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Article

Complementary Genomic Screens Identify SERCA as a Therapeutic Target in *NOTCH1* Mutated Cancer

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SUMMARY

Notch1 is a rational therapeutic target in several human cancers, but as a transcriptional regulator, it poses a drug discovery challenge. To identify Notch1 modulators, we performed two cell-based, high-throughput screens for small-molecule inhibitors and cDNA enhancers of a *NOTCH1* allele bearing a leukemia-associated mutation. Sarco/endoplasmic reticulum calcium ATPase (SERCA) channels emerged at the intersection of these complementary screens. SERCA inhibition preferentially impairs the maturation and activity of mutated Notch1 receptors and induces a G_0/G_1 arrest in *NOTCH1*-mutated human leukemia cells. A small-molecule SERCA inhibitor has on-target activity in two mouse models of human leukemia and interferes with Notch signaling in *Drosophila*. These studies "credential" SERCA as a therapeutic target in cancers associated with *NOTCH1* mutations.

INTRODUCTION

Selective expression of transcription factors directs the hierarchical specification of the hematopoietic lineage, and acquired mutations that perturb the function of these factors have a central role in leukemia pathogenesis. A prime example involves Notch1, a surface receptor that is essential for T-cell progenitor specification and maturation. Acquired mutations that activate Notch1 are found in 40% to 70% of childhood and adult T-cell acute lymphoblastic leukemia (T-ALL) (Lee et al., 2005; Mansour et al., 2006; Weng et al., 2004). Moreover, recent reports identified *NOTCH1* activating mutations in 10%–15% of chronic lymphocytic leukemia (CLL) (Di Ianni et al., 2009; Puente et al., 2011) and mantle cell lymphoma (Kridel et al., 2012).

Notch receptors regulate many aspects of normal development and tissue homeostasis (reviewed in Kopan and Ilagan,

Significance

Notch1 is aberrant in many malignancies, with both gain and loss-of-function mutations reported, highlighting the need for therapies selectively targeting mutant Notch1 receptors. T cell acute lymphoblastic leukemia (T-ALL), a high-risk leukemia in need of better treatment approaches, is one disease notable for frequent, activating mutations in *NOTCH1*. In this study, we identify SERCA inhibition as an approach to selectively impair the maturation of mutant Notch1 receptors in T-ALL and demonstrate the antileukemia activity of this strategy both in vitro and in vivo. With increasing evidence of SERCA mutations in hereditary diseases and cancer, our study also suggests that aberrant SERCA activity might contribute to diseases linked to altered Notch signaling.

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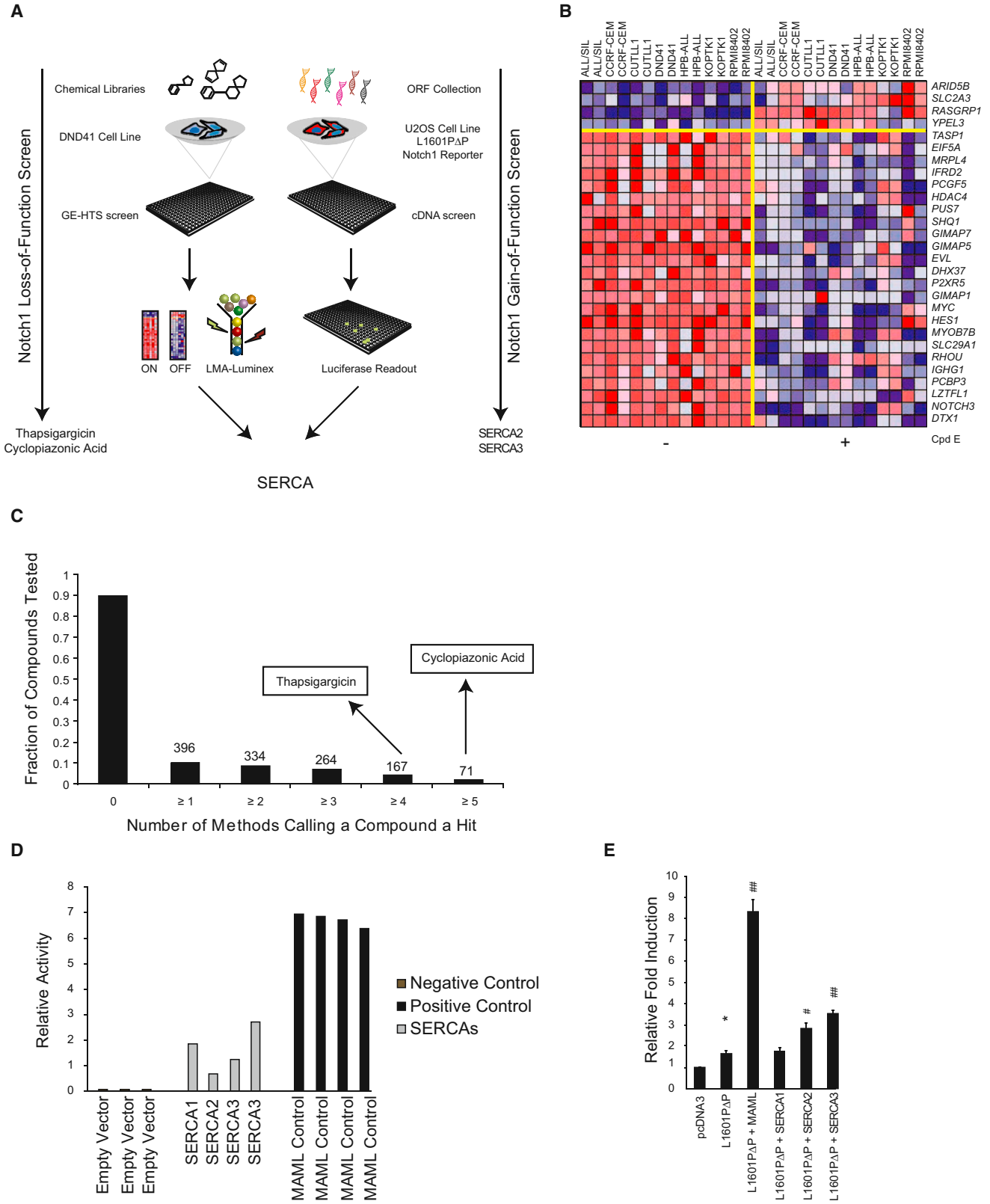

