ultrabithorax (Tsai et al., 2017; Tsai et al., 2019) are present in *foci* or "hubs" in the nucleus. By creating high local concentrations, hubs are proposed to enable the activation of target enhancers under conditions where the overall nuclear concentration of the TF is low, as for NICD-CSL complexes. A hint that NICD could promote the formation of hubs comes from the observation, in live imaging experiments, that CSL becomes concentrated around a target locus *in vivo* (Fig. 2AB) (Gomez-Lamarca et al., 2018). Further experiments will be needed to determine whether this is a general property of NICD-CSL complexes and whether other factors colocalize. Interestingly, all TF hubs analyzed to date remain highly dynamic and only transiently interact with nascent sites of transcription, suggesting a "hit and run" mechanism for initiating transcription (Mir et al., 2018), rather than a model where TFs become integrated with stable PolII/Med clusters. However, as clustering of PolII/Med has not been examined in the same context as TFs, this remains speculative. It will be important to follow both types of cluster/hub in the same nuclei to distinguish the models.

A "hit and run" mechanism would be attractive in the context of Notch signaling. In Notch active cells, there is an increase in the local concentration of both active and repressive complexes at a target locus. In these conditions CSL binding to DNA remains highly dynamic (Gomez-Lamarca et al., 2018) suggesting that, if the concentration of NICD determines PolII/Med cluster sizes, it must do via a transient interactions. As a consequence, the transcription burst size changes, based on experiments using the MS2 system for live imaging of Notch-regulated transcription in *Drosophila* and *C. elegans*. At the molecular level, assuming a

two state model of transcription, this would indicate NICD increases the rate of transcriptional initiation and keeps the promoter in an active state for longer (Falo-Sanjuan et al., 2019; Lee et al., 2019). This contrasts with other signals, like estrogen (Fritzsch et al., 2018), which appear to have the converse effect, modulating the frequency rather than the size of transcriptional bursting. How NICD concentration changes burst size remains to be shown, but one possibility is that NICD determines the size of PolII clusters that are formed.

In summary, the dynamic binding of NICD-CSL complexes to DNA and their ability to operate at extremely low concentrations of NICD might be explained by a model in which dynamic condensation of transcription factors, co-activators, elongation factors and Pol II complexes contributes to gene expression by producing a microenvironment that compartmentalizes transcription reactions within the nucleus. Such clustering by NICD with other factors would increase local concentration of NICD at target enhancers and could produce longer lasting clusters of Med/PolII, whose size and lifetime would determine transcription burst size (Fig. 2B).

How do different genes "see" Notch activity?

Despite the simplicity of the pathway, Notch activity is known to promote many different outcomes depending on the cell type and developmental stage. Sometimes it can even promote activation of genes with opposing effects in the same context (Krejcí et al., 2009). The final outcome of the pathway, *e.g.* whether it promotes differentiation or stem cell maintenance, relies on the set of target genes that are deployed in each context. Genes must therefore respond to NICD through the guidance of other factors, either in a directly combinatorial manner or through a more hierarchical mechanism. In addition to other transcription factors that confer a tissue specific response, other 'features' such as signal levels, time exposed to signal or signal patterns, can also influence whether a target gene responds. This means that the enhancers must be able to read and decode this information.

Tissue specificity

The Notch pathway promotes very different outputs depending on the target genes that are activated. These may differ according to the tissue, cell-type, developmental stage or pathological state. Two striking statistics illustrate the major role for context/tissue specificity in setting the Notch response. First, only small fractions of potential Notch targets are shared between two cell types in *Drosophila* (slightly more than 10%) (Krejcí et al., 2009), NOTCH1-driven T-ALL and Small B Cell Lymphomas (Ryan et al., 2017) or human and murine T-ALL cell lines (50%) (Wang et al., 2011). Indeed, even though most signaling contexts result in activity of genes of the *HES/HEY* family amongst the primary targets, the precise family member differs widely. Amongst other targets, only *c-Myc*, *Deltex* and *NRARP* are widely detected (Ryan et al., 2017). Second, only 1% of all CSL motifs in the *Drosophila* genome are bound by CSL/SuH in a given cell type (Skalska et al., 2015) and there are relatively few enhancers that are occupied in more than one cell type, of those examined to date. Even in cases where genes respond widely, as with *c-Myc*, different enhancers are activated in different cell-types (Herranz et al., 2014; Ryan et al., 2017; Yashiro-Ohtani et al., 2014).

