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# Notch Signaling in Acute Promyelocytic Leukemia

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## Abstract

Acute promyelocytic leukemia (APL) is initiated by the *PML-RARA* fusion oncogene and has a characteristic expression profile that includes high levels of the Notch ligand *JAG1*. In this study, we used a series of bioinformatic, *in vitro*, and *in vivo* assays to assess the role of Notch signaling in human APL samples, and in a *PML-RARA* knockin mouse model of APL *(Ctsg-PML-RARA)*. We identified a Notch expression signature in both human primary APL cells and in Kit+Lin −Sca1+ (KLS) cells from pre-leukemic *Ctsg-PML-RARA* mice. Both genetic and pharmacologic inhibition of Notch signaling abrogated the enhanced self-renewal seen in hematopoietic stem/progenitor cells (HSPCs) from pre-leukemic *Ctsg-PML-RARA* mice, but had no influence on cells from age-matched wildtype mice. In addition, 6 of 9 murine APL tumors tested displayed diminished growth *in vitro* when Notch signaling was inhibited pharmacologically. Finally, we found that genetic inhibition of Notch signaling with a dominant negative MAML protein reduced APL growth *in vivo* in a subset of tumors. These findings expand the role of Notch signaling in hematopoietic diseases, and further define the mechanistic events important for *PML-RARA*-mediated leukemogenesis.

### Keywords

Notch; Acute Promyelocytic Leukemia; Self-renewal



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N.R.G., J.M.K, and T.J.L. designed the research, analyzed data and wrote the paper; T.L. A.M.V, L.D.W. and S.M.S. performed experiments.

**Conflict of interest.** The authors declare no conflict of interest.

## Introduction

The t(15;17)(q22;q11.2) translocation produces the *PML-RARA* fusion gene, which has been shown to be the initiating event for acute promyelocytic leukemia (APL, FAB M3) in several mouse models of the disease[1-3]. The long latency to APL development in these models (frequently over 1 year) suggested the requirement for secondary/cooperating events in leukemogenesis[4-8]. In our murine model, a human *PML-RARA* cDNA is knocked into the murine cathepsin G locus (*Ctsg-PML-RARA*; also referred to as *mCG-PR*) [3]. We and others recently demonstrated that bone marrow cells from young, pre-leukemic *Ctsg-PML-RARA* mice have increased colony forming and replating ability *in vitro*, and have a competitive advantage over wild type cells *in vivo*[9-13], implying that *PML-RARA* expression alone can alter hematopoiesis. Collectively, these results suggest that *PML-RARA* initially acts in a multipotent progenitor cell to increase self-renewal; the molecular pathways underlying this activity are not yet fully understood.

The Notch signaling cascade is a well-characterized pathway that is important for the self-renewal of several types of stem cells, including HSPCs (reviewed in Sandy et al[14]). Hematopoietic malignancies frequently demonstrate abnormalities in the Notch cascade, most notably T lymphoblastic leukemias (T-ALLs), where *NOTCH1* mutations are found in approximately 60% of cases[15]. The Notch pathway is also an attractive candidate for involvement in APL, based on several lines of evidence: *1)* primary human APL samples overexpress the Notch ligand *JAG1*, compared to other AML subtypes[16,17], to promyelocytes[17,18] and to CD34+ cells[19], *2) JAG1* mRNA and protein increase after *PML-RARA* expression is induced in the PR-9 cell line[19], *3) JAG1* is rapidly downregulated by all trans retinoic acid (ATRA) treatment of NB4 cells and primary APL blasts [19,20], and *4) PML-RARA* expression activates a *Hes1* promoter reporter construct, a known target of Notch signaling[19]. To date, there are no published studies of the role of JAG1 and Notch signaling in APL pathogenesis.

In this report, we show that Notch signaling is important for the pathogenesis of APL. We provide bioinformatic evidence for activation of a known Notch signature in both human APL cells, and in pre-leukemic Kit+Lin−Sca1+ (KLS) cells from *Ctsg-PML-RARA* mice. Using both pharmacologic and genetic approaches, we also found that Notch blockade abrogates the enhanced self-renewal observed in pre-leukemic cells from *Ctsg-PML-RARA* mice, but not in bone marrow cells expressing the *AML1-ETO* fusion gene. Finally, we show that dependence on Notch signaling is retained in a subset of fully transformed murine APL tumors. These findings suggest that Notch signaling is a key downstream effector of *PML-RARA*, with roles in both early leukemogenesis and in fully transformed cells.

## Methods

### Expression microarrays and GSEA

Human and murine expression arrays have been previously described elsewhere [17,21,22] and have been deposited in GEO: GSE12662, GSE10358 and GSE24728. Gene set enrichment analysis (GSEA) was performed using phenotype permutation analysis with signal to noise

gene ranking (http://www.broad.mit.edu/gsea). Gene sets were considered to be significantly enriched at an FDR < 0.25, according to **http://www.broadinstitute.org/gsea/index.jsp.**

## RNA-seq analysis

RNA-seq data from 176 AML patients with known FAB subtypes were obtained as part of The Cancer Genome Atlas project and analyzed using a custom pipeline based on the Tophat and Cufflinks software packages[23]. Sequencing reads were mapped with TopHat (version 1.3.1) to the reference genome sequence (GRCh37) using ENSEMBL human gene annotations (release 58). Accepted read alignments were processed with Cufflinks (version 1.1.0) using default parameters to filter alignments to rDNA and mitochondrial sequences and calculate fragments per kilobase of exon per million fragments mapped (FPKM) values for each transcript isoform. An aggregate FPKM value for *JAG1* was calculated by summing the values obtained for three annotated *JAG1* isoforms.

## Cell lines and antibodies

The PR-9 cell line was a kind gift of P. Pelicci of the European Institute of Oncology, Milan, Italy; *PML-RARA* expression was induced as described [17]. OP-9 cells were purchased from ATCC. Cells, including primary APL samples, were lysed directly in SDS sample buffer (final concentration of 0.83% SDS). Antibodies raised against Rara (C-20, Santa Cruz), Jag1 (H-114, Santa Cruz), cleaved-Notch1 Val1744 (Cell Signal Technologies) and actin (C-4, Millipore) were utilized for western blots. Murine APL cells were stained with either FITC-αGr-1 or APC-αc-Kit (eBioscience) for flow cytometry. For intracellular staining, cells were fixed and permeabilized following surface staining, using the FoxP3 Intracellular Staining Buffer Set (eBioscience), and then stained for PEJag-1 (eBioscience).

## Mice

The *Ctsg-PML-RARA* mice have been previously described[3], and were back-crossed to the C57BL/6 strain (Taconic) for at least 10 generations. 129SvJ/B6 F1 hybrid animals were generated by mating 129SvJ males with C57BL/6J females (both parental strains obtained from Jackson Laboratory). All animal care and experimental protocols were done in accordance with institutional guidelines and approved by the Animal Studies Committee of Washington University School of Medicine in accordance with current NIH policy.

## Retroviral transductions of murine bone marrow cells

The MSCV-DNMAML1-GFP plasmid [24] was provided by Dr. Jon Aster; MSCV-DNMAML1-YFP was generated by replacing GFP with YFP. The MSCV-*Jag1* plasmid [25] was a gift from Dr. Rafael Kopan. Dr. Timothy Graubert provided MSCV AML1-ETO ires GFP[26]. Retrovirus production, transduction of murine bone marrow, and transplants were performed as described previously [8,27]. GFP positive or GFP/YFP double positive cells were sorted into liquid media 24-48 hours after spinfection and then plated into methylcellulose media, as previously described [13].

