RESEARCH COMMUNICATION

Notch activation stimulates transient and selective binding of Su(H)/CSL to target enhancers

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The CSL [CBF1/Su(H)/Lag2] proteins [Su(H) in *Drosophila*] are implicated in repression and activation of Notch target loci. Prevailing models imply a static association of these DNA-binding transcription factors with their target enhancers. Our analysis of Su(H) binding and chromatin-associated features at 11 E(spl) Notch target genes before and after Notch revealed large differences in Su(H) occupancy at target loci that correlated with the presence of polymerase II and other marks of transcriptional activity. Unexpectedly, Su(H) occupancy was significantly and transiently increased following Notch activation, suggesting a more dynamic interaction with targets than hitherto proposed.

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The Notch signaling pathway plays an important role in a wide diversity of developmental contexts (Artavanis-Tsakonas et al. 1999; Schweisguth 2004; Bray 2006). As the pathway impinges directly on the nucleus, it is important to determine the mechanisms that underlie the transcriptional response and how this response is tailored appropriately to different developmental contexts.

Activation of Notch precipitates a proteolytic cleavage, catalyzed by the γ-secretase/presenilin complex, which releases an intracellular fragment Nicd (Mumm and Kopan 2000). In the nucleus, Nicd forms a complex with the DNA-binding protein CSL [CBF1/Su(H)/Lag2] and the coactivator Mastermind (Mam) (Nam et al. 2006; Wilson and Kovall 2006). Together, these recruit histone acetyltransferases (HATs) and other cofactors required for transcriptional activation (Wallberg et al. 2002; Fryer et al. 2004). In the absence of Notch activation, CSL contributes to repression of target genes complexed with corepressors including Hairless in *Drosophila* (e.g., Kao et al. 1998; Morel et al. 2001; Zhou and Hayward 2001). These act as tethers for additional repressors, such as Groucho and CtBP, along with histone deacetylases (HDACs) (Kao et al. 1998; Hsieh et al. 1999; Morel et al. 2001; Barolo et al. 2002; Nagel et al. 2005; Oswald et al. 2005).

According to current models, CSL remains bound to the DNA at its targets, and the switch between repression and activation is mediated through exchange of associated proteins (Barolo et al. 2002; Bray 2006). However, other transcription factors that participate in both repression and activation, such as the glucocorticoid receptors, appear to have a much more dynamic interaction with DNA (Agresti et al. 2005; Bosisio et al. 2006). This equilibrium is altered by the presence of ligands, and it has been proposed that transcriptionally productive complexes have slower dissociation kinetics (Bosisio et al. 2006). Such a model suggests that there is a rapid exchange of DNA-bound factors on and off the DNA, with stabilization occurring only as a consequence of recruiting secondary factors. If this were to apply in the case of CSL, it opens up the possibility that there could be distinct complexes (activation and repression) formed off the DNA, alleviating the need for Nicd to actively dissociate a stable interaction between corepressors and CSL.

Here, we set out to monitor CSL/Su(H) occupancy at target enhancers under different conditions to ascertain whether it changes after Notch activation as predicted by the more dynamic models. We also investigated whether epigenetic modifications at target-genes correlate with inducibility and/or activation. To address these questions, we used a simple procedure to activate Notch in *Drosophila* cells, allowing stringent analysis of chromatin before and after activation, and assayed the 11 well-characterized Notch target genes within the *E(spl)* complex, whose functional Su(H) sites have been mapped (Fig. 1A; Bailey and Posakony 1995; Lecourtois and Schweisguth 1995; Nellesen et al. 1999; Cooper et al. 2000; Lai et al. 2000; Castro et al. 2005). Our results demonstrate that Su(H) is only present at the subset of *E(spl)* enhancers that are transcriptionally active in a given cell type. More importantly, we detect a dramatic and transient increase in Su(H) occupancy after Notch activation, in agreement with dynamic models of gene regulation.

