Molecular Cancer Research

Notch Represses Transcription by PRC2 Recruitment to the Ternary Complex

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Abstract

It is well established that Notch functions as a transcriptional activator through the formation of a ternary complex that comprises Notch, Maml, and CSL. This ternary complex then serves to recruit additional transcriptional cofactors that link to higher order transcriptional complexes. The mechanistic details of these events remain unclear. This report reveals that the Notch ternary complex can direct the formation of a repressor complex to terminate gene expression of select target genes. Herein, it is demonstrated that $p19^{Arf}$ and *Klf4* are transcriptionally repressed in a Notch-dependent manner. Furthermore, results indicate that Notch recruits Polycomb Repressor Complex 2 (PRC2) and Lysine Demethylase 1 (KDM1A/LSD1) to these promoters, which leads to changes in the epigenetic landscape and repression of transcription. The demethylase activity of LSD1 is a prerequisite for Notch-mediated transcriptional repression. In addition, a stable Notch transcriptional repressor complex was identified containing LSD1, PRC2, and the Notch ternary complex. These findings demonstrate a novel function of Notch and provide further insight into the mechanisms of Notch-mediated tumorigenesis.

Implications: This study provides rationale for the targeting of epigenetic enzymes to inhibit Notch activity or use in combinatorial therapy to provide a more profound therapeutic response. *Mol Cancer Res; 1–11. ©2017 AACR.*

Introduction

Transcription factors mediate the cellular programs instructed by extracellular cues. These extracellular cues direct complex transcriptional cascades that result in the activation and repression of genes that govern all cellular processes including cell type specification, proliferation, and differentiation (1–3). In many cases, there is a strict separation between transcription factors that assemble activation complexes and those that assemble repression complexes. In other cases, transcription factors can direct both activation and repression complexes based on complexes' components and/or the epigenetic landscape, setting the context for transcriptional response (4, 5).

Notch is a receptor-based transcription factor activated by ligand engagement through cell-to-cell contact. Notch signaling regulates key cellular functions such as proliferation, differentiation, and apoptosis in a context-dependent manner. Deregula-tion of Notch activity is important in driving the neoplastic phenotype in many human malignancies (6–8). Upon activation, the Notch intracellular domain (Notch^{ic}) is released from the plasma membrane and translocates into the nucleus, where it forms a ternary complex with Mastermind (Maml) and CSL. This ternary complex then serves as a scaffold to recruit higher-order transcriptional machinery to initiate a downstream transcriptional cascade (9–12).

Although it is clear that Notch is a transcriptional activator, many expression-profiling experiments reveal that there are genes repressed by Notch activity (13–21). The mechanisms by which these genes are repressed by Notch signaling are underexplored, as it is thought that repression is an indirect mechanism. As part of the transcriptional cascade initiated by Notch, expression of the Hes/Hey family of transcriptional repressors is induced (22). In many cases, transcriptional repression directed by activation of Notch signaling is a secondary effect mediated by this family of transcriptional repressors (15, 17, 23). Examples of this mechanism include, among others, the regulation of PTEN (19). However, in many cases, repression by Notch signaling cannot be attributed to Hes/Hey proteins and the mechanisms underlying these events are not known (16, 18, 20, 21). This indicates that there are distinct mechanisms of gene repression upon activation of Notch signaling that contribute to the Notch-induced transcriptional cascade.

Previously, we reported that in Notch-induced T-cell leukemogenesis, Notch suppresses p53 activity through repression of *Arf* expression (24). However, the detailed mechanism by which Notch mediates *Arf* repression was not clear. In this study, we demonstrate that Notch directs transcriptional repression of Arf through direct binding of the Notch ternary complex to the *Arf* promoter region. Upon binding the *Arf* promoter region, Notch

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serves to recruit the polycomb repressive complex 2 (PRC2) in an LSD1-dependent manner. Recruitment of these factors leads to the enrichment of the repressive mark H3K27me3 and the loss of the active mark H3K4me3 and subsequent termination of *Arf* expression. In addition, we provide evidence for a Notch repressor complex bound to DNA that contains LSD1 and PRC2 and therefore, provide a novel mechanism of gene regulation by Notch. Importantly, we also observe this activity in the regulation of *Klf4* by Notch indicating that direct repression of transcription by Notch is a common mechanism in Notch signaling. These data also provide rationale for the use of epigenetic therapy in combination with Notch inhibition for treating Notch-dependent cancers.

Materials and Methods

Cell culture

Primary lymphomas tumors and the lymphoma cell lines were collected and established in our laboratory as described previously between 2004 and 2005 (24). HPB-ALL, ALL-SIL, and 293T cell lines were obtained from the ATCC and cultured under recommended condition for less than 6 months. Mouse embryonic fibroblasts (MEF) were prepared from wild-type C57Bl/6 embryos at day E13.5 following a standard MEF isolation protocol (25) and were cultured in complete DMEM. Animal experiments were approved by the University of Miami Institutional Animal Care and Use Committee. The number of the passages of the cell lines used in this study did not exceed 15. All cell lines were maintained at 37°C in 5% CO_2 and tested for mycoplasma contamination.

Retroviral infections, qRT-PCR analysis, nuclear extraction, and Western blotting

Retroviral Infections, qRT-PCR analysis, nuclear extraction, and Western blotting were performed as described previously (26, 27). Cell samples were collected 72 hours postinfection. The following antibodies were used in Western blotting: anti-Notch1 (1:1,000, Abcam), anti-CSL (1:1,000; Polyclonal), anti-MAML1 (1:1,000, Cell Signaling Technology), anti-LSD1 (1:1,000, Cell Signaling Technology), and anti-EZH2 (1:1,000, Cell Signaling Technology).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed as described previously (28). Briefly, chromatin samples were immunoprecipitated with the following antibodies: rabbit IgG (Abcam), anti-Notch1 antibodies (Bethyl Laboratories), anti-Maml1 (Cell Signaling Technology), anti-LSD1 (Cell Signaling Technology), anti-EZH2 (Cell Signaling Technology), anti-SUZ12 (Abcam), anti-H3K4me3 (Abcam), or anti-H3K27me3 (Abcam). DNA was cleaned using PCR purification kit (Qiagen) and target sequences were amplified by qPCR. Primer sequences are available upon request.

DNA affinity precipitation

The conjugation of biotinylated dsDNA and streptavidin agarose beads was performed as described previously (26). For DNA affinity precipitation experiments from 4084 nuclear extraction, the supernatant that contained the nuclear protein fraction was incubated with DNA streptavidin beads (preblocked with 1 mg/mL BSA and 100 µg/mL sonicated salmon sperm DNA) for 1 hour at 4°C on a rotator and beads were washed three times with nonsupplemented DNA lysis buffer. Proteins bound to the beads were analyzed by SDS-PAGE and Western blot analysis using the appropriate antibodies.