### Cryopreserved murine APL samples

Cryopreserved murine APL samples were collected and frozen as described previously[28]. Cells were rapidly thawed and resuspended in RPMI media supplemented with 10% FBS, IL-3 (6 ng/mL), IL-6 (10 ng/mL) and SCF (100 ng/mL). For experiments with compound E and compound IX (Calbiochem), 200,000 cells were cultured with or without inhibitors for 48 hours. Cells were then plated in methylcellulose media and colony formation was scored at 7 days. Alternatively, cells were thawed and grown on irradiated (2000 cGy) OP9 stromal cells in the presence of murine cytokines TPO (10 ng/ul), SCF (100 ng/ul), IL3 (6 ng/ul) and FLT3-ligand (10 ng/ul) for 24 hrs. Cells were then treated with Compound E (2 μM) for 48 hours. Dead cells were removed by density centrifugation (Histopaque 1083, Sigma) and lysed in SDS sample buffer as described above. For transduction assays, cells were first cultured on OP9 stroma, and non-adherent cells were collected and transduced with MSCV-DN-MAML1-GFP or GFP control retroviral supernatants. Cells were analyzed for GFP positivity after 48 hours, and 1 million cells were engrafted in syngeneic mice via retro-orbital injection. Mice were sacrificed at the first sign of illness (typically 4 weeks).

## Results

### JAG1 is dysregulated in APL cells

We previously reported a signature of genes with altered expression in APL cells; the Notch ligand Jagged-1 (*JAG1*) was among this set[17]. Using gene expression profiling, we examined the expression of *JAG1* in bone marrow samples collected from a set of 180 *de novo* AML patients[21] and in purified normal myeloid populations (CD34+ cells, promyelocytes, and neutrophils) from 5 normal human bone marrow samples[17]. *JAG1* expression is somewhat variable in AML samples, but is expressed at significantly higher levels in FAB M3/APL samples compared to all other FAB subtypes, as well as normal myeloid populations (**Figure 1A** and data not shown). This pattern of *JAG1* expression was validated by RT-PCR in a subset of the patients (Figure S1) and by using RNA-seq data from 176 AML patients (that completely overlap with the patient cohort with microarray expression studies) with known FAB subtypes that were part of The Cancer Genome Atlas (TCGA) project on AML (**Figure 1B).** Further validation was also performed using an independent set of *de novo* AML samples from the Cancer and Leukemia Group B (CALGB) Cooperative group (**Figure S1)**. In addition, genes encoding the components of Notch activation, including the Notch receptors and various genes involved in processing and transcriptional activation are also expressed in APL cells, indicating that the essential components of Notch signaling are present in APL cells **(Figures 1C and S2)**.

Although an association between *FLT3-ITD* and *JAG1* expression has been noted in other studies[29,30], there was no difference in *JAG1* expression within APL cases when segregated by *FLT3-ITD* status (Figure 1D). Using both expression platforms (microarray and RNA-seq) we also found that *JAG1* was consistently over-expressed in APL cells relative to other fusion oncogene-driven AML cells (**Figures 1E and 1F)**. Similar findings were observed for another Notch ligand, *DLL1*, although the levels of expression are much lower than that observed for *JAG1* **(Figures 1G and 1H)**. These results are similar to multiple other AML gene expression profiling studies [16,18,19,29-31], and strongly suggest that overexpression of

*JAG1 (*and *DLL1)* is a characteristic of APL. Because *JAG1* is a well-characterized Notch ligand, and the dominant Notch ligand in APL cells, we decided to investigate the role of *JAG1* and Notch signaling in APL.

## Bioinformatic evidence that Notch signaling is present in APL cells

Increased Notch signaling is a major component of T-ALL due to activating mutations in *NOTCH1* [15], and several studies have reported dysregulated gene expression due to this aberrant Notch signaling in T-ALL cells [32-34]. We used gene set enrichment analysis (GSEA) with three Notch signatures identified in T-ALL, including 1) 'GSI-Notch' (comprised of genes whose expression changes in T-ALL cells upon treatment with gamma secretase inhibitors [32]), 2) 'Notch-Targets' (comprised of genes previously reported to be transcriptional targets of NOTCH1 [33]), and 3) 'Notch-GSIDNMAML' (comprised of transcriptional targets that are inhibited by both GSI treatment and DNMAML expression [34]). All these signatures are enriched in APL cells compared to normal promyelocytes (**Figures 2 and S3**), providing strong bioinformatic evidence that Notch signaling is activated in human APL cells.

## Notch signaling is present in APL cell lines

The PR-9 cell line, which contains a zinc inducible *PML-RARA* cDNA, is frequently used to study early events following *PML-RARA* expression. Consistent with previous reports[19], JAG1 protein levels increased following induction of *PML-RARA*; JAG1 protein was detected by intracellular flow cytometry but not by conventional extracellular staining (**Figure S4**). JAG1 levels decreased following treatment with ATRA (data not shown), which has been reported previously [20]. Cleaved Notch-1 protein levels peaked soon after JAG1 protein levels reached a maximum (**Figure 3A**), suggesting that Notch activation is a direct result of JAG1 upregulation. To determine whether downstream transcriptional targets of Notch signaling are induced with *PML-RARA* expression, we examined gene expression data from resting and $ZnSO_4$-treated PR-9 cells. We found that the Notch signatures enriched in primary APL cells (**Figure 2**) have overall increased expression at 8-16 hours after zinc induction of *PML-RARA* expression (**Figure 3B and Figure S5**). Several known Notch targets (*HSPC111, TASP1, PHB, GSPT1*) in T-ALL [32-34], as well as *JAG1*, showed increased expression over time **(Figure S6)**. These results demonstrate that activation of Notch signaling occurs as a consequence of *PML-RARA* induction in PR-9 cells, where it activates a similar transcriptional program to that found in primary APL cells.

## Jag1 overexpression and Notch signaling are found in a murine model of APL

We next examined Notch signaling in the previously described *Ctsg-PML-RARA* mouse model of APL[3], which produces a lethal leukemia that responds to ATRA both *in vitro* and *in vivo* and which has a similar gene expression signature as human APL [17,35]. We examined the expression of *Jag1* using previously published gene expression profiles of 21 murine APL samples and wildtype $Lin^{-}Sca^{+}$ (LS) progenitor cells undergoing 7 days of G-CSF induced myeloid differentiation (**Figure 4A**) [35]. *Jag1* expression was detectable in the majority of tumors and was higher than that of Lin−/Sca+ cells (d0) or promyelocytes (d2). Additionally, *Jag1* mRNA levels remained below a signal intensity of 500 for the entire 7

day *in vitro* differentiation, suggesting that *Jag1* is not significantly expressed at any stage of normal myeloid development, similar to the expression data obtained with normal human myeloid cells (**Figure 1A-B**). We then tested for expression of Jag1 protein and activated Notch1 in primary murine APL tumor samples. Cleaved Notch1 was detected by western blotting and the activated Notch signal in these tumors was sensitive to GSI inhibition (**Figure 4B**). Further, Jag1 protein was detected by flow cytometry in all tumors tested, with a range of 19% to greater than 90% of the cells containing Jag1 (**Figure 4C and** Supplemental **Table 1**). Similar to induced PR-9 cells, Jag1 protein was detectable only by intracellular (and not extracellular) flow cytometry (**Figure S7)**. Therefore, like human APL and APL cell lines, murine APL cells both overexpress Jag1 and have activated Notch signaling, providing a rationale for utilizing the *Ctsg-PML-RARA* model to investigate the role of Notch signaling in leukemogenesis.

## Overexpression of Jag1 does not lead to increased self-renewal in vitro or AML in in vivo

We next sought to determine if overexpression of *Jag1* is sufficient for transformation by transducing wildtype C57BL/6 bone marrow cells with retroviruses expressing either a murine *Jag1* cDNA [25] or a GFP cDNA (**Figure 5A**). We found that *Jag1* overexpression did not lead to increased colony forming ability compared to GFP or mock-transduced controls in serial plating experiments (**Figure 5B**), and the percentage of GFP-expressing cells was similar in cultures of *Jag1* and GFP transduced marrow (**Figure 5C)**. We transplanted irradiated syngeneic host animals with either *Jag1* or GFP transduced bone marrow. GFP positivity in the peripheral blood was similar in the *Jag1* and GFP control groups over time (**Figure 5D-E)**. After 300 or more days post-transplant, none of the mice had developed leukocytosis (**Figure 5F-G**), anemia, thrombocytopenia or splenomegaly (data not shown). Collectively, these results suggest that while *Jag1* overexpression and Notch signaling are present in APL cells, *Jag1* overexpression in wildtype cells is not sufficient to induce self-renewal or initiate APL.