Results and Discussion

EDTA triggers Notch activation in S2-N cells

To investigate changes in chromatin that accompany Notch activation, we needed to establish conditions where receptor activation could be temporally controlled. It has been reported that exposing cells to EDTA stimulates shedding of the Notch ectodomain (Rand et al. 2000; Gupta-Rossi et al. 2001). This renders the residual transmembrane fragment a substrate for γ-secretase cleavage and, hence, results in Notch activation. Despite results suggesting that cell surface Notch in *Drosophila* would not be susceptible (Kidd and Lieber 2002), we have found that EDTA causes robust activation of *E(spl)* Notch-target genes in a Notch-expressing *Drosophila* S2 cell line (S2-N) (Fig. 1A,B). No effect was seen when S2 cells that do not express Notch were treated with EDTA (Fig. 3A, below; data not shown).

All 11 *E(spl)* genes were induced following EDTA treatment. Most were expressed at very low levels before

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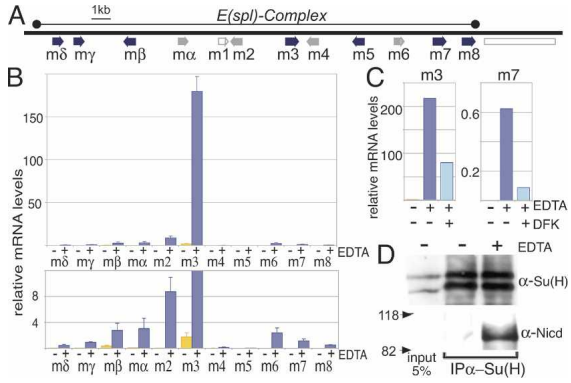

Figure 1. EDTA elicits Notch activation in S2-N cells. (*A*) Diagram of *E(spl)* complex. Genes are indicated by arrows: basic helix–loop–helix genes (blue); Bearded-type (gray); not Notch responsive (white). (*B*) mRNA levels of *E(spl)* genes before and 30 min after EDTA treatment of S2-N cells. *Bottom* panel shows results plotted on a more sensitive scale. (*C*) *m3* and *m7* RNA levels in EDTA-treated S2-N cells ± γ-secretase inhibitor DFK-167 (300 μM). (*D*, *top* panel) Su(H) is detected as two bands in the total input and in α-Su(H) immunoprecipitates with and without EDTA treatment. (*Bottom* panel) Nicd is only present in the immunoprecipitated sample from EDTA-treated cells. Positions of molecular weight markers (in kilodaltons) are indicated for the *bottom* panel.

activation and, although stimulated following EDTA treatment, their absolute levels of expression remained low. One gene, *m3*, was expressed at intermediate levels prior to activation and was induced 50 times by EDTA treatment to expression levels that were ~100 times higher than other *E(spl)* genes. There was no change in expression of the housekeeping genes or nontarget loci analyzed (e.g., Supplementary Fig. S1A). A qualitatively similar effect on *E(spl)* gene expression was obtained in S2 cells transfected with a plasmid expressing Nicd (Supplementary Fig. S1B).

To ascertain whether EDTA activates *E(spl)* genes through its effects on Notch, cells were treated in the presence of γ-secretase inhibitors (e.g., DFK-167). This compromised the induction of *m3* and *m7* expression by EDTA (Fig. 1C). In addition, by immunoprecipitating Su(H) from cell extracts and probing for coprecipitation of Nicd (Fig. 1D), we confirmed that there is a robust association of Nicd with Su(H) in EDTA-treated, but not in control cells.

In summary, EDTA treatment provides a method to rapidly activate Notch in a temporally controlled manner throughout a cell population (and is subsequently referred to here as Notch activation). By activating Notch in this way we may potentially be providing a larger, more concerted burst of Notch activity than occurs during normal signaling in the animal. Nevertheless, this makes it possible to analyze the chromatin state before and after Notch activation under carefully timed conditions.