Transcription factors that confer tissue specificity to Notch targets have only been identified in a few contexts so far. One example is the Runx family transcription factors, which are involved in determining the cohort of Notch-regulated genes in both T-ALL cancer cells and *Drosophila* blood cells (Terriente-Felix et al., 2013; Wang et al., 2014). Runx motifs are found in proximity to CSL motifs and are necessary for the full activity of the blood cell enhancers, where tested. Depletion of Runx interferes with target gene activity and its ectopic expression can promote activity of blood-cell genes in a heterologous cell-type (Skalska et al., 2015). Similar properties are manifest by the bHLH TF Twist in muscle precursors, where a subset of genes involved in keeping the progenitor state can only be transcribed in the presence of both NICD and Twist (Bernard et al., 2010). Other examples include GATA-like factors, which cooperate with Notch in the

regulation of *ref-1* in the *C. elegans* endoderm (Neves et al., 2007). Co-regulation with proneural bHLH transcription factors is also a common feature in neurogenesis, where the *HES/E(spl)* gene family frequently contain a proneural binding motif closely associated with SPS motifs in their enhancers (Culí and Modolell, 1998; Nakao and Campos-Ortega, 1996; Parras et al., 1996).

There are several different models to explain how TFs can confer specificity. One possibility is that co-binding is required for cooperative responses (Fig. 3A). In the majority of examples there does not appear to be any clear spacing or arrangement of motifs, making it unlikely that co-binding involves very stereotyped protein-protein interactions. Nevertheless, the proximity could increase the valency for recruiting other key factors, such as mediator complex. A second possibility is that, because the tissue specific transcription factors are present prior to Notch activation, their role is to set a permissive chromatin state for CSL recruitment. Indeed, it is generally accepted that enhancers need to be rendered accessible by TFs, and that only a subset of TF types are efficiently able to "pioneer" because they can bind DNA even when wrapped around histones (Fig. 3A). Thus, it is likely that some of the cooperating TFs will be "pioneers" that render a certain set of enhancers responsive in a given cell type. For example, GATA factors appear to have qualities of pioneers (Cirillo et al., 2002).

Several of these examples indicate that the cell-specific regulatory inputs change the way enhancers perceive NICD. This was also evident in live-imaging experiments with the *m5/m8* mesectoderm enhancers in the early *Drosophila* embryo. Binding motifs for two localized transcription factors, Twist and Dorsal, were important to confer a fast and robust response to NICD (Falo-Sanjuan et al., 2019). This implies that these other TFs in some way prime the enhancers so they are poised for activation once NICD is released. Another example of hierarchical programming occurs during T-cell commitment, which is driven by the Notch target *Bcl11b*. By tagging the two *Bcl11b* alleles with different fluorescent proteins, it was shown that each is switched on in an independent and stochastic manner over a period of days. This relies on two parallel events, a stochastic switch *'in cis'* caused by a distal enhancer and 'trans-acting' Notch activation, that will promote *Bcl11b* transcription only from those alleles that have been previously activated *in cis* (Ng et al., 2018). During neural lineage specification in *Drosophila*, the cell types determined by Notch mediated lateral inhibition depend on which other factors, from a temporal cascade, are present in the neural stem cell at that specific moment (Li et al., 2013).

The well-characterised roles of Notch in T-cell and neuronal specification also highlight how the response must be reprogrammed at different steps in the lineage. For example, the presence of Eyeless/Pax6 prevents key enhancers from responding to Notch signalling in intermediate neural progenitors and in doing so prevents tumorigenesis from ectopic pathway activity (Farnsworth et al., 2015). We have proposed that chromatin remodelling mechanisms decommission enhancers to enable a switch from one type of response (*e.g.* to maintain progenitors) versus another (*e.g.* to promote a specific cell-type differentiation) (Fig. 3A) (Zacharioudaki et al., 2019). In neural stem cell lineages, this decommissioning requires the Mi-2/NuRD (Nucleosome Remodeling Deacetylase) complex. In the absence of Mi-2, the stem cell progeny no-longer properly switch-off the genes involved in maintaining their stem cell properties, so these remain responsive to Notch and revert the progeny to stem cell behaviours. As this type of reprogramming is integral to developmental transitions in cell states it will be important to discover how widely NuRD complex is deployed to mediate these transitions and/or whether there are several different ways that responsive enhancers can be shut down or remodeled.