## Inhibition of Notch signaling reduces self-renewal in marrow cells from Ctsg-PML-RARA mice

We next investigated the role of Notch signaling in *PML-RARA* induced leukemogenesis. Marrow cells from pre-leukemic *Ctsg-PML-RARA* animals have increased colony forming ability *in vitro* and a competitive advantage over wildtype cells *in vivo* [9-13]. We and others[10,22] have shown that *PML-RARA* is expressed in the KLS cells of these mice. To determine whether Notch signaling is activated in *Ctsg-PML-RARA* KLS cells, we performed GSEA on KLS cells from young (6-8 week) pre-leukemic *Ctsg-PML-RARA (PR)* mice and wildtype (WT) controls; the Notch target signature [33] that was enriched in human APL (**Figure 2**) and induced PR-9 cells (**Figure 3**) was also present in KLS cells derived from *Ctsg-PML-RARA* animals (**Figure 6A and Figure S8)**.

To examine the functional role of Notch signaling in early leukemogenesis, we cultured bone marrow cells from young (6-8 week old) pre-leukemic *Ctsg-PML-RARA* mice (or wildtype C57BL/6 mice) in methylcellulose media supplemented with IL3, IL6, and SCF, and assessed colony formation in the presence of GSIs (compound E or compound IX) or DMSO control. As expected, marrow derived from wildtype C57BL/6 animals did not

serially replate; this was not influenced by either compound [36] (**Figure S9**). In contrast, after 1 week in culture, colony formation by *Ctsg-PML-RARA* bone marrow grown in media containing GSIs was significantly reduced (**Figure 6B and 6C**). This effect was further enhanced after 2 rounds of replating. These data suggest that Notch signaling may be partially responsible for the abnormal replating phenotype observed with *Ctsg-PML-RARA* progenitor cells.

We further validated the role of Notch signaling in serial replating with a dominant-negative fragment of Mastermind-like 1 (MAML1) fused to GFP; this portion of MAML contains the domains necessary to interact with cleaved Notch, but lacks the domains needed to recruit transcriptional machinery [24,37,38]. We retrovirally transduced wildtype C57BL/6 or *Ctsg-PML-RARA* bone marrow with either DNMAML-GFP or GFP control virus, sorted GFP+ cells to >95% purity, and plated them in methylcellulose media. DNMAML-transduced *Ctsg-PML-RARA* bone marrow significantly reduced colony-forming activity at weeks 1, 2 and 3, similar to that observed with pharmacological inhibition of Notch signaling (**Figure 6D-E**). In contrast, expression of DN-MAML1 had no impact on the growth and colony forming activity of the *RUNX1-RUNX1T1/AML1-ETO* fusion in mouse bone marrow cells (**Figure 6F**), suggesting that the ability of Notch to regulate self-renewal is not a shared feature among all AML-initiating oncogenic fusion proteins. As previously shown[36], genetic inhibition of Notch signaling did not impair colony formation by wildtype cells (**Figure S9**).

## Inhibition of Notch signaling reduces colony formation by primary murine APL cells

To investigate the role of Notch signaling in murine APL tumors, we cultured cells in the presence of DMSO, ATRA, compound E or compound IX for 48 hours in liquid culture, followed by 7 days in methylcellulose without inhibitors **(Figure 7A)**. As a control, 129/B6 F1 total bone marrow was subjected to the same assay. Of 13 tumors evaluated, 9 formed colonies using vehicle control conditions (Supplemental **Table 1**). As expected, ATRA exposure resulted in significantly fewer colonies in 9/9 tumors (Figures 7B and S10 for individual tumors and Figure 7C for summarized data). Six of the 9 assayed tumors formed significantly fewer colonies after exposure to GSIs, although neither compound E nor compound IX significantly altered colony formation by wild type marrow cells (**Figure S9**). In many of the GSI responsive tumors, the reduction in colony formation was similar to that observed with ATRA treatment. On average, exposure to compound E or compound IX resulted in colony formation that was 62.2% (SD 35.0%) or 45.2% (SD 24.4%) of the DMSO controls, respectively. In addition, the degree of inhibition of colony formation by compound E and compound IX were significantly correlated ($p<0.01$ for compound E, and $p<0.001$ for compound IX, **Figure 7D**), which suggests that Notch signaling plays a role in the survival or self-renewal of some APL tumors *in vitro*. Exposure of four different primary human APL samples to compound IX (using a recently characterized *in vitro* stromal co-culture system [39]) also resulted in a significant dose-dependent inhibition of growth in 2 of 4 samples (**Figure S11**).

Finally we investigated whether Notch signaling is required for growth of APL cells *in vivo*. We transduced 6 tumors (including samples that were either susceptible or resistant to GSIs) with MSCV-DNMAML1-GFP or a GFP control virus, and engrafted 1 million transduced

cells into syngeneic 129/B6 host animals 48 hrs after transduction (**Figure 7E-F**). At the time of engraftment, the tumors had similar degrees of GFP positivity (DNMAML1-GFP, mean, 37.8%, range, 27%-47.8%; vs. GFP control, mean, 28.5%, range, 18%-48.6%). GFP positivity in the CD117+ splenic tumor population was then assessed when the animals became moribund, typically at about 4 weeks. In some animals engrafted with DNMAML1-GFP tumors, very few of the recovered tumor cells were GFP positive, despite having high GFP levels at the time of engraftment. These results suggest that inhibition of Notch signaling with DNMAML1 reduced the ability of some tumors to expand in mice, which could be caused by reduced engraftment, diminished growth, and/or reduced self-renewal of cells expressing this inhibitor.

## Discussion

In this report we extended the observation that the Notch ligand *JAG1* is overexpressed in APL cells [16,18,19,29-31] by performing a functional study of the role of Notch signaling in both human and murine APL samples. Not only do human APL cells express the core components of the Notch pathway, but they also show enrichment in Notch signaling, and are sometimes sensitive to gamma secretase inhibitors. Pharmacological and genetic inhibition of Notch signaling resulted in a loss of serial replating by *Ctsg-PML-RARA* progenitor cells, demonstrating that the Notch pathway is necessary for the increased self-renewal found in this mouse model. Finally, most murine APL samples retain sensitivity to Notch inhibition *in vitro* and *in vivo*, suggesting that Notch signaling is also relevant for the growth or survival of some fully transformed APL cells.

Our data suggest that *JAG1* overexpression is characteristic of APL, but not absolutely restricted to this AML subtype (Figure 1A), suggesting that there may be multiple distinct pathways leading to high *JAG1* expression. Despite the data presented here and elsewhere [19,20], the mechanism by which *PML-RARA* regulates *JAG1* expression and Notch activation is unclear. Three independent whole genome chromatin immunoprecipitation studies failed to find evidence of PML-RARA binding in the *JAG1* promoter in either cell lines or primary APL cells [40-42], despite the presence of an inverted repeat and combined direct repeat-PU.1 motif sites similar to known PMLRARA binding sites [20,40] in the proximal *JAG1* promoter. In addition, cotransfection of a *JAG1* promoter-reporter construct (1.5 kb of 5' flanking sequence) with *PML-RARA* did not result in *JAG1* promoter activation (data not shown). Clearly, the relationship between *PML-RARA* expression and *JAG1* activation is indirect, and additional experiments will be required to define this pathway.