Su(H) binding correlates with Notch inducibility

To determine the relationship between Su(H) occupancy and enhancer activity, we compared the distribution of Su(H) and C-terminal domain (CTD)-phosphorylated RNA polymerase II (Pol II) across the *E(spl)* complex in two cell lines; S2-N (derived from hemocytes) and DmD8 (derived from wing imaginal discs) (Fig. 2A,B). The DmD8 cells have Notch present on the cell surface and, like S2-N cells, could be activated with EDTA (Fig. 2; data not shown). Enrichment of DNA fragments encompassing the Su(H)-binding sites, the promoters (transcription start site), and the open reading frames of all the genes (Supplementary Fig. S2) were analyzed in chromatin immunoprecipitates (ChIP) from the two cell types before and after activation.

Two regions, the *m3* and *m*β enhancer fragments, were strikingly enriched in Su(H) ChIPs from S2-N cells before and after Notch activation (Fig. 2A). This correlated well with inferred transcriptional activity, as fragments corresponding to the ORFs of both *m3* and *m*β were highly enriched in the α-Pol II ChIP after activation (Fig. 2B). However, the only mRNA present at high levels 30 min post-activation was *m3* (Fig. 1C). One explanation for this apparent discrepancy is that *m*β mRNA is unstable in these cells. This was borne out by experiments comparing *m3* and *m*β mRNA levels under conditions where both were expressed from a heterologous metallothionein promoter (Supplementary Fig. S1C). The fact that Su(H) recruitment is restricted to the two most highly transcribed genes, *m3* and *m*β, suggests that other *E(spl)* enhancers do not require recruitment of a

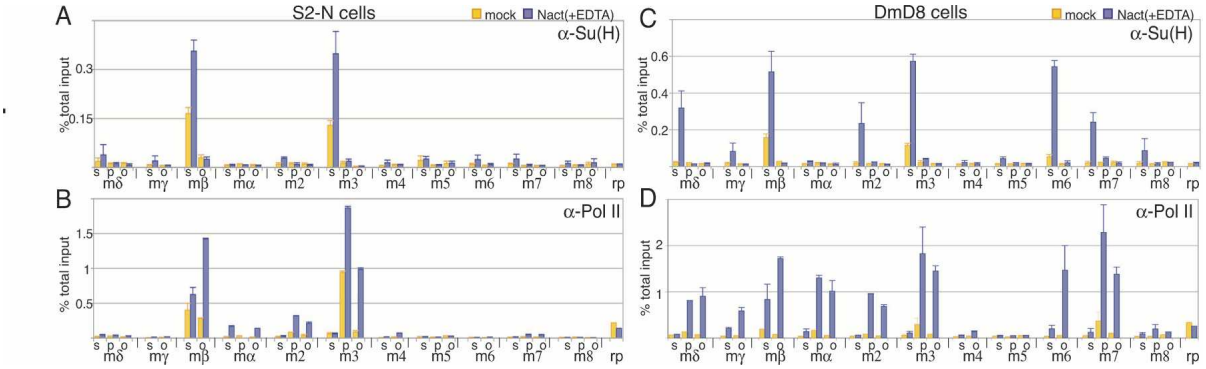

Figure 2. Su(H) occupancy increases after Notch activation. ChIP with α-Su(H) (*A*,*C*) or α-Pol II (phosphorylated CTD) (*B*,*D*) antibodies in S2-N (*A*,*B*), and DmD8 (*C*,*D*) cells before (orange) or 30 min after (blue) EDTA. Precipitated DNA was quantified by real-time PCR. Each gene is represented by two or three fragments. (s) Su(H)-binding/enhancer region; (p) promoter; (o) ORF. A single 5′ region was amplified when Su(H) sites were close to the promoter. Control was *rp49* ORF (rp). Results are average of three independent experiments (error bars indcate standard error of the mean). Increase in enrichment after activation of *m*β-s ($P = 0.03$) and *m3*-s ($P = 0.04$) in S2-N cells and of *m*δ-s ($P = 0.04$), *m*β-s ($P = 0.04$), *m3*-s ($P = 0.003$), *m6*-s ($P = 0.002$), and *m7*-s ($P = 0.02$) in DmD8 cells is significant.