Signal features

Unlike many signaling pathways, which involve intermediate steps that can modify or amplify the signal profile, the activity is manifest in the levels and dynamics of cleaved NICD. It is likely that these signal "features" will vary depending on the conditions. For example, the levels of Notch at the membrane, the amounts and type of ligand presented, the size of the contact area and the overall size of the cell could all

influence how much NICD is released and its temporal profile (*e.g.* continuous versus pulsatile). This very direct connection between receptor activation and nuclear NICD levels may explain why the system is very sensitive to changes in the genetic dose of different elements in the pathway. For example, there are clear phenotypes in flies heterozygous for mutations in *Drosophila* Notch or Delta. Likewise, heterozygous mutations in *Notch1* predispose to heart valve and endothelium defects in mice (Nigam and Srivastava, 2009), whereas *Notch2* heterozygotes present reduced numbers of marginal zone B cells (Witt et al., 2003). In humans loss of an allele of *NOTCH2* has been associated with Hajdu-Cheney syndrome (Simpson et al., 2011), which causes bone loss, and Serpentine Fibula-Polycystic Kidney Syndrome (Isidor et al., 2011). Heterozygous mutations in *JAG1* or in a small proportion of cases in *NOTCH2* cause Alagille syndrome, a developmental disorder with varied manifestations (McDaniell et al., 2006; Oda et al., 1997). Since half the amount of the ligand or receptor is enough to cause a phenotype, this suggests that some Notch target genes are sensitive to changes in the amounts of NICD, either due to the threshold needed for activation or to the subsequent effects on transcriptional output.

One way signal features might be detected by enhancers is if there is a critical amount of NICD required to trigger their activation, which we refer to here as the signal "threshold". Individual enhancers could be configured to have a specific response threshold and would only become operational once the levels of NICD exceeded a particular concentration (Fig. 3B). For example, both *Hes5* and *Hey1* are expressed in response to Notch activity during the development of the inner ear, where *Hes5* appears to require a higher level of signaling than *Hey1* for its activation and therefore can only be activated from Delta1, which induces higher levels of signaling than Jagged1 (Petrovic et al., 2014). Evidence to support this model comes from experiments analyzing the response of two enhancers (*m5/m8* and *sim*) to ectopic Notch activity in the *Drosophila* embryo. Their onset times shift in the presence of ectopic Notch, but to a differing extent, consistently with them becoming active at different thresholds of NICD concentration (Falo-Sanjuan et al., 2019). Experiments in the same system using optogenetic control of Notch activity (with the inhibitory opto-Delta) also support this model, as inhibition during different time periods determines when the *sim* enhancer starts to respond, consistent with it responding to a specific threshold of NICD (Viswanathan et al., 2019). In both cases, the overall probability of cells switching on transcription also differed according to the extent of signaling (Falo-Sanjuan et al., 2019; Viswanathan et al., 2019). One model to reconcile these two aspects is that the threshold of response is not 'all or none' but of probabilistic nature, where cells releasing higher NICD levels than threshold have a higher probability of responding than those at threshold levels.

At the DNA level, the numbers, strength and arrangement of CSL binding motifs could be important in conferring response to a particular threshold of NICD. Although there is evidence from *in vitro* and cell culture experiments that differences in the sequence of the CSL motif impacts on the affinities and activity (Cave et al., 2005), the relevance to response thresholds *in vivo* has not been explicitly explored and responsive enhancers often differ greatly in these properties. However, one common feature of several highly responsive enhancers is the presence of SPS motifs, which achieve higher expression levels than a similar number of unpaired motifs in luciferase assays (Cave et al., 2005; Nam et al., 2007). However, modifications in SPS sites did not change the onset of the response in live imaging experiments (Falo-Sanjuan et al., 2019), suggesting that the threshold of NICD required for transcription to initiate from the enhancer was likely unchanged, although the subsequent bursting properties. were affected. On the other hand, mutations in the motifs for the context specific transcription factors (Twist and/or Dorsal) delayed the onset of transcription, suggesting they modulate the threshold of NICD required for activation (Falo-Sanjuan et al., 2019). These results indicate that any influence from the configuration of CSL motifs on the response threshold for NICD will be entwined with binding of other transcription factors.