Size exclusion chromatography

Size exclusion chromatography was performed as described previously (27). In brief, 10 mg of the nuclear extracts from 4084 and 6780 were used. A 500-µL portion of the sample was loaded onto the Superose 6 HR 10/30 resin column (GE Healthcare Life Sciences) which was equilibrated with column buffer [150 mmol/L NaCl, 40 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 0.5 mmol/L DTT, 5% glycerol, 0.001% NP-40] and collected into 350-µL fractions; fractions were then subjected to Western blotting analysis or individual fractions were pooled for next step experiment.

DNA affinity chromatography

The Hitrap streptavidin column was washed with PBS and then introduced to previously annealed biotinylated dsDNA containing two high-affinity CSL-binding sites (2× CSL). Indicated samples containing 10 µg/mL salmon sperm DNA were subjected to the column equilibrated with column buffer. The column was then eluted with column buffer containing 250 mmol/L or 350 mmol/L NaCl. Each sample was collected into 500-µL fractions and concentrated accordingly to ideal volume.

Cell viability assay

Cell viability assays were performed with CellTiter-Glo (G7572, Promega), as described in the product manual.

Results

Notch represses *Arf* expression

Previously, we reported that in a mouse model of Notch-driven T-cell lymphoma, p53 levels are suppressed by the repression of *Arf* expression (24). Consistent with this observation, in T-cell lymphoma cell lines 4084 and 3749 which are derived from the Top-Notch mouse, we observed lower *Arf* expression both at the mRNA and protein levels compared with the Myc-driven T-cell lymphoma cell line 6780 (29), which is derived from Tet-off Myc transgenic mouse (Fig. 1A and B). To investigate whether the repression of *Arf* is directly regulated by Notch signaling, we ectopically expressed $Notch^{ic}$ in primary MEFs and determined *Arf* mRNA and protein levels (Supplementary Fig. S1). Expression of $Notch^{ic}$ in MEFs led to a dramatic reduction of *Arf* mRNA and protein compared with MEF infected with GFP control or mock. To demonstrate the specificity for the *Arf* locus, $p16^{INK4a}$ expression was analyzed and, no effects were observed on expression of $p16^{INK4a}$ (Fig. 1C and D). As *Arf* and $p16^{INK4a}$ share an overlapping locus with distinct regulatory regions, these data indicate that Notch specifically governs the transcriptional repression of *Arf*.

Repression of *Arf* expression is a direct transcriptional effect of Notch and is accompanied by changes in the epigenetic landscape on the *Arf* promoter

We next sought to determine the mechanism by which Notch mediates transcriptional repression of *Arf*. The current model suggests that Notch drives a transcriptional network through the activation of gene expression. The HES and HEY proteins are canonical targets of Notch signaling and act as transcriptional
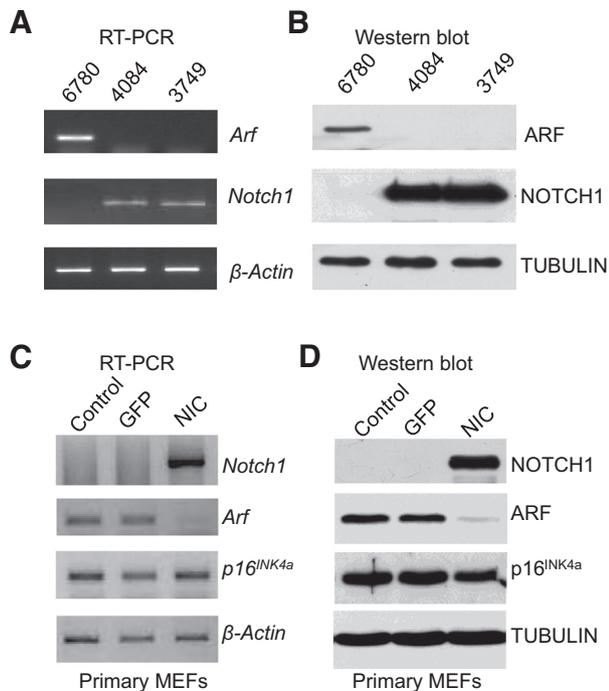
Figure 1.
Notch represses Arf gene expression. RT-PCR (**A**) and Western blot analysis (**B**) of Arf and Notch in mouse lymphoma cell-lines. RT-PCR (**C**) and Western blot analysis (**D**) of Arf p16^{INK4a} and Notch in primary MEFs infected with GFP or Notch^{ic} (NIC) encoding vector. β-Actin and Tubulin are used as loading controls.

repressors (22, 23). Therefore, we reasoned that these proteins, among others, were plausible candidates for Notch-directed repression of Arf. However, neither overexpression nor siRNA-mediated knock down of Hes/Hey members had any effect on Arf levels (Supplementary Fig. S2). Therefore, we thought that repression of Arf might be directly regulated by Notch.

To test this hypothesis, we carried out a chromatin immunoprecipitation (ChIP) experiment in primary lymphomas generated from TOP-Notch^{ic} mice. Using primers spanning approximately 7 kb on the promoter region (Fig. 2A) to investigate Notch binding, quantitative PCR analysis of the ChIP samples revealed that Notch binds to the Arf promoter primarily in the proximal region upstream of the transcription start site (Fig. 2B). To determine the events subsequent to Notch binding, we examined epigenetic marks on the Arf promoter. In primary T-cell lymphomas derived from the Top-Notch mouse, the repressive mark H3K27me3 is more abundant compared with the active mark H3K4me3 (Fig. 2C). This is consistent with the repressive state of the Arf locus. Similarly, we detected lower levels of H3K4me3 and higher levels of H3K27me3 on the Arf promoter in Notch driven T-cell lymphoma cell line 4084 compared with a Myc-driven T-cell lymphoma cell line 6780 (Fig. 2D and E; Supplementary Fig. S3).

To recapitulate the molecular mechanism, we performed ChIP experiments in primary MEFs that ectopically expressed Notch^{ic} (NMEF). When Notch^{ic} is expressed in primary MEFs, we readily detect the occupancy of Notch, Maml, and CSL on the Arf locus in comparison with control-infected MEF cells (CMEF; Fig. 2F–H).

These data demonstrate that the Notch ternary complex directly localizes to the Arf locus.

Taken together, these results indicate that repression of Arf transcription is a direct transcriptional effect of Notch. Notch-mediated repression is through the binding of Notch ternary complex to the Arf promoter, which induces the reduction of active mark H3K4me3 and the accumulation of repressive mark H3K27me3.