Su(H) repressor complex for their repressed state. In agreement, only *m3* and *m*β were markedly derepressed when Su(H) or the corepressor Hairless were depleted using RNA interference (Supplementary Fig. S1D).

The distributions of Su(H) and Pol II were much more widespread across the *E(spl)* complex in DmD8 cells. In general, enhancers that were enriched in the Su(H) ChIP corresponded to genes where Pol II was recruited (Fig. 2C,D). Two genes, *m4* and *m5*, were not enriched in the Su(H) ChIP nor the Pol II ChIP, and neither did we detect any mRNA increase after Notch stimulation (Fig. 2D; data not shown). We note that *m*α was significantly enriched in the Pol II, but not the Su(H) ChIP. This discrepancy may indicate that our PCR-amplified fragment does not encompass the functional Su(H) site for *m*α. Otherwise, as in S2-N cells, there was a good correlation in DmD8 cells between Su(H) recruitment and the presence of Pol II after activation. What was surprising, however, was that there was little enrichment for Su(H) at the inducible genes prior to Notch activation. Most enhancers were only detectably enriched in the Su(H) ChIP after activation.

Su(H) occupancy is enhanced after Notch activation

Current models imply that Su(H) levels at target loci will be unchanged on Notch activation, as they postulate an exchange between Nicd and corepressors. The significant increase in Su(H) occupancy following Notch activation was therefore unexpected (Fig. 2A,C, cf. blue and orange bars). In S2-N cells, there was a 2–2.5 times increase in enrichment for *m3* and *m*β enhancer fragments after activation. Similar results were obtained using α-GFP to perform ChIP in S2-N cells expressing a GFP-Su(H) fusion (data not shown). In contrast, no increase in occupancy was detected following EDTA treatment of S2 cells without Notch (Supplementary Fig. S3) nor in ChIPs against other chromatin-associated factors (e.g., H3, H2A.v) (Fig. 4, below; Supplementary Fig. S4) in S2-N cells, confirming that the increased ChIP of the enhancers with Su(H) is a specific effect. In DmD8 cells, the increase in Su(H) occupancy after activation was even more dramatic (Fig. 2C). Enrichment at five of the enhancers increased significantly in the ChIP after activation, with a >10-fold increase in occupancy at *m*δ, *m6*, and *m7* (Fig. 2C).

The increased enrichment of target loci following Notch activation suggests that the activation complex is more stably associated with chromatin. A time course of *m3* mRNA levels in S2-N cells post-induction showed that expression declines once activation ceases (Fig. 3A). To ask whether the increase in Su(H) occupancy following Notch activation was also transient, we compared chromatin from activated S2-N cells (30 min) and from cells after activation had ceased (180 min) (Fig. 3B). There was a robust increase in enrichment of the *m3* enhancer in ChIP from activated cells at 30 min, but by 180 min, levels returned to those in uninduced cells. These data indicate that the association of Su(H) with its target enhancers in S2-N cells is more dynamic than has been proposed and is stimulated by Notch activation.

To confirm that the increased Su(H) occupancy is also transient in DmD8 cells and to determine whether it can be attributed to the formation of complexes with Nicd, we performed α-Su(H) and α-Nicd ChIP at different times after activation and assayed the levels of the *m7* enhancer fragment (Fig. 3C,D). In both ChIPs there was a transient increase in enrichment of the *m7* enhancer at 30 min, with the levels declining over subsequent time points. Furthermore, analysis of the amount of Nicd that coimmunoprecipitated with Su(H) showed peak levels at 30–60 min post-activation (Fig. 3E). Interestingly, Nicd appeared as a smear of increasing size, suggesting that it acquires post-translational modifications with time. Together, these data support the hypothesis that the increased Su(H) occupancy post-activation occurs as a consequence of its association with Nicd.