After transcription is initiated in response to Notch, levels of NICD above the threshold alter bursting size (duration and amplitude) from different target genes and enhancers both in *C. elegans* and *Drosophila* (Fig. 3A) (Falo-Sanjuan et al., 2019; Lee et al., 2019). This indicates that the activity of Notch enhancers can be

tuned according to the amount of NICD, possibly by altering the size or duration of Pol II initiation complexes (hubs) as discussed above. Notably however, they appear to reach a maximum so that, beyond a certain level of NICD, mean transcription levels appear to saturate rather than continuously increasing with higher NICD levels (Falo-Sanjuan et al., 2019; Schroeter et al., 1998). Saturation could occur because other factors are at limiting levels (epigenetic, coactivators, etc.) or because there is a physical limit to the valency of the polymerase complexes that can form and/or can transcribe one gene at the same time.

Another intriguing possibility is that enhancers respond differently depending on the temporal kinetics of NICD. For example, it has been proposed that *Hes1* responds to a pulse of Notch activity while *Hey1* and *HeyL* require sustained Notch for their activation (Fig. 3B). These two behaviours correlated with activation by different ligands: DLL1 produced pulsed NICD release from the membrane whereas DLL4 produced sustained activation. In these synthetic experiments, the differences resided ligand intracellular domain, which affected membrane clustering (Nandagopal et al., 2018). How different temporal dynamics of nuclear NICD are generated and how in turn this has differential effects on target enhancers remains to be further investigated.

Conclusions:

Because of the simplicity of the Notch pathway, the diverse range of outcomes it can promote remains one of the outstanding questions in the field. As the different outcomes depend on the set of genes that are deployed in each context, genes must be able to respond to a variety of inputs in combination with Notch. This implies that the properties of the response to NICD rely not only on the Notch response elements (CSL sites) but also on other factors. The mechanisms in place to promote this can be very different: a distal enhancer is responsible for the stochastic decision of whether *Bcl11b* can be activated in response to Notch in T-cells; priming factors at a responsive enhancer in the *Drosophila* midline switch its response to Notch from stochastic to deterministic. Additionally, genes can respond to extremely low amounts of nuclear NICD, suggesting specific mechanisms ensure accurate responses. In this review we have focused on mechanistic details of the transcriptional response to Notch, and highlighted examples where genes are known to decode different aspects of the Notch signal. More work will be essential to understand which of these 'modes' of decoding are universal or specific to some Notch targets and how they are regulated, to be able to fully understand what 'context-specificity' means at the molecular level for all Notch target genes.