Notch recruits polycomb repressive complex-2 to the Arf promoter

As an enrichment of H3K27me3 was detected on the Arf promoter, we next investigated the putative mediators responsible for the methylation of H3K27. One of the well-studied histone methyl transferases that methylates H3K27 is Polycomb repressor complex-2 (PRC2; refs. 30–34). To investigate the involvement of PRC2 in Notch-mediated transcriptional repression, we first examined the mRNA levels of its core components, Ezh2 and Suz12, in lymphoma cells. Cell lines that have higher levels of activated Notch (4084 and NA1) express greater levels of both factors compared with the Myc-driven lymphoma cell line 6780, which has no detectable Notch activity (Supplementary Fig. S3A and S3B). In primary MEFs, Notch^{ic} expression also results in increased transcriptional levels of Ezh2 and Suz12 (Fig. 3A). We then conducted ChIP experiments to investigate whether PRC2 is colocalized to the Arf promoter together with the Notch ternary complex. In Notch^{ic}-infected MEFs, occupancy of the proximal Arf promoter by EZH2 and SUZ12 mirrors the occupancy by Notch^{ic} (Fig. 3B and C). In contrast, EZH2 and SUZ12 were not detected on the Arf locus in control infected MEFs. These data indicate that PRC2 localizes to the Arf locus in a Notch-dependent manner. Moreover, when Notch^{ic}-infected MEFs are treated with the EZH2 inhibitor EPZ-6438, the transcriptional activity of Arf is rescued from Notch repression (Fig. 3D; refs. 35, 36). In comparison, ChIP analysis on the Hey2 promoter, which is transcriptionally activated by Notch, reveals that the ternary complex but not the PRC2 complex is readily detected (Fig. 3E). These data clearly indicate that Notch-mediated PRC2 recruitment leading to transcriptional repression is a specific and distinct Notch event.

LSD1, a histone demethylase, is required for the assembly of the Notch repressor complex

In addition to increased levels of the repressive mark H3K27me3, there is a significant decrease in the levels of the active mark H3K4me3 on the Arf promoter in Notch-induced T-cell lymphoma (Fig. 2D); therefore, we sought to determine the enzyme that directs this epigenetic change. LSD1 is a histone demethylase that has been shown to account for demethylation of H3K4 in many cases (37). ChIP experiments performed in Notch^{ic}-infected primary MEFs revealed that Notch induces the binding of LSD1 to the Arf promoter compared with control MEFs that show no enrichment. These data demonstrate that LSD1 colocalizes with the Notch ternary complex and PRC2 on the Arf promoter (Fig. 4A). Moreover, when Notch^{ic}-infected MEFs were treated with the LSD1 inhibitor tranylcypromine (TCP), repression of Arf transcription by Notch was blocked (Fig. 4B; refs. 36, 38). We next examined the occupancy of these factors on the Arf locus in Notch^{ic} MEF cells treated with TCP. Compared with Notch^{ic} MEF cells treated with vehicle, lower occupancy levels of LSD1 and Notch on the Arf promoter were observed in the TCP-treated cells (Fig. 4C and D). Consequently, the binding of EZH2

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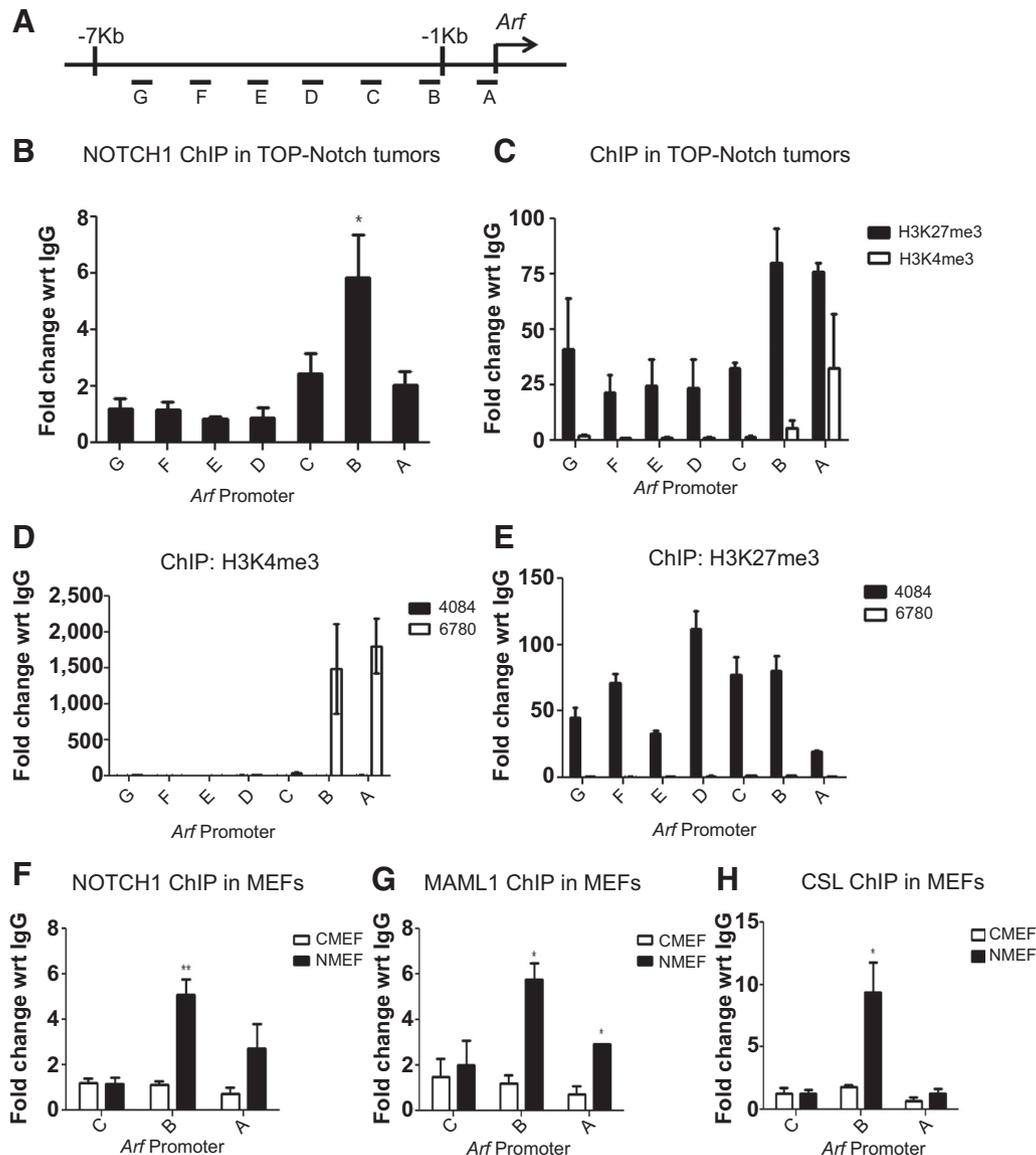
Figure 2.
Downregulation of *Arf* is a direct transcriptional effect of Notch and is accompanied by changes in the epigenetic landscape on the promoter. **A,** Schematic representation of the primers spanning the *Arf* promoter used for ChIP-PCR analysis. **B,** ChIP-PCR analysis showing Notch binding on the *Arf* promoter in primary mouse lymphoma tumors (three individual biological repeats). **C,** ChIP-PCR analysis depicting the amount of H3K4me3 and H3K27me3 binding on the *Arf* promoter in primary mouse lymphomas tumors. H3K4me3 (**D**) and H3K27me3 (**E**) binding in mouse lymphoma cell lines (4084 and 6780). In Notch[ic]-infected MEFs (NMEF) compared with control MEFs (CMEF), ChIP-PCR analysis showing: Notch (**F**), Maml1 (**G**), and CSL (**H**) binding in the proximal region on *Arf* promoter. Results are represented as fold change with respect to IgG. Data are shown as means $\pm$ SEM ($n = 3$; *, $P \leq 0.05$).