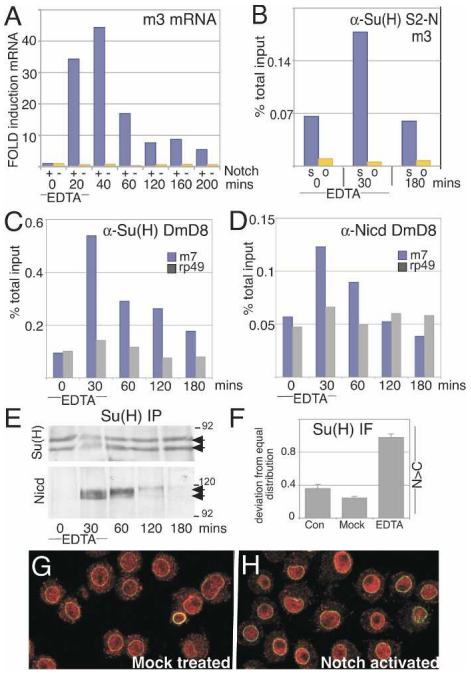

Figure 3. Time course of Su(H) occupancy and Nicd recruitment. (*A*) *m3* mRNA levels in S2-N (blue) and S2 cells (orange) at the indicated times relative to EDTA treatment. (*B–D*) α-Su(H) (*B,C*) or α-Nicd (*D*) ChIP at the indicated times relative to EDTA treatment in S2-N (*B*) or DmD8 (*C,D*) cells. Enrichment of *m3* enhancer (blue) and ORF (orange) (*B*) or *m7* enhancer (blue) and *rp49* ORF (control, gray) (*C,D*) were quantified. (*E*) Su(H) was immunoprecipitated at the indicated times and immunoprecipitates were probed to detect Su(H) (*top* panel, arrows) or Nicd (*bottom* panel, arrows). Approximate positions of molecular weight markers (in kilodaltons) are indicated. (*F–H*) Su(H) immunofluorescence (IF) (α-Su(H), red) (*G,H*) in S2-N cells treated as indicated. In *F*, distribution was quantified as described in the Supplemental Material. Differences between EDTA and control or mock cells were significant ($P < 0.001$) in S2-N cells. α-DMO (green) marks the nuclear envelope in *G* and *H*.

In previous studies, high levels of the Nicd fragment were found to promote nuclear accumulation of Su(H) (Fortini and Artavanis-Tsakonas 1994; Kidd et al. 1998). We therefore investigated whether the more physiological levels of Nicd produced by EDTA treatment could influence Su(H) distribution in S2-N cells. Staining of cells before and after EDTA treatment revealed a significant shift toward nuclear localization of Su(H) in EDTA-treated S2-N cells (Fig. 2F,H) compared with mock treated cells (Fig. 2F,G). No such shift was seen in control cells (Fig. 2F; Supplementary Fig. S3). Activation of Notch was accompanied therefore by an increase in the nuclear concentration of Su(H) as well as by increased occupancy of target enhancers. This brings into question whether some interactions between Nicd, Su(H)/CSL,

and Mam could occur before nuclear entry, explaining why the activation complex has been detected in cytoplasmic fractions (Jeffries et al. 2002).

Chromatin marks associated with highly inducible genes

Reversible post-translational modifications on histone tails and exchange of histone variants play an important role in the regulation of transcription. We therefore investigated whether the difference in Su(H) binding across the 11 *E(spl)* genes could be related to specific chromatin modifications. ChIP was performed on chromatin from S2-N and DmD8 cells before and 30 min after Notch activation using antibodies directed against histone H3, against histone modifications primarily associated with active chromatin (trimethylation at Lys 4 of histone H3 [$^{tri\text{-}me}$K4-H3] and acetylation of histone H4 [^{Ac}H4]), and against the histone variant H2A.v. In S2-N cells there was a clear correlation between the chromatin modifications and high Su(H) occupancy (Fig. 4A,B; Supplementary Fig. S4A,B). In DmD8 cells this correlation was less robust, although many of the same trends were evident (Fig. 4C,D; Supplementary Fig. S4C,D).