We initially hypothesized that overexpression of *Jag1* alone would be leukemogenic in wildtype marrow, since other studies have found that *Jag1* overexpression in stromal cells results in myeloproliferation [43,44]. Surprisingly, we found no detectable hematopoietic alterations in mice transplanted with *Jag1* overexpressing marrow cells, nor did we observe increased colony formation nor replating of *Jag1* transduced cells *in vitro*. Importantly, over-expression of *Jag1* in our study was confined to hematopoietic cells rather than the bone marrow stroma. Further, in both of the stromal overexpression studies, increased *Jag1* expression was caused indirectly by another genetic event[35,43].

Our work adds to the increasing body of literature demonstrating that the cellular context, as well as the level of Notch activation, can influence the resulting phenotype[14,45-48]. Klinakis et al [49] demonstrated that loss of Notch signaling results in an expansion of GMPs and eventual development of a CMML-like myeloproliferative disease, which can be rescued by reacquisition of Notch signaling. However, the leukemia-initiating cell in these CMML models may be a more differentiated progenitor than that of *Ctsg-PMLRARA* mice, where *PML-RARA* is first expressed in the KLS compartment [10,22]. Our findings suggest that Notch signaling induced by *PML-RARA* can promote the self-renewal of myeloid progenitors without affecting the downstream commitment to the myeloid lineage, since preleukemic *Ctsg-PML-RARA* animals initially have increased self-renewal in the absence of any block in myeloid differentiation[9-13]. The results presented here also suggest that canonical Notch activation and high *JAG1* expression may be specific for *PML-RARA*, since these findings were not observed in cells expressing *AML1-ETO* (Figs 1 and 6). In addition, recent studies have suggested that the Notch pathway may behave as a tumor suppressor in some myeloid disorders, including those driven by MLL-AF9[50]. Long-term experiments designed to determine the consequences of genetic inhibition (or promotion) of Notch signaling on leukemogenesis in *Ctsg-PML-RARA* mice are currently in progress, and should further clarify the role of this pathway for leukemic transformation.

Not only is Notch signaling active in pre-leukemic *Ctsg-PML-RARA* stem/progenitor cells, but some fully transformed APL samples (both human and murine) are sensitive to Notch inhibition. Surprisingly, murine tumors that were either sensitive (3430 and 13499) or resistant (3673 and 2972) to GSIs in our replating assays (Figure 7 and Supplemental Table 1) had constitutive cleavage of Notch1 that was blocked by GSI treatment (Figure 4B). There was no correlation between *Jag1* expression and GSI sensitivity in these tumors, suggesting that some APL tumors rely on Notch1-independent mechanisms for their progression. The *Myc* gene is a known target of Notch signaling that is required for *PML-RARA* induced APL[7] ; it is possible that some APL tumors utilize the Notch pathway to activate *Myc*, while others find alternative pathways (e.g. gene amplification). In addition, our previous studies have identified significant genetic heterogeneity among APL[8] tumors, with different mutations cooperating with *PML-RARA* to cause APL in each tumor.

In summary, we have demonstrated a role for Notch signaling in the pathogenesis of APL. The expression of *JAG1* and activation of Notch signaling are common events in the development of both murine and human APL. Collectively, our results demonstrate that activation of Notch signaling is at least partially responsible for increased self-renewal in progenitors that express *PML-RARA*. We also show that dependence on Notch signaling is retained by some tumors *in vitro* and *in vivo*. Further investigation will be necessary to demonstrate the precise requirements for Notch signaling during *PML-RARA* induced leukemogenesis. Regardless, the demonstration that this pathway is active in some APL tumors may result in new approaches for treating patients with relapsed or refractory APL.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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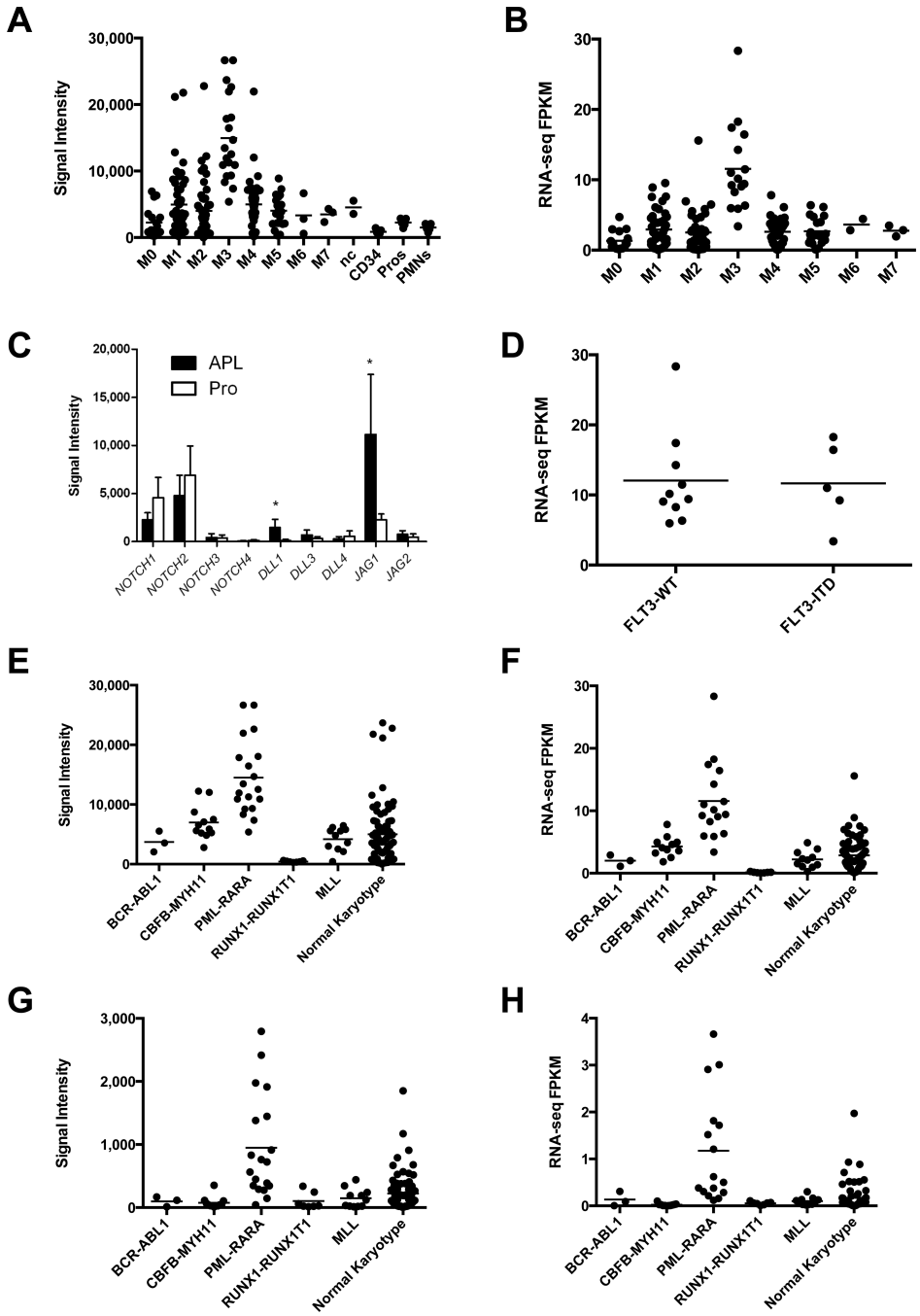



**Figure 1. Expression of *JAG1* and Notch signaling components in APL, other AML and normal myeloid cells**

A). Expression array-derived data for *JAG1* in a set of 180 de novo AML samples, and flow sorted normal CD34+ cells, promyelocytes, and neutrophils. B). *JAG1* expression data from 178 *de novo* AML samples with known FAB subtypes obtained from The Cancer Genome Atlas RNA-Seq database for AML. Each data point in A and B represents one patient sample or one normal sample. C). Expression of Notch receptors and ligands in APL (closed bars) and normal promyelocytes (open bars). D). Expression of *JAG1* by RNA-seq in APL cases separated by *FLT3-ITD* status. E and F). Expression of *JAG1* in fusion-driven AML

samples by expression arrays (E) and RNA-seq (F); G and H). Expression of *DLL1* in fusion-driven AMLs by expression arrays (G) and RNA-seq (H). For panels A, B and E-H, mean expression in M3/PML-RARA cases is statistically different than all other comparison groups as determined by one-way ANOVA with Tukey's multiple comparison test (alpha 0.05). For panel C, *, $p<0.05$.