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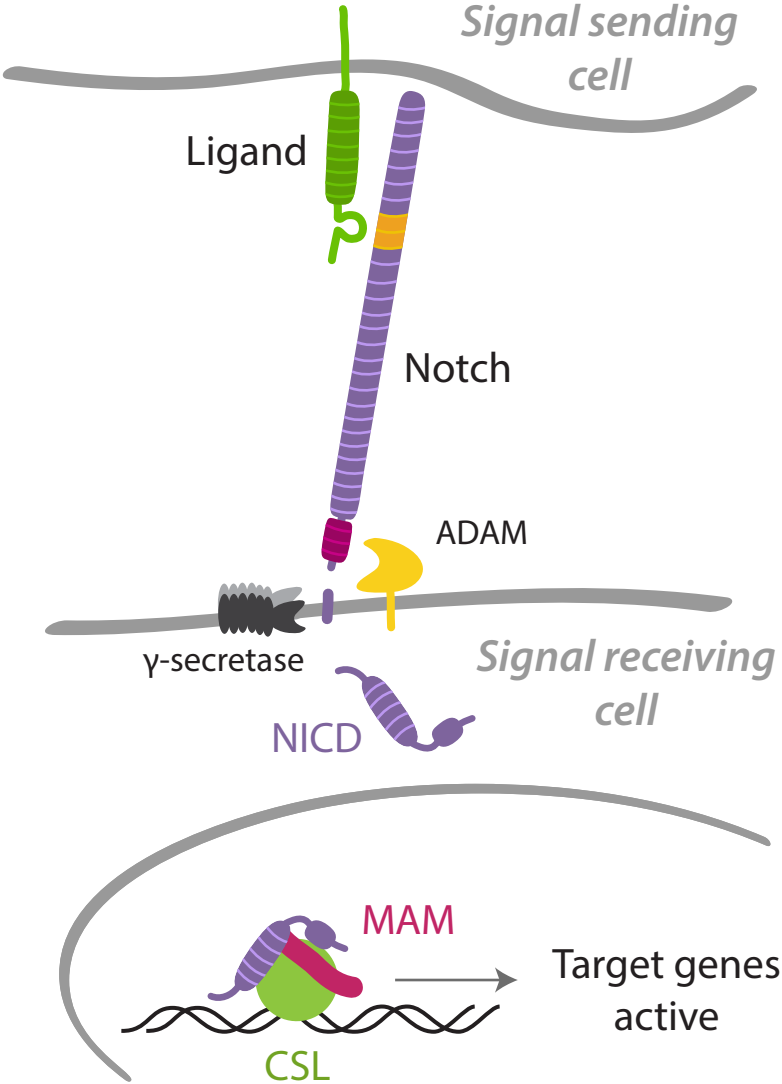
Figure Legends:

Figure 1 – Schematic representation of the Notch pathway Interaction of the transmembrane receptor Notch with transmembrane receptors in adjacent cells triggers two consecutive cleavages of Notch by ADAM metalloproteases and γ-secretase. The intracellular domain of Notch (NICD) is released and enters the nucleus, where it activates transcription of target genes via interaction with the DNA-binding CSL (CBF1, Su(H) and LAG-1) transcription factor and co-activator Mastermind (Mam).

Figure 2 – Transcriptional regulation by the Notch pathway at the molecular level A) Summary of Notch OFF and ON conditions illustrating the repressing and activating complexes, based on their crystal structures, and the observed occupancy in different assays. In Notch OFF conditions CSL is bound to co-repressors (Hairless, H, in *Drosophila*). CSL does not accumulate in specific genome loci in live imaging experiments and exhibits low occupancy (small "peaks") in ChIP experiments. In Notch ON conditions the activating complex NTC (NICD, CSL and MAM) binds DNA and recruits co-activators. Local accumulations of CSL in the *E(spl)* locus can be detected *in vivo* in *Drosophila* salivary glands and CSL occupancy at target enhancers is increased in ChIP experiments (large "peaks"). **B)** Model of dynamic CSL-DNA interactions driving transcriptional bursting over different timescales. Hubs of CSL are formed via assisted loading in response to NICD. These contain CSL both in activator and repressor complexes. Interactions through LCDs could, as shown in other systems with different TFs, recruit and stabilize clusters of Mediator and PolII, which have been correlated with the size of transcriptional bursts.