One characteristic observed in both cell types was a reduced association of H3 and by implication of nucleosomes with the active genes. In S2-N cells this was evident prior to Notch activation, with a lower than average enrichment of both *m3* and *m*β enhancer/promoter in the H3 ChIP (Fig. 4A). In DmD8 cells these two genes also had lower H3 coverage prior to activation (Fig. 4C), and there was a further decrease in H3 at these and at five other genes (*m*γ, *m*α, *m2, m6, m7*) after activation. Thus, activation is accompanied by a decrease in H3, and by implication, with a loss of nucleosomes from the enhancer/promoter regions. Both cell types also showed a large increase in the proportion of ^{Ac}H4 at the active genes, following Notch activation (Fig. 4B,D). This fits with reported recruitment of a HAT (p300) to the Nicd/CSL/Mam complex (Oswald et al. 2001; Wallberg et al. 2002). However, in both cell-types, ^{Ac}H4 encompassed adjacent loci that were not highly expressed, suggesting either that this complex can contact histones at a distance from its initial site of recruitment or that there is secondary recruitment of other HATs.

Two other chromatin marks that correlated strongly with activated genes in S2-N cells were $^{tri\text{-}me}$K4-H3 (Supplementary Fig. S4A) and enrichment for the histone variant H2A.v/H2A.Z (Supplementary Fig. S4B), which is suggested to mark transcriptionally poised genes (Raisner et al. 2005; Zhang et al. 2005). In DmD8 cells the correlation with $^{tri\text{-}me}$K4 was less evident, but four out of eight induced genes showed significant enrichment (Supplementary Fig. S4C). Likewise, there was no specific enrichment for H2A.v prior to activation (Supplementary Fig. S4D). However, in both S2-N and DmD8 cells, the proportion of H2A.v decreased at activated loci following EDTA (Supplementary Fig. S4E). A similar decrease in H2A.Z was found to accompany activation of the mammalian *c-myc* gene, where removal of H2A.Z was proposed to facilitate activation (Farris et al. 2005). The observed decrease in H2A.v following Notch activation is in agreement with this model, although more widespread analysis will be needed to determine whether it is indicative of a general mechanism at Notch targets.

Concluding remarks and mechanistic implications

Our results demonstrate that there is a significant increase in Su(H) occupancy at target genes following EDTA/Notch activation. This increase is transient and correlates with the presence of Nicd, implying that the kinetics of binding differ when Su(H) is complexed with Nicd, and that the association between Su(H) and its cognate sites is much more dynamic than expected. We also note that there are differences in Su(H) occupancy between genes prior to activation, suggesting the possibility of gene-specific modes of regulation [i.e., at some there may be constitutive recruitment of Su(H), whereas at others Su(H) binding is only signaling induced].

Several mechanisms could account for the increased Su(H) occupancy after Notch activation. One possibility is that the activation complex has a higher affinity for DNA. Structural analysis of the CSL/Nicd/Mam tertiary complex did not reveal any novel interactions between