and repressive mark H3K27me3 to the promoter are also decreased, consistent with the reduction of Notch-binding activity (Fig. 4E and F). Western blot analysis indicates that there is no difference in expression of Notch1, LSD1, and EZH2 in Notch[ic] MEFs and the TCP-treated Notch[ic] MEFs (Fig. 4G). Therefore, the lower occupancy levels observed on the *Arf* promoter are not a result of the protein loss. Collectively, these data indicate that the demethylation activity of LSD1 is a prerequisite for the Notch repressor complex formation.

Interestingly, on the *Hey2* promoter, we also detect LSD1 binding, which is induced by Notch (Fig. 4H). To address the specificity

of LSD1 recruitment to repressed genes, we analyzed the effect of TCP treatment on *Hey2* expression. Similar to the results obtained for the repression of *Arf* expression by Notch, TCP also blocked Notch in transcriptional activation. That is, the treatment of Notch[ic]-infected MEFs with TCP resulted in a decrease in transcription of *Hey2* and loss of Notch and LSD1 occupancy from the promoter (Fig. 4I and J). Taken together, these data indicate that LSD1 is required for both activation and repression by Notch.

The Notch-driven lymphoma cell line NA1 and 4084 has undetectable levels of *Arf* mRNA compared with the Myc-driven lymphoma cell line 6780 (Fig. 4K). When NA1 and 4084 cells are
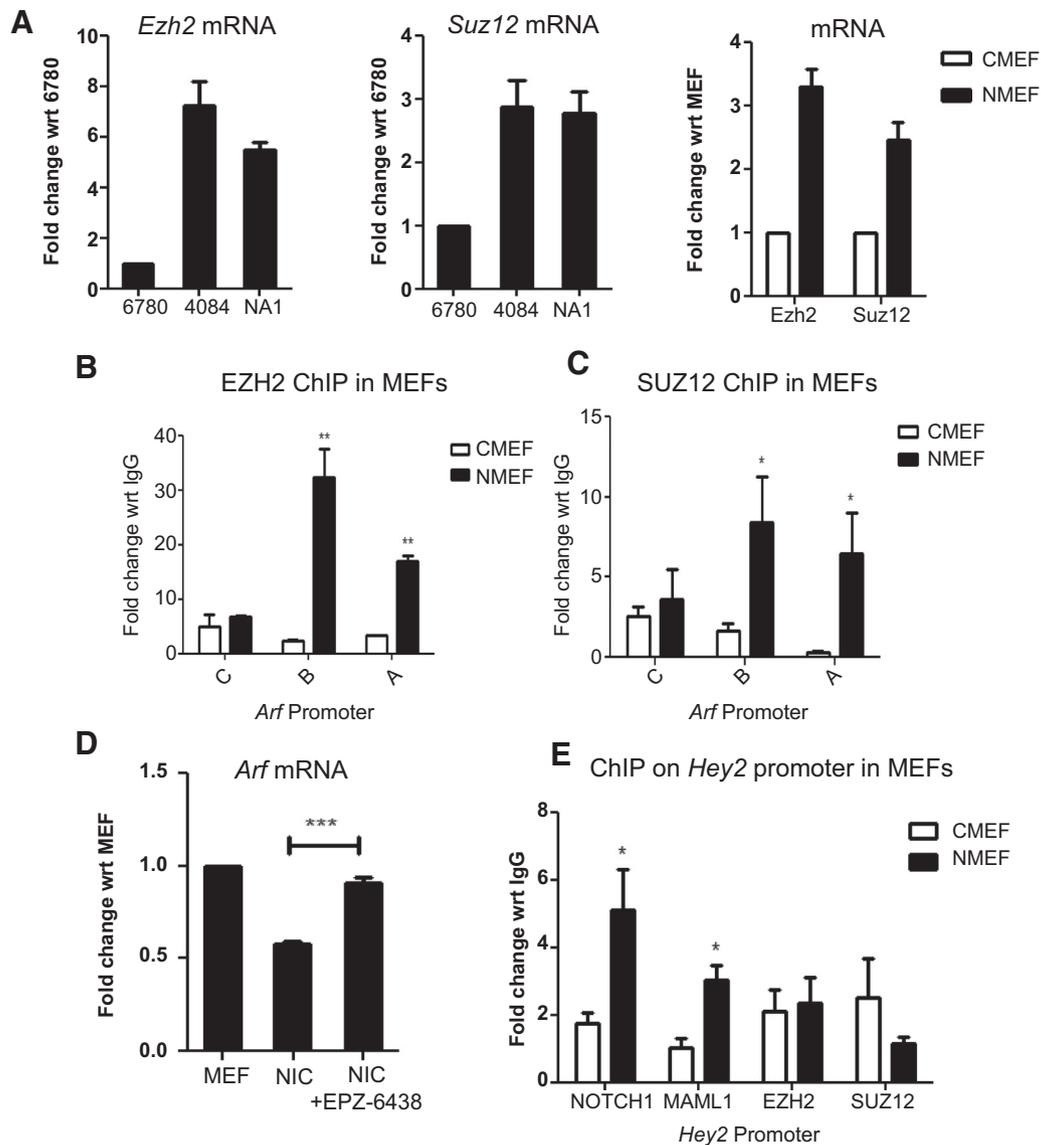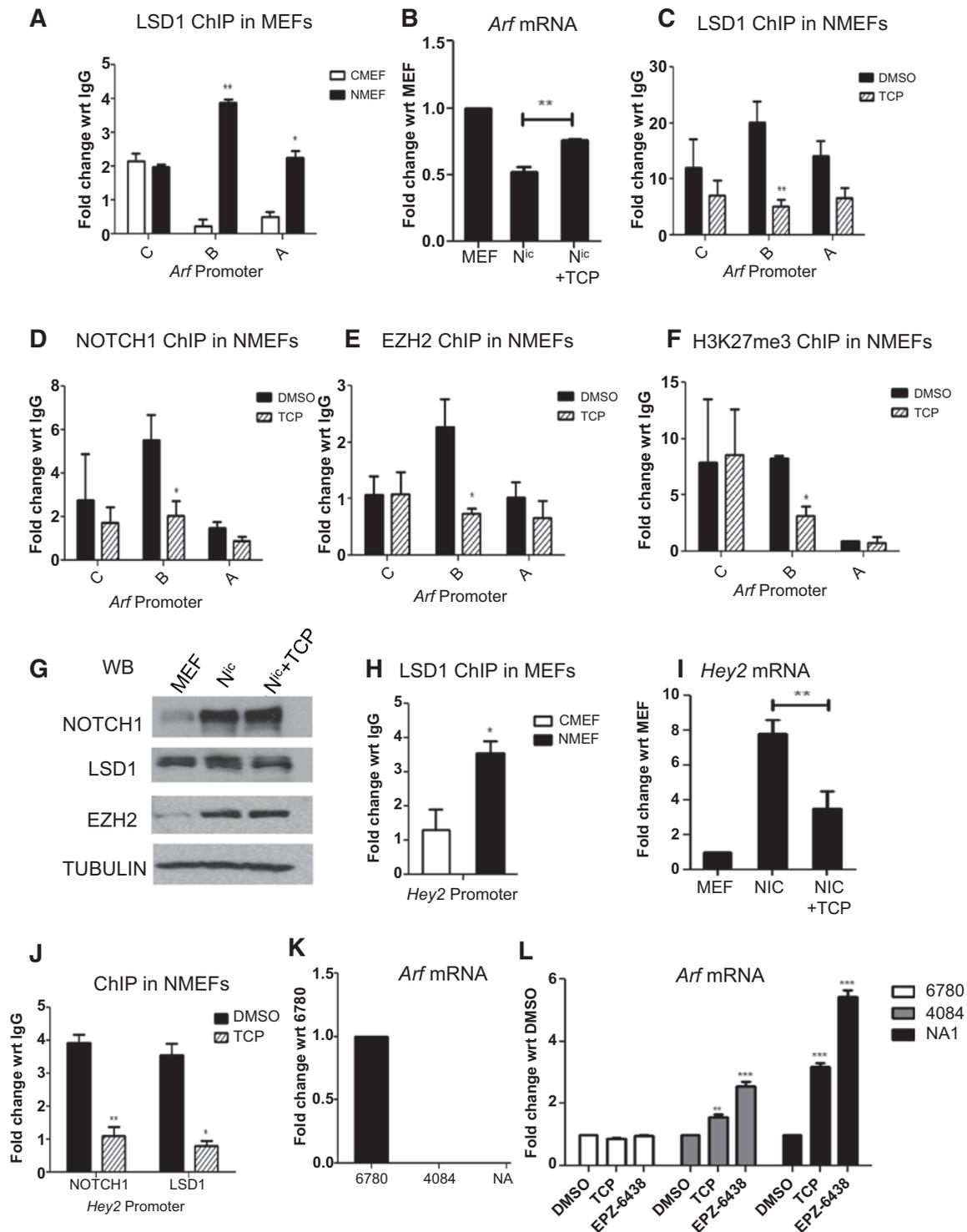
Figure 3.
Notch recruits polycomb repressive complex-2 (PRC2) to the *Arf* promoter. **A,** qRT-PCR analysis of *Ezh2* and *Suz12* in mouse lymphoma cell lines and primary MEFs infected with control and Notch[ic] retrovirus. ChIP-PCR analysis showing EZH2 (**B**) and SUZ12 (**C**) binding to the *Arf* promoter in control and Notch[ic]-infected MEFs. Results are expressed as fold change with respect to IgG. Shown are means ± SEM (*n* = 3). **D,** qRT-PCR analysis of *Arf* in primary MEFs, Notch[ic]-infected MEFs and Notch[ic]-infected MEFs further treated with EPZ-6438 (5 μmol/L, 48 hours). Data are shown as means ± SEM (*n* = 3). **E,** ChIP-PCR analysis showing Notch, Maml, EZH2, and SUZ12 binding level on the *Hey2* promoter in control and Notch[ic]-infected MEFs. Results are expressed as fold change with respect to IgG. Shown are means ± SEM (*n* = 3; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