Figure 3 – Different modes in which targets 'see' Notch activity A) Models of tissue specific responses to NICD conferred by other transcription factors. NTC can bind combinatorically with other factors (left). Pioneers or priming factors can promote changes in chromatin accessibility or epigenetic state that allow subsequent transcriptional activation in response to Notch (middle). Enhancer decommissioning ensures Notch target genes are no longer susceptible to activation following cell fate decisions (right). **B)** Differential target gene expression in response to different features of the Notch signal. Genes can respond to different levels of NICD via thresholds of activation, such that at low NICD levels only genes with low thresholds are active. In addition to thresholds, increasing levels of NICD increase the transcription bursting size (amplitude and length) rather than the frequency (top). Genes can also respond to different temporal patterns of NICD production, *e.g.* sustained vs pulsed activation (bottom).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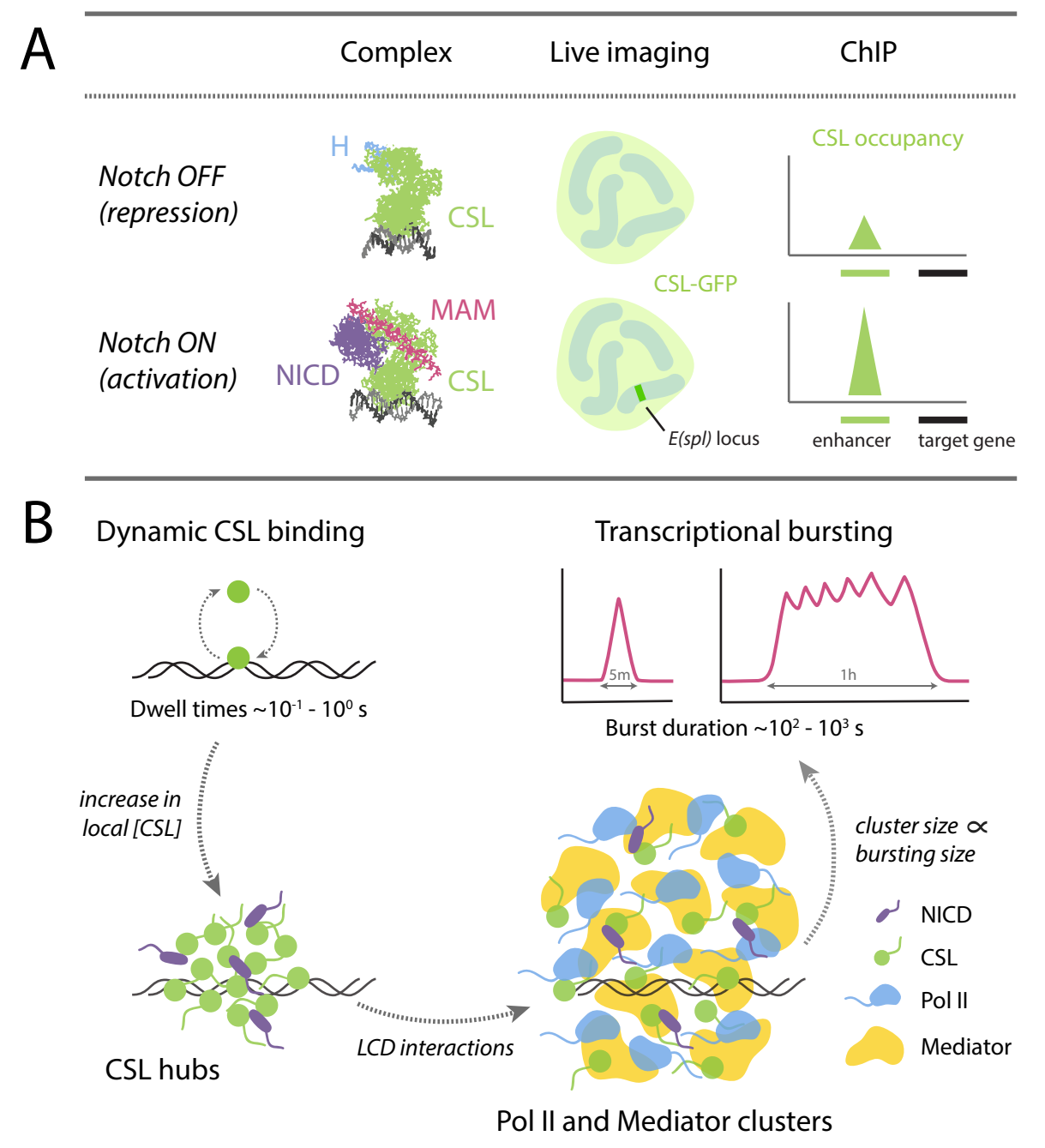
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Dynamic CSL binding
Transcriptional bursting
Dwell times ~10-1 - 100 s
5m
1h
Burst duration ~102 - 103 s
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Pol II
Mediator
CSL hubs
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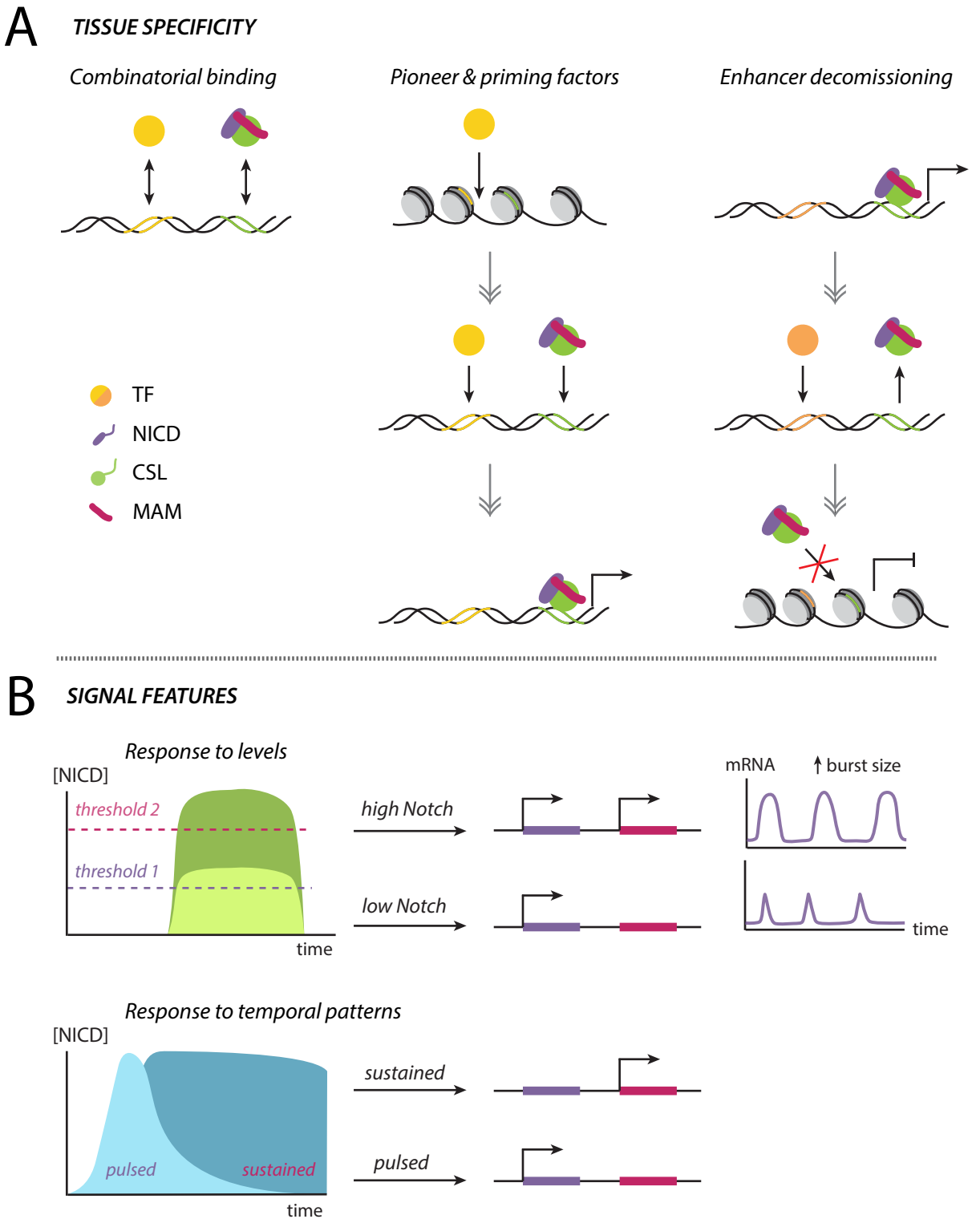
Pol II and Mediator clusters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Combinatorial binding
Pioneer & priming factors
Enhancer decomissioning
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SIGNAL FEATURES
Response to levels
[NICD]
threshold 2
threshold 1
time
high Notch
low Notch
mRNA
burst size
time
Response to temporal patterns
[NICD]
pulsed
sustained
time
sustained
pulsed