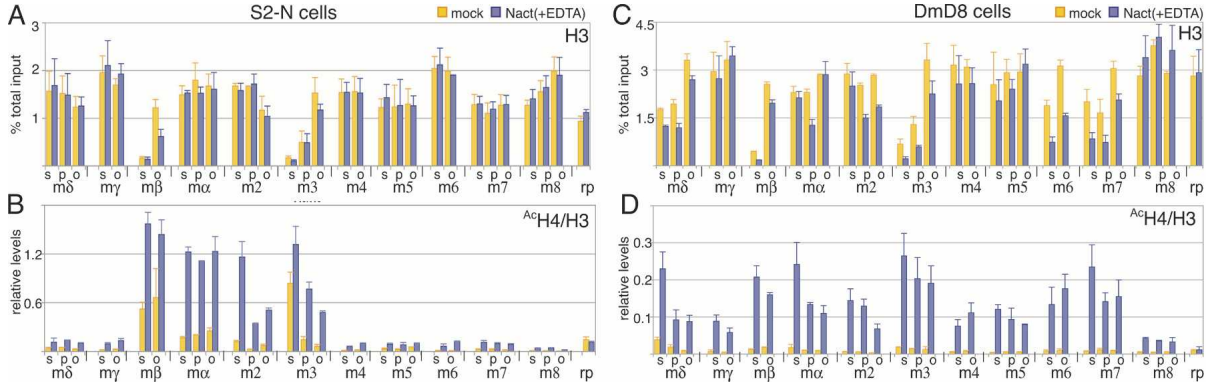

Figure 4. Chromatin modifications across *E(spl)* genes before and after Notch activation in S2-N and DmD8 cells. ChIP was performed with the indicated antibodies in S2-N (*A*,*B*) and DmD8 (*C*,*D*) cells before (orange) and 25 min after (blue) EDTA and bound fragments were quantified by real-time PCR. Results are an average of three independent experiments (error bars indicate standard error of the mean). (*B*,*D*) For each column, levels were first calculated relative to input and then as a ratio to the equivalent sample from the α-H3 ChIP. Controls were *rp49* (rp). In α-H3 ChIP, the decrease in enrichment of *m*β-o ($P = 0.02$) and *m3*-o ($P = 0.05$) in S2-N cells and of *m*δ-s ($P = 0.006$), *m*δ-p ($P = 0.03$), *m*β-s ($P = 0.002$), *m*α-p ($P = 0.02$), *m2*-p ($P = 0.008$), *m3*-s ($P = 0.03$), *m3*-p ($P = 0.03$), *m6*-s ($P = 0.004$), and *m7*-s ($P = 0.03$) in DmD8 cells are all significant.

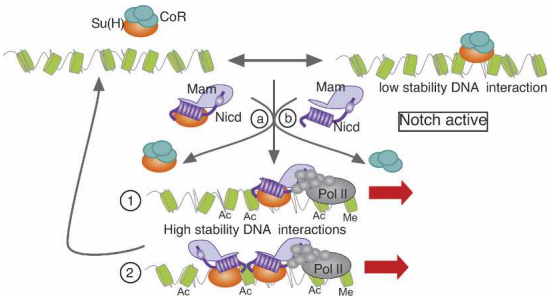

Figure 5. Dynamic model for Su(H) recruitment. Fast exchange and low residency of Su(H) (orange) when complexed with corepressors (turquoise). Following Notch activation, Su(H) complex with Nicd (dark purple) and Mam (light purple) is more stably associated with the DNA, possibly due to (1) interactions with other factors (gray, Pol II and associated factors) and/or (2) cooperative binding. This is accompanied by nucleosome (green) loss and histone modifications (Ac, me). Su(H) may form a complex with Nicd and Mam prior to DNA binding (a) or exchange could occur on transiently bound Su(H) (b). After recruitment, Nicd is subsequently modified and most likely degraded (Fryer et al. 2004), so Su(H) reverts to a lower occupancy state.

CSL and DNA that could account for an increase in affinity per se (Nam et al. 2006; Wilson and Kovall 2006). However, the affinity could be increased by cooperative interactions between two activation complexes on the DNA. Recent analysis demonstrates that Nicd-containing complexes can bind cooperatively to DNA with appropriately arranged paired sites (Nam et al. 2007). This may therefore be a significant factor in the enhanced Su(H) occupancy that we detect after activation. However, not all the enhancer fragments we analyzed contain paired Su(H) sites, suggesting that additional mechanisms are involved.