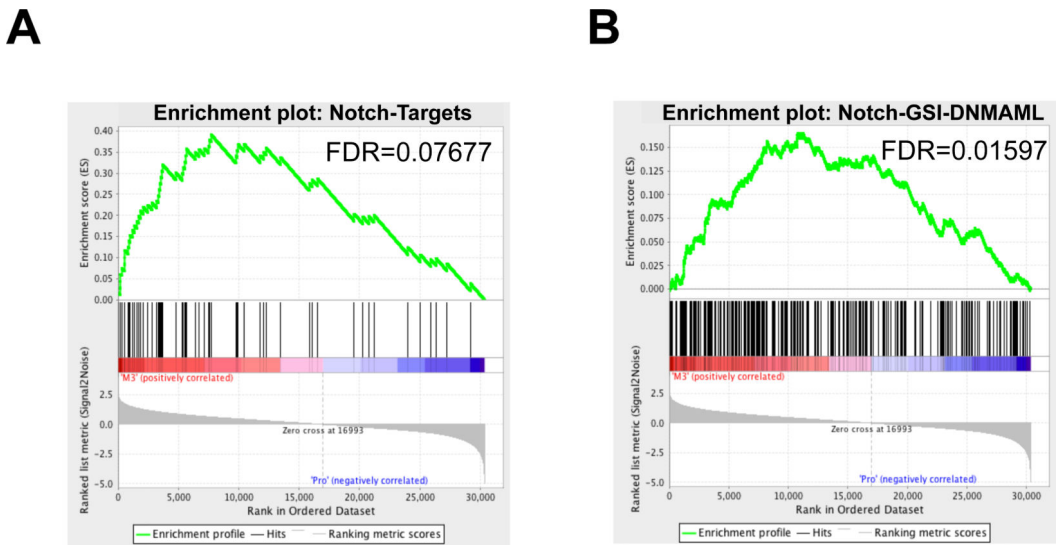



**Figure 2. Notch target gene signatures are enriched in human APL cells**

GSEA plots of 22 APL samples compared to 5 normal promyelocyte (Pro) samples demonstrate significant enrichment in APL of A) a previously described set of Notch transcriptional targets in T-ALL[33] and B) a previously published set of transcriptional targets of Notch whose expression is inhibited by both gamma secretase inhibitor treatment and DNMAML expression[34]. Normalized enrichment scores-A: 1.6523; B: 1.1921.

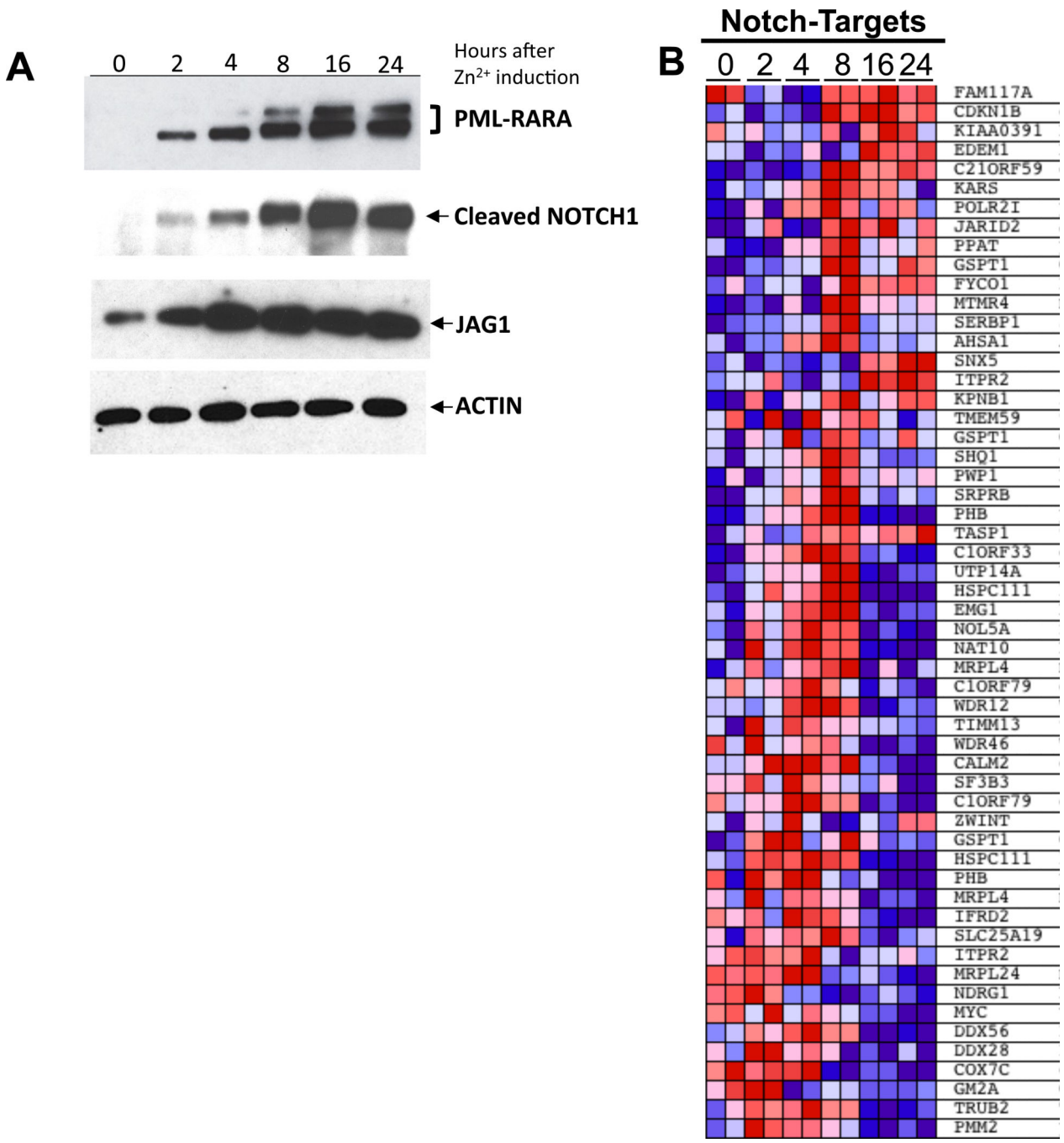



**Figure 3. Increased JAG1 and activation of Notch signaling are found in induced PR-9 cells**

A). Western blots showing protein levels of PML-RARA (using an anti-RARA antibody), JAG1, cleaved Notch-1 (ICN1), and actin in PR-9 cells at 0, 2, 4, 8, 16 and 24 hours after $Zn^{2+}$ induction. Data are representative of three independent inductions. B) Heat map showing induction of the Notch-Targets[33] signature in PR-9 cells 0, 2, 4, 8, 16 or 24 hours after $Zn^{2+}$ induction of *PML-RARA* expression. Panels A and B were generated from different induction experiments.