treated with TCP and EPZ-6438, transcriptional activity is released from Notch-directed repression. In contrast, no effect on *Arf* transcription is observed in 6780 cells treated with either TCP or EPZ-6438 (Fig. 4L). Moreover, both inhibitors only affect NA1 cell line viability but not 6780 cell line (Supplementary Fig. S4). Similar finding has been observed in human leukemia cell line ALL-SIL that *Arf* transcriptional level is elevated by both inhibitors (Supplementary Fig. S5). Taken together, the inhibition of LSD1 or EZH2 contributes to the rescue of ARF expression in Notch[ic]-expressing MEFs and Notch-driven T-cell lymphoma cell lines, indicating that both LSD1 and EZH2 activities are critical for Notch-directed transcriptional repression of *Arf* expression.

**Transcriptional repression is likely a common mechanism of Notch-directed transcriptional programming**

To extend the Notch repressor paradigm, we sought to investigate other genes repressed by Notch. Previous studies in mouse gastrointestinal tract, human colon cancer cell lines and human esophageal adenocarcinoma cells have shown lower levels of *KLF4* expression when Notch activity is high (20, 21). Consistent with these studies, *Klf4* expression in Notch-driven T-cell lymphoma cell lines and Notch[ic]-infected MEFs is relatively low when compared with 6780 cells and control-infected MEFs (Fig. 5A and B). To determine whether *Klf4* is repressed directly by Notch as in the case of *Arf*, we performed ChIP analysis on the *Klf4* promoter.

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Figure 4.
LSD1, a histone demethylase, is required for the assembly of the Notch repressor complex. **A,** ChIP-PCR analysis showing LSD1 binding to the *Arf* promoter in control and Notch[ic]-infected MEFs. **B,** qRT-PCR analysis of *Arf* in primary MEFs, Notch[ic]-infected MEFs and Notch[ic]-infected MEFs further treated with TCP (10 μmol/L, 48 hours). Shown are means ± SEM ($n = 3$; **, $P < 0.01$). **C–F,** ChIP-PCR analysis of Notch1 (**C**), LSD1 (**D**), EZH2 (**E**), and H3K27me3 (**F**) binding to *Arf* promoter between control and TCP treatment group. **G,** Western blot analysis revealed protein levels of Notch1, LSD1, and EZH2 in primary MEFs, Notch[ic]-infected MEFs, and TCP-treated Notch[ic]-infected MEFs. Tubulin is used as a loading control. **H,** ChIP-PCR analysis showing LSD1 binding to the *Hey2* promoter in control and Notch[ic]-infected MEFs. **I,** qRT-PCR analysis of *Hey2* in primary MEFs, Notch[ic]-infected MEFs and Notch[ic]-infected MEFs further treated with TCP (10 μmol/L, 48 hours). Shown are means ± SEM ($n = 3$; **, $P < 0.01$). **J,** ChIP-PCR analysis of Notch and LSD1 binding to the *Hey2* promoter between control and TCP treatment group. **K,** qRT-PCR analysis of *Arf* in lymphoma cell lines 6780, 4084, and NA1. **L,** qRT-PCR analysis of *Arf* in the three lymphoma cell lines treated with LSD1 inhibitor TCP (10 μmol/L, 48 hours) or EZH2 inhibitor EPZ-6438 (5 μmol/L, 48 hours). ChIP results are represented as fold change with respect to IgG. Data are shown as means ± SEM ($n = 3$; *, $P \leq 0.05$; **, $P < 0.01$).