Figure 1. Identification of SERCA at the Intersection of Two High-Throughput Screens

(A) Notch1 inhibitory modulators were identified using GE-HTS in DND41 cells, and these results were integrated with results from a cDNA library screen for factors enhancing the signaling activity of the leukemogenic *NOTCH1* allele, L1601PΔP. ORF, open reading frame; LMA, ligation-mediated amplification.

(legend continued on next page)

2009). Mammalian Notch receptors are processed during maturation by a furin-like protease, leading to the formation of two, noncovalently associated subunits. Signaling is normally initiated by binding of the Notch ectodomain to a ligand of the DSL family expressed on a neighboring cell. This interaction triggers two additional, successive proteolytic cleavages in the Notch transmembrane subunit. The first, mediated by ADAM-10 or ADAM-17 (Brou et al., 2000), occurs within a juxtamembrane negative regulatory region (NRR) at a site that is protected in the Notch *off* state (Gordon et al., 2009; Gordon et al., 2007). This cleavage within the Notch transmembrane domain creates a short-lived intermediate that is primed for secondary cleavage by the γ-secretase complex, an event that liberates the intracellular domain of Notch1 (ICN). ICN translocates to the nucleus, associates with the DNA-binding factor RBPJ, and recruits coactivators of the Mastermind-like (MAML) family to activate expression of target genes.

Each of the proteolytic steps involved in the activation of Notch receptors is a potential therapeutic target. Indeed, γ-secretase inhibitors (GSIs) have anti-T-ALL activity in vitro (Weng et al., 2004) and in vivo (Cullion et al., 2009; Real et al., 2009). The GSI MK-0752 was tested in a phase I clinical trial in patients with relapsed acute leukemia (DeAngelo et al., 2006). This trial was halted, however, due to gastrointestinal toxicity thought to be related to chronic pan-Notch receptor inhibition in gut progenitor cells (Wong et al., 2004). Thus, other approaches to Notch1 inhibition are desirable.

Historically, it has been difficult to develop high-throughput assays for small molecules that disrupt protein-DNA or protein-protein interactions (Darnell, 2002). Recently, there has been renewed interest in cell-based screening to address the problem of ''undruggable'' targets using various approaches (Carpenter, 2007; Inglese et al., 2007; Stegmaier et al., 2004). Gene expression-based high-throughput screening (GE-HTS) is a chemical genomic approach in which gene expression signatures serve as surrogates for cellular states (Hahn et al., 2008; Stegmaier et al., 2004). One application of GE-HTS is for the identification of small molecules that modulate transcriptional signatures produced by aberrantly activated transcription factors (Corsello et al., 2009). A limitation of cell-based assays is that identifying the biologically relevant target of the small molecule can be a daunting task. One way to overcome this challenge is to design multiple, integrated cell-based screens and then to focus on common emerging hits. With the availability of genome-scale cDNA collections, overexpression screens have also proven to be powerful tools to probe biological pathways and to identify the protein targets of small molecules. Here, we used complementary GE-HTS and cDNA overexpression screens to search for small-molecule modulators of Notch1 signaling in T-ALL.

RESULTS

Development of a GE-HTS Assay for Notch1 Inhibitors

Figure 1A outlines our approach. We first defined a robust Notch1 transcriptional signature for the GE-HTS assay. We selected a set of genes that defined the Notch1 activation state from genomewide expression profiling of seven *NOTCH1*-mutated T-ALL cell lines treated with the GSI compound E (Cpd E) (Palomero et al., 2006b). From a set of approximately 500 genes with differences of $p < 0.01$ by two-sided Student's t test, 28 genes with mean fold changes > 1.5 between the Notch1 *on* versus *off* states (Figure 1B) and four invariant control genes were selected and validated (Figure S1A available online). To confirm that the signature reports on Notch1 inhibition and not GSI treatment per se, we transduced DND41 cells with Notch1-specific shRNA and demonstrated induction of the Notch1 *off* signature (Figures S1B–S1D). This is consistent with prior work in which we showed that GE-HTS identified a Notch1 *off* signature in T-ALL cells treated with a Notch1-specific inhibitory antibody (Aste-Amézaga et al., 2010). To ensure that the signature does not identify generic growth inhibitors or cellular toxins, we treated DND41 cells with drugs known to be active against T-ALL cells. These drugs inhibited growth but did not induce the Notch1 *off* signature (Figures S1E–S1F).

Identification of Small Molecules that Modulate Notch1

We screened 3,801 drugs or drug-like compounds in the human T-ALL cell line DND41. GE-HTS data were collected after 72 hr of treatment as previously described (Peck et al., 2006). Because true-positives are more likely to score by multiple scoring metrics, we applied a consensus classification system requiring hits to score in multiple algorithms: summed score, weighted summed score, K-nearest neighbor, naive