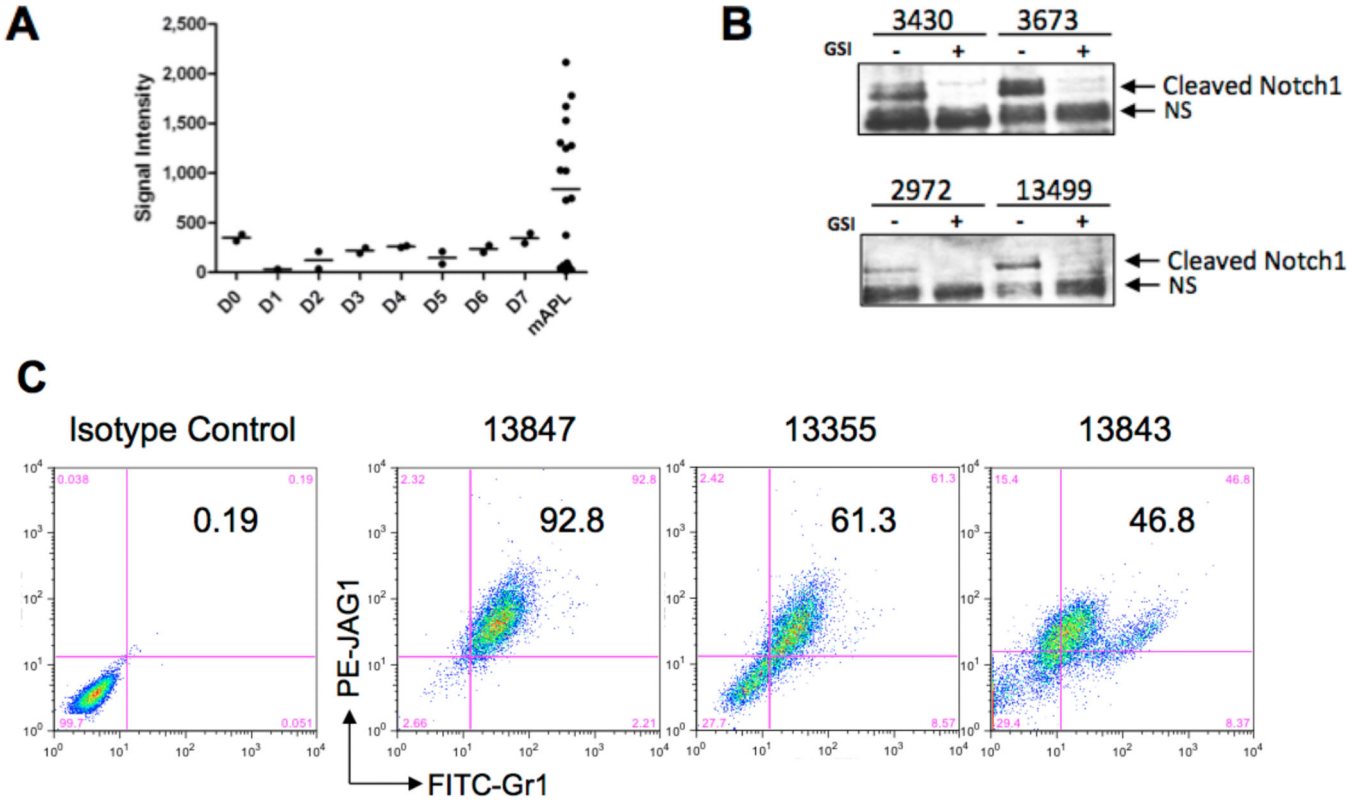



**Figure 4. Jag1 and activated Notch signaling are found in murine APL samples**

A). Microarray expression data for *Jag1* in murine $Lin^{-}Sca^{+}$ cells undergoing *in vitro* differentiation with G-CSF, and in 22 murine APL samples. Promyelocytes are highly enriched on days 2 and 3 of *in vitro* differentiation[33] B) Western blot showing cleaved Notch1 in murine APL tumors treated for 48 hrs with either DMSO vehicle, or 2 μM compound E. The faster migrating band is non-specific (NS) and not sensitive to GSI treatment. 13499 and 3430 are GSI-sensitive tumors (Figure 7, Supplemental Table 1); 2972 and 3673 are insensitive. C). Intracellular flow cytometry detection of Jag1 in representative murine APL samples. Numbers correspond to percentage of cells co-expressing Gr-1 and Jag1.

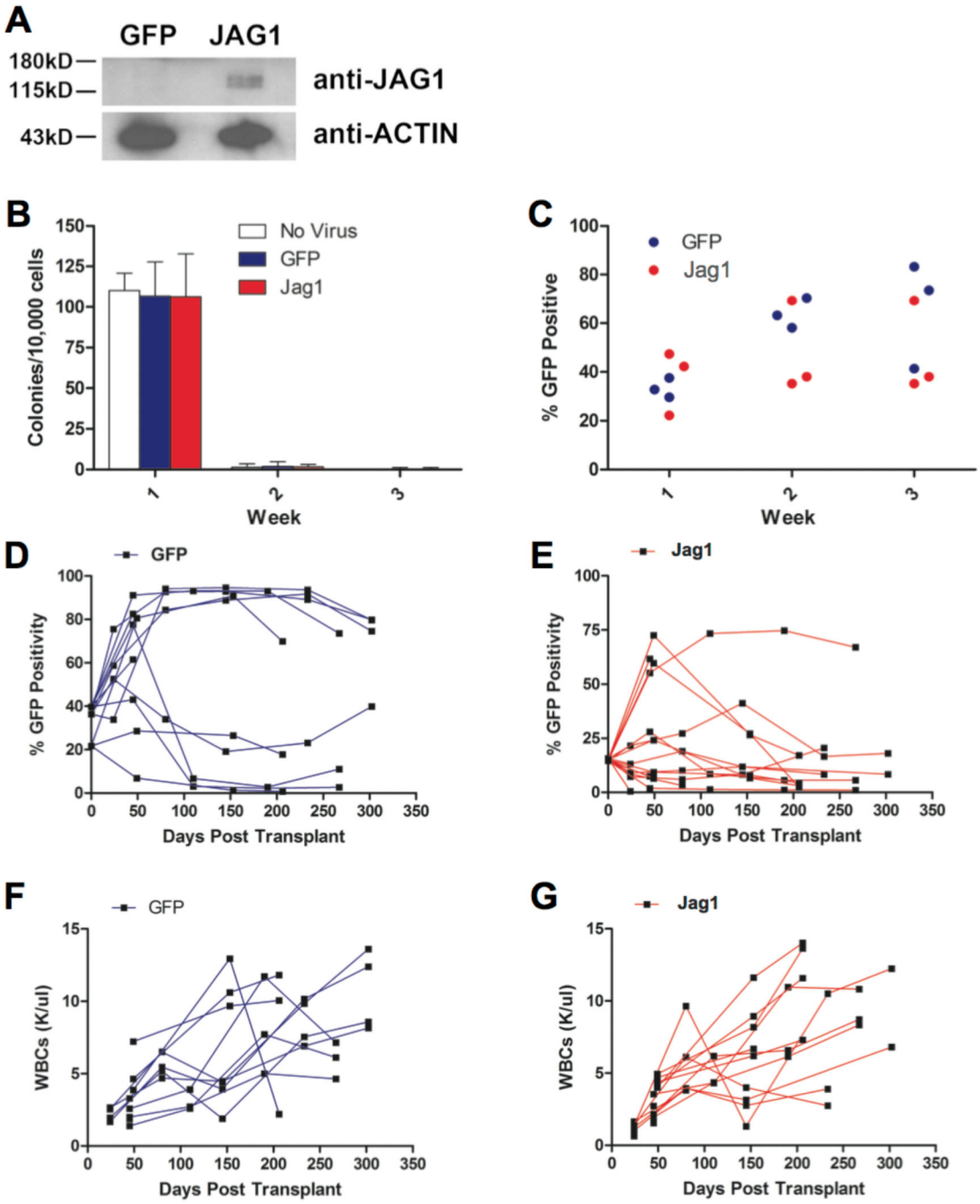



**Figure 5. Jag1 overexpression does not lead to changes in self-renewal *in vitro* nor cause AML *in vivo***

A) Western blot analysis of GFP sorted bone marrow transduced with either control MSCV-IRES-GFP virus (GFP) or *Jag1* expressing virus (Jag1), expanded for 1 week on OP-9 stroma and sorted for GFP positivity, demonstrating overexpression of Jag1 in the transduced cells. B) Colony formation for wildtype C57BL/6 marrow that was mock transduced, or transduced with either MSCV-*Jag1*-IRES-GFP or MSCV-IRES-GFP control, and plated in methylcellulose media containing IL-3, IL-6 and SCF. Data shown are means

+/− standard deviations. C) GFP positivity in marrow transduced with *Jag1* virus or GFP vector control during 3 weeks of growth in methylcellulose media. Each data point represents the average of three plates. There was no statistically significant difference in % GFP+ cells at any time point. (D, E) GFP positivity in the peripheral blood of mice transplanted with whole marrow transduced with either the GFP control (panel D) or the *Jag1* vector (panel E). (F, G) Peripheral blood WBC counts in animals transplanted with the GFP control (panel F) or the *Jag1* vector (panel G) transduced marrow.