A second explanation is that interactions with other cofactors help to stabilize the Su(H) activation complex on the DNA. Studies of nuclear receptors suggest that in the resting state they rapidly exchange on and off the DNA, and that the formation of transcriptionally competent complexes slows this exchange (Agresti et al. 2005; Metivier et al. 2006). We envisage that the interactions of Su(H)/CSL with its cognate sites have similar change in dynamics; a fast exchange and low residency occurring when Su(H)/CSL is complexed with corepressors, a slow exchange and longer residency occurring when it is complexed with Nicd and competent to recruit productive transcription complexes and/or to make cooperative interactions (Fig. 5).

Materials and methods

Notch activation by EDTA

S2-N cells are a stable Notch-expressing S2 cell line containing a Cu^{2+}-inducible (metallothionein promoter) pMT-Notch construct (Fehon et al. 1990) and grown under permanent selection with 10 µM methotrexate (Sigma). Expression of full-length Notch was induced overnight by 600 µM $CuSO_4$ (Sigma) in cell culture medium. DmD8 cells were obtained from the *Drosophila* Genomics Resource Center (http://dgrc.cgb.indiana.edu). Activation in S2-N and DmD8 cells was achieved by exposing cells to 2 mM EDTA in PBS for 30 min. This was followed in some cases by replacement of cell culture medium and a further period of incubation. Control cells were treated with PBS without the addition of EDTA (mock-treated).

RNA isolation and quantification

RNA was isolated by Trizol (Ambion). Reverse transcription was performed with M-MLV RT (Promega) and oligo-dT primers or random hexamers (Promega). cDNA levels were quantified by real-time PCR using QuantiTec Sybr Green PCR mix (Qiagen) and the AbiPrism machine. The calibration curve was constructed from serial dilutions of genomic DNA, and values for all genes were normalized to the levels of rp49. To allow comparison among primer sets, a constant amount of genomic DNA (standard DNA) was used in each PCR run for additional normalization.

ChIP

Cells were cross-linked with 2% formaldehyde, the reaction was quenched by 0.125 M glycine, and the cells were harvested, washed with PBS, and resuspended in nuclear lysis buffer (the composition of ChIP buffers is provided in the Supplemental Material). Lysate was diluted 10 times in immunoprecipitation dilution buffer, sonicated by Bioruptor (Diagenode), and precleared with rabbit IgG (Sigma) and protein G agarose (Santa Cruz Biotechnology). ChIP reactions were performed overnight at 4°C with 700 µL of lysate and 1–2 µg of specific antibodies. Immunocomplexes were isolated with Protein G agarose, washed twice with wash buffer1 and twice with wash buffer2, and then de-cross-linked at 65°C in 0.25 M NaCl. Samples were treated with 0.2 mg/mL proteinase K, extracted by phenol/chloroform, and ethanol-precipitated. Pellets were resuspended in 35 µL of water and 0.5 µL was used for real-time PCR (primer sequences available on request). To allow comparison among primer sets, a constant amount of total input genomic DNA was used in each PCR run for normalization. The following antibodies were used: acetylated H4 (Upstate Biotechnology, 06-866), histone H3 (Abcam, ab1791), trimethyl K4 (Abcam, ab8580), CTD-phosphorylated Pol II (Abcam, ab5408), Su(H) (Santa Cruz Biotechnology, sc15813), Nicd (Developmental Studies Hybridoma Bank, C17.9C6), and H2A.v (gift from R.L. Glaser, New York State Department of Health, Albany, NY).

Immunostaining

Cells were grown on glass coverslips, with or without Cu induction, and mock-treated or activated by EDTA, then fixed for 30 min with 4% PFA. After blocking with PBT (1× PBS, 0.2% Triton X-100, 5% BSA) for 30 min, cells were stained with primary antibody for 90 min, washed with PBT, and stained with secondary antibody for 90 min.

Western blotting

Immunoprecipitated proteins were resolved by 7% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with specific antibodies as indicated. Bound antibodies were detected using horseradish-conjugated secondary antibodies and the ECL detection system (Amersham).

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