Figure 5.
Assembly of the Notch repressor complex on the *Klf4* promoter. **A,** qRT-PCR analysis of *Klf4* in lymphoma cell lines 6780, 4084, and NA1. **B,** qRT-PCR analysis of *Klf4* in primary MEFs, Notch^{ic}-infected MEFs, and Notch^{ic}-infected MEFs treated with TCP (10 µmol/L) or EPZ-6438 (5 µmol/L) for 48 hours. **C,** Schematic representation of the CSL-binding region on the *Klf4* promoter and ChIP analysis of Notch, Maml1, EZH2, and SUZ12 on that region. Results are expressed as fold change with respect to IgG. Data are shown as means ± SEM ($n = 3$; *, $P \leq 0.05$; **, $P \leq 0.01$).
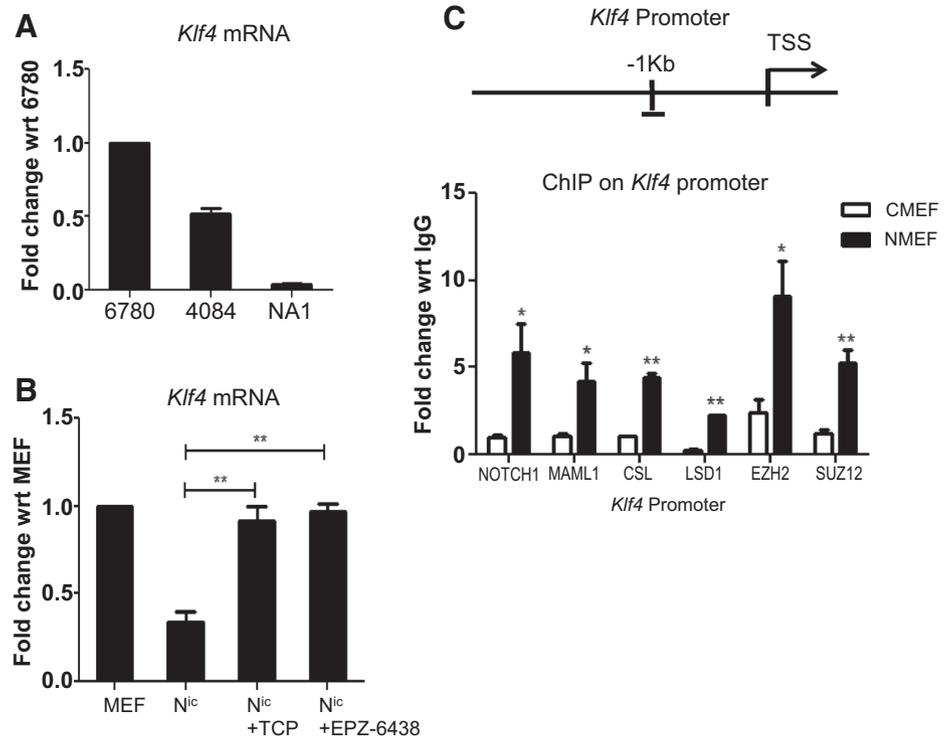
As observed with the *Arf* locus, the Notch ternary complex and PRC2 are present on the *Klf4* locus in Notch^{ic}-infected MEFs and absent in control-infected MEFs (Fig. 5C). When Notch-expressing MEFs are treated with LSD1 or EZH2 inhibitors, the transcriptional levels of *Klf4* are similarly rescued from Notch-directed repression (Fig. 5B). These data reveal that the recruitment of PRC2 complex by Notch is likely an essential mechanism for Notch-mediated gene repression and is a more general component of a Notch-induced transcriptional cascade.

Identification of a Notch transcriptional repressor complex

We provide compelling evidence that Notch directs the transcriptional repression of *Arf* by the recruitment of the epigenetic enzymes EZH2 and LSD1 accompanied by changes in histone methylation. To determine whether Notch in fact forms a stable transcriptional repression complex on the Notch ternary complex scaffold, we carried out a DNA affinity precipitation assay using oligonucleotides, harboring CSL-binding sites (CSL) conjugated to agarose beads and nuclear lysates derived from 4084 cells. DNA oligonucleotides harboring mutant CSL-binding sites (Mut) were used as a control for specificity. When nuclear lysates from 4084 cells were subjected to the CSL DNA affinity precipitation, we can readily detect the Notch ternary complex along with LSD1 and EZH2. However, in the CSL mutant pull-down, neither the Notch ternary complex nor EZH2 and LSD1 are isolated (Fig. 6A). These results indicate that the Notch ternary complex is associated with EZH2 and LSD1 and furthermore, that the binding of this complex is CSL-dependent.

To purify the Notch-repressor complex, we utilized CSL-DNA affinity FPLC. We applied 4084 nuclear lysate to a CSL-DNA affinity column, washed extensively, and eluted bound proteins using 350 mmol/L NaCl. In this analysis, both EZH2 and LSD1 coelute with the Notch ternary complex, consistent with the batch

DNA affinity precipitation. To demonstrate that these coeluted proteins were complexed we reloaded the eluate onto the CSL-DNA affinity column and observed that EZH2 and LSD1 coeluted with Notch again. In this experiment, no proteins were detected in the flow-through (FT). In contrast, if we reload the primary FT, which is largely depleted of CSL, we observe EZH2, LSD1 along with unbound Notch and Maml in the FT and no proteins detected in the eluate. Moreover, the input lane in the FT reload clearly shows the loss of CSL from the primary run (Fig. 6B and C). These data support the hypothesis that a stable Notch repression complex can be purified.

To further prove the existence of a Notch repressor complex, we employed a two-step chromatography scheme and compared nuclear extracts from 4084 and 6780 cells (Fig. 6D). Western blot analysis of the nuclear lysates from both cell lines demonstrate that unlike 4084 cells, NOTCH and MAML protein levels are undetectable in 6780, whereas other components including CSL are equivalent (Fig. 6E). Nuclear lysates from 4084 and 6780 were fractionated by size exclusion chromatography on a Superose 6 column. On the basis of migration profiles (Supplementary Fig. S6) we prepared two sets of pooled fractions. Pool A refers to the largest complexes, which contain the higher-molecular-weight Notch-containing complexes (A); and pool D that refers to the smallest complex (D), in which monomeric CSL is enriched. Pools A and D were then separately subjected to CSL DNA affinity FPLC followed by stepwise elution at 250 mmol/L NaCl and 350 mmol/L NaCl. Analysis of the pool A derived from 4084 cells revealed that EZH2 and LSD1 coelute with the Notch ternary complex under 250 mmol/L NaCl condition, whereas the negative control SP1 failed to coelute (Fig. 6F). As described above, monomeric CSL in the pool D eluted at 350 mmol/L NaCl (Supplementary Fig. S7). Analysis of pool D derived from 6780 cell lysates reveals that monomeric CSL is readily purified,
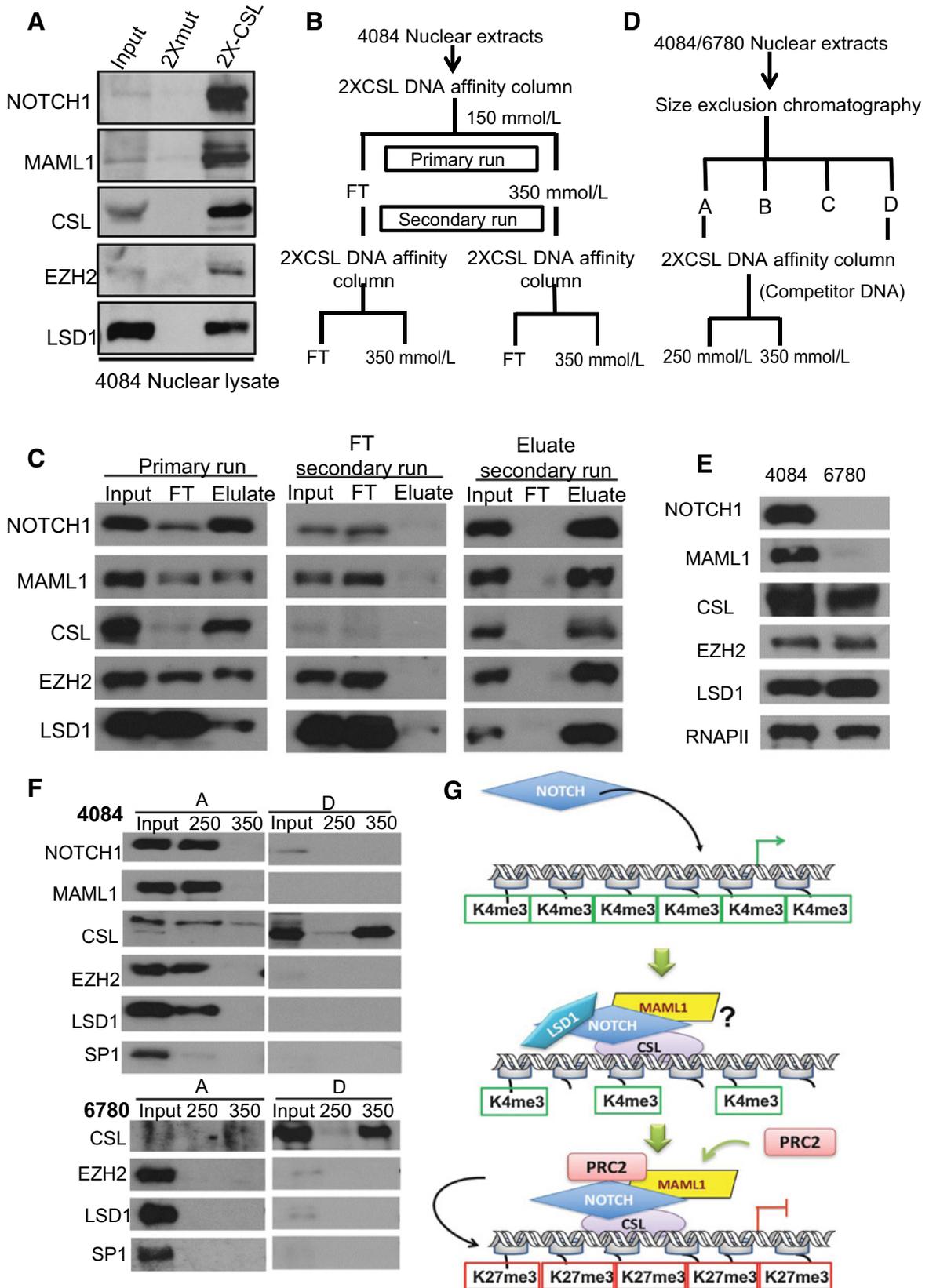
Table 1. MS analysis showed significant enrichment of PRC2 and LSD1 along with the Notch ternary complex from 4084 lysates in comparison with 6780-derived lysates

Complex/protein names	Gene names	MS/MS Count 4084A250	MS/MS Count 6780A250	Intensity fold change
Notch ternary complex	Notch1	10	0	2.38E+08
	Maml1	9	0	1.77E+08
	Rbpj	12	0	3.84E+08
PRC2	Ezh2	24	1	4.50E+02
	Suz12	24	2	3.40E+02
	Eed	17	3	3.41E+01
	Rbbp4	37	17	1.38E+01
LSD1	Kdm1a	34	4	5.90E+01

however, no CSL, EZH2 or LSD1 were detected in pool A (Fig. 6F). Samples containing the pool A/250 mmol/L eluate were analyzed by LC-MS/MS. Comparison of 4084 and 6780 purifications by MS analysis showed significant enrichment of PRC2 and LSD1 from 4084 lysates along with the Notch ternary complex but no enrichment from 6780-derived lysates (Table 1). These data indicate that copurification of PRC2 and LSD1 is dependent on the presence of the Notch ternary complex. Taken together, these data reveal the existence of a stable transcriptional repressor complex comprising EZH2, LSD1, and the Notch ternary complex.

Discussion

Many expression analysis studies have reported that induction of Notch signaling results in both the activation and repression of gene expression. It has long been thought that Notch functions as a transcriptional activator that initiates a transcriptional cascade. In such a model, Notch would repress gene expression by first activating the expression of transcriptional repressors, such as the Hes/Hey family (canonical Notch target genes), which in turn would repress transcription of their target genes. For example, induction of Notch represses the expression of PTEN and Fbw7. In this case, repression is directly mediated by Hes/Hey, which is a secondary effect of Notch (17, 19, 22). In other cases, such as the regulation of Sox9 in chondrogenesis and miR-155 during inflammation, Notch does not invoke Hes/Hey repressors to turn these genes off but rather it appears to be a direct effect of Notch as evidenced by ChIP analysis (16, 18). However, to date, this "direct" effect is only supported by a circumstantial evidence, as no mechanism for repression by Notch has been reported. Herein, we present evidence that reveals a novel mechanism by which Notch directly represses gene transcription through binding to target promoters. We demonstrate that the downregulation of *Arf* and *Klf4* by Notch is a direct effect and is accompanied by changes in the epigenetic landscape to favor repression. Furthermore, we

demonstrate that PRC2, the histone methyl transferase complex responsible for H3K27me3, is recruited by Notch to these repressed promoters. Data also indicate that recruitment of PRC2 and the subsequent enrichment of H3K27me3 require the activity of LSD1. Furthermore, using a two-step chromatography purification scheme, we have identified a stable Notch repressor complex comprising EZH2, LSD1, and the Notch ternary complex. On the basis of these data, we propose the following model for the assembly of a Notch-directed repressor complex on target promoters: in that, Notch binding to the target promoters recruits cofactors Maml and PRC2, together with LSD1, to drive the reduction of H3K4me3 and the accumulation of H3K27me3 resulting in repression of transcription (Fig. 6G).

PRC2 is a component of the Notch transcriptional cascade

It has been established that several epigenetic modifiers including EZH2 are aberrantly expressed in multiple human malignancies (34, 39). EZH2 is known as a core enzymatic component of PRC2 which mediates the methylation of H3K27 and functions as a transcriptional repressor complex (33). Our data demonstrates that by recruiting PRC2 to target promoters, Notch directly mediates target gene repression. Is PRC2 a direct transcriptional target of Notch? Preliminary data suggest that Notch activation leads to direct transcriptional induction of both Ezh2 and Suz12. Although there is no evidence indicating that Ezh2 and Suz12 are direct Notch target genes, our results suggests that they are indeed part of the transcriptional cascade, as are Hes and Hey genes. We have demonstrated that NACK is a direct transcriptional target of Notch and acts as a critical coactivator in Notch-mediated transcription (26). Considering this, a model emerges that suggests that upon activation of Notch there is a series of target genes that are activated and in turn function as cotranscriptional regulators of Notch-mediated transcription. This mechanism serves to enforce a Notch transcriptional profile of activated and repressed genes. Therefore, an interesting question is what determines how Notch becomes an activator or a repressor of

Figure 6.
Identification of the CSL-dependent Notch transcriptional repressor complex. **A,** DNA affinity precipitation analysis using nuclear lysate from 4084 cell line. **B,** Purification scheme for reloading the flow-through (FT) and the eluate back to the 2× CSL column. **C,** According to the purification scheme shown in **B**, 4084 nuclear lysate was subjected to a 2×-CSL affinity binding column twice, Notch, Maml1, CSL, EZH2, and LSD1 proteins in FT (flow-through) and eluate were visualized by Western blot analysis. Input represents 1% of the total protein loaded onto the column, FT and Eluate represent 4% of the total collection. **D,** The 2-step purification scheme for 4084 nuclear extracts. **E,** In nuclear lysate from 4084 compared with 6780, protein levels of Notch, Maml1, CSL, EZH2, and LSD1 were determined by Western blot analysis, RNA polymerase II was used as loading control. **F,** Section A and section D from 4084 and 6780 fractionation were subjected to 2×-CSL affinity binding column and eluted with column buffer containing 250 mmol/L and 350 mmol/L NaCl. Notch, Maml, CSL, EZH2, and LSD1 proteins were visualized by Western blotting. Sp1 was used as negative control. Input represents 1% of the total protein loaded onto the column, 10% of the eluate was loaded as sample. **G,** The transcriptional status of the target promoters is active prior to Notch activation. The binding of Notch ternary complex recruits corepressors including, LSD1 and EZH2 resulting in changes in epigenetic landscape and leads to the transcriptional repression of target genes.

transcription. The answer likely involves the local environment and specific modifications to the Notch ternary complex in a context-dependent manner.

LSD1 is required for both Notch transcriptional activation and repression

LSD1 is a histone demethylase that has been reported to be involved in Notch-mediated transcriptional activation. However, a mechanism of action for this role of LSD1 has not been resolved. Mulligan and colleagues have also suggested that LSD1 serves as a repressor for Notch target genes by forming a complex with CSL and that induction of Notch replaces LSD1 from the target promoters (40). However, data from Benkirane and colleagues indicates that upon Notch activation, LSD1 switches its function from a corepressor to a coactivator by modifying different histone marks (3, 41). In this study, we sought to investigate the role of LSD1 in Notch-mediated gene repression. Our data have revealed that LSD1 is recruited by Notch to the *Arf* promoter and its demethylase activity is required for the assembly of the Notch repressor complex. Inhibiting LSD1 activity with TCP dissociates the repressor complex from the *Arf* promoter and rescues *Arf* transcription from Notch repression. However, we have also detected the binding of LSD1 to the *Hey2* promoter, which is a gene activated by Notch. Inhibition of LSD1 activity with TCP also blocks activation of Hey2 transcription by Notch and therefore is not specific to either of these activities. Taken together, our study indicates that LSD1 is required for both Notch-mediated activation and repression, perhaps through influencing assembly of the ternary complex on DNA.

Assembly of the Notch repressor complex on target promoters

In this study, we demonstrate a link between Notch, LSD1 and PRC2 in Notch-mediated gene repression. Moreover, protein purification analysis has identified a stable Notch repressor complex comprising EZH2, LSD1 and the Notch ternary complex. The mechanistic details describing how the complex assembles, however, remain to be fully understood. Maml, a core factor in Notch transcriptional complexes, is acetylated by p300 during Notch-mediated transcriptional activation (42). Unpublished data from our laboratory demonstrate that this modification is important for recruiting the coactivator NACK and activating transcription (Ke and colleagues, submitted). Evidence derived from this study reveals that Maml is also involved in the repressor complex. Therefore, one possibility is that posttranslational modifications on Maml by distinct epigenetic enzymes dictate whether Notch activates or represses transcription by the subsequent recruitment of secondary complexes. How is PRC2 recruited to the Notch

ternary complex? Mechanisms for the recruitment of PRC2 to transcription factors remain elusive, but several models have been proposed (43–47). Jaird2 has been shown to link PRC2 to chromatin in ES cells (48). Histone deacetylases (HDAC) 1 and 2 associate with PRC2 core complex to drive gene repression (49). The long noncoding RNA HOTAIR has been shown to serve as a bridge between LSD1 and PRC2 (50). Whether or not any of these factors contribute to the Notch repressor complex assembly and activity is yet to be explored.

On the basis of data presented herein, we propose a novel regulatory mechanism of Notch. That is, in addition to activating gene expression by forming a transcriptional activation complex, Notch directs the assembly of a repressor complex and acts as a transcriptional repressor. As ARF is an important tumor suppressor that triggers the induction of p53 activity, repression of ARF transcription by Notch is fundamental to its role in driving tumorigenesis. Therefore, this study reveals potential new targets in the attack on Notch-dependent tumors. Specifically, chemical attack on the PRC2 enzyme EZH2 in combination with inhibition of Notch might provide an effective therapeutic approach.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Han, P. Ranganathan, C. Tzimas, A.J. Capobianco
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Han, P. Ranganathan, C. Tzimas, W. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Han, P. Ranganathan, C. Tzimas, L. Astudillo, W. Zhou, D.J. Robbins
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