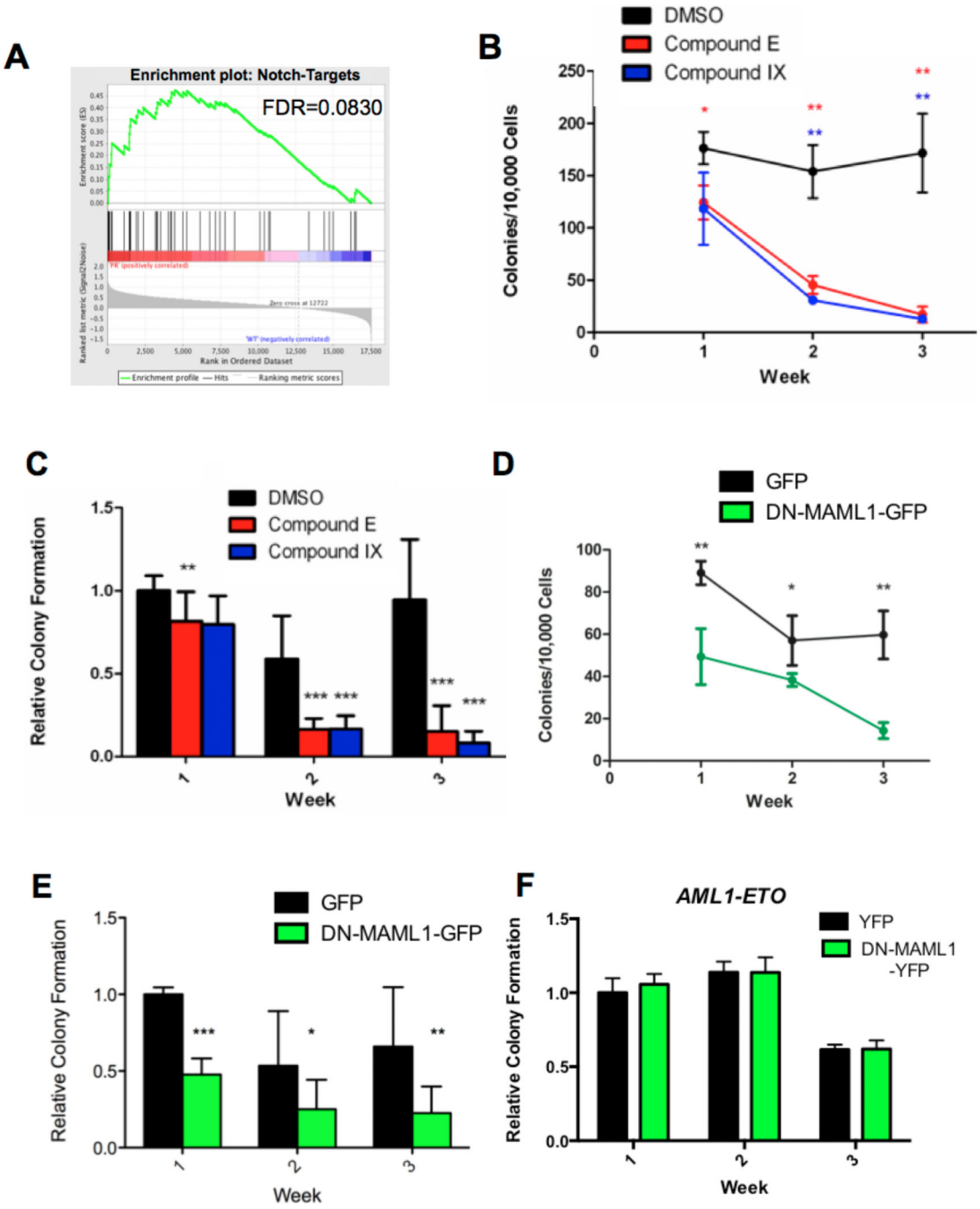
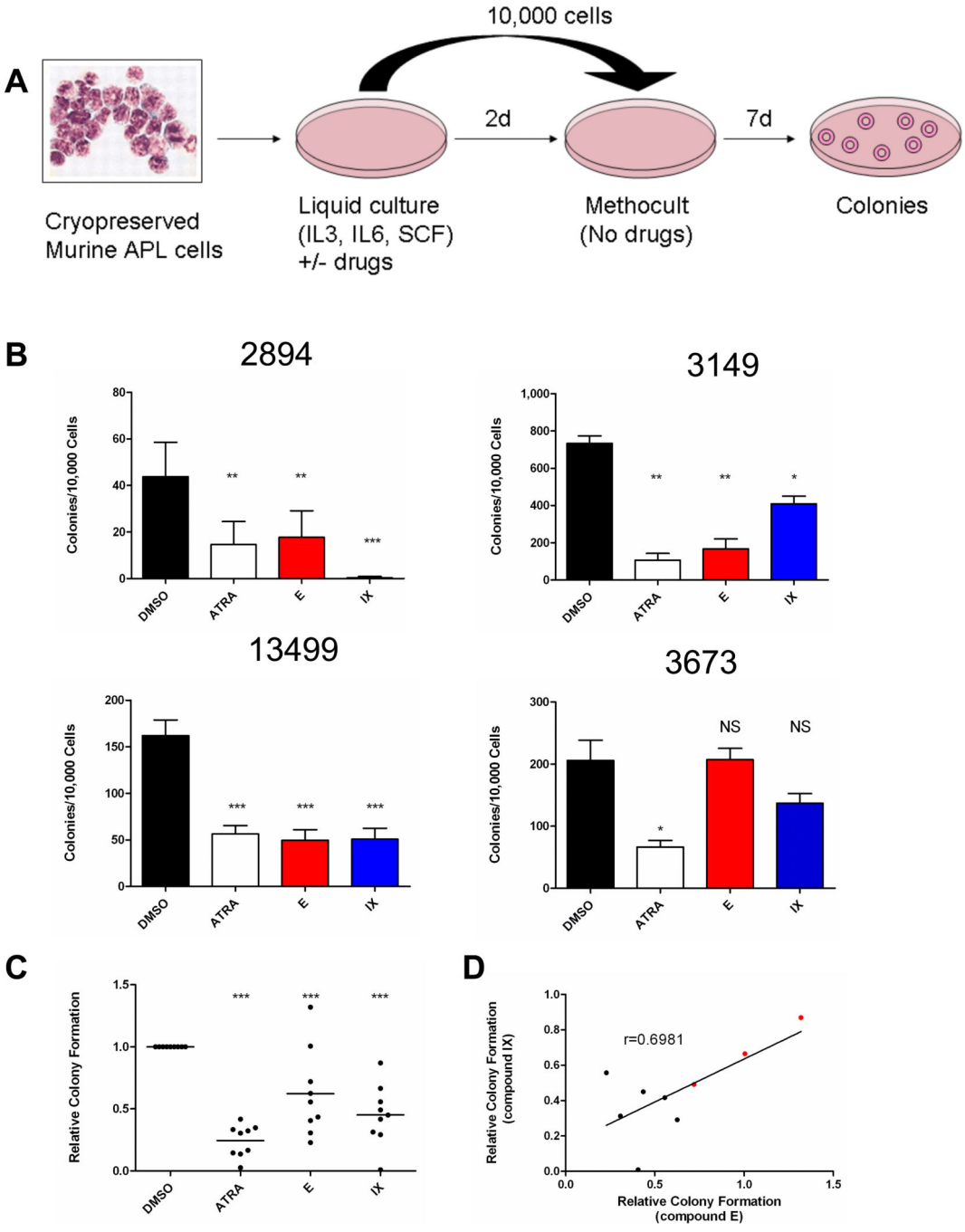



**Figure 6. Notch signaling in pre-leukemic progenitor cells from *Ctsg-PML-RARA* mice**

A) GSEA plot of 6 KLS samples sorted from pre-leukemic *Ctsg-PML-RARA* (PR) mice compared to 4 wildtype C57BL/6 KLS (WT) samples. The data demonstrates significant enrichment of the previously described Notch-Targets (normalized enrichment score 1.5395)[33] signature in *Ctsg-PML-RARA* KLS cells. B) Colony formation from total bone marrow cells from a representative *Ctsg-PML-RARA* mouse plated in methylcellulose media containing IL-3, IL-6 and SCF and supplemented with either DMSO, 2 μM compound E, or 25 μM compound IX. Colonies were counted and cells replated weekly for 3 weeks. Data

shown are means +/− standard deviation. C) Summarized colony formation data from 5 independent *Ctsg-PML-RARA* marrow samples treated as shown in C. Data shown are means +/- standard deviation, and are normalized to the week 1 DMSO treated control colony counts. D). Colony formation from total bone marrow from a representative *Ctsg-PML-RARA* animal transduced with either MSCV-DN-MAML1-GFP or a GFP control virus, sorted for GFP+ cells, and plated in methylcellulose media supplemented with IL-3, IL-6 and SCF. E) Summarized colony formation data from 4 independent *Ctsg-PML-RARA* marrow samples treated as shown in Panel D. Data shown are means +/− standard deviation and are normalized to the week 1 GFP control. In all experiments shown, cells from each animal were plated in triplicate for each condition. Summarized data are representative of 3-5 animals per group. F. Relative colony formation from total bone marrow cells transduced with *AML1-ETO* and either YFP or DN-MAML1-YFP. GFP/YFP double positive cells were sorted and plated in methylcellulose media supplemented with IL-3, IL-6 and SCF. A representative example from 3 independent transductions is shown. In all graphs, one asterisk (*) indicates $p<0.05$, two asterisks (**) indicates $p<0.01$, and three asterisks (***) indicates $p<0.001$.



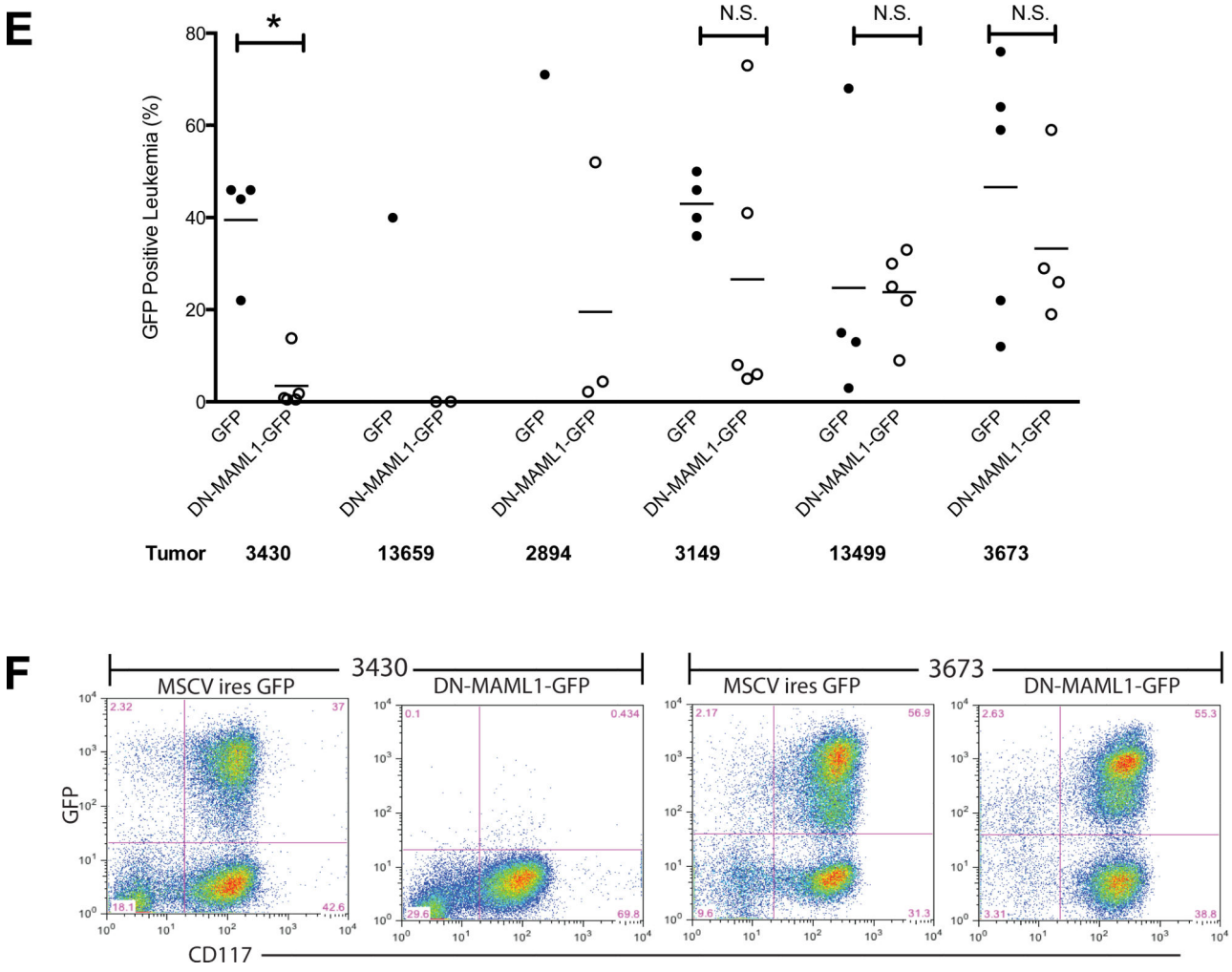



**Figure 7. Inhibition of Notch signaling in murine APL cells *in vitro* and *in vivo***

A). Schematic diagram of experimental protocol. B). Representative colony formation data for 4 murine APL samples treated for 48 hours with 1 μM ATRA, 2 μM compound E, 25 μM compound IX, or DMSO vehicle control, and then plated in methylcellulose media supplemented with IL-3, IL-6 and SCF in triplicate, as described in Panel A. Data bars are the mean numbers of colonies per 10,000 cells plated. Error bars represent standard deviations. C). Summarized data for 9 independent tumors treated as described in Panels A and B. Each data point represents the average of three plates for a single tumor, normalized to the average colony numbers for DMSO vehicle controls for that tumor. In all graphs, one asterisk (*) indicates $p<0.05$, two asterisks (**) indicates $p<0.01$ and three asterisks (***) indicates $p<0.001$. D) Relative colony numbers with compound E plotted against relative colony numbers with compound IX for the 9 tumors shown in Panel C. Each data point represents a single tumor, and colony formation was normalized to DMSO vehicle controls. The three GSI-resistant tumors are highlighted in red. E). Leukemia cells derived from 6 *Ctsg-PML-RARA* tumors were transduced with MSCV DN-MAML1-GFP or MSCV-ires-GFP control virus, and engrafted in C57BL/6 × 129SvJ F1 mice. After 4 weeks, total splenic cells were analyzed for GFP and CD117 (c-Kit) positivity by flow cytometry. Data shown

are the percentage of cells positive for both GFP and CD117. *, $p<0.05$; N.S., not significant. Data points represent individual recipient animals. F) Flow cytometry plots from tumors 3430 and 3673, showing GFP and CD117 positivity in the spleens of animals transplanted with either DNMAML-GFP or GFP control transduced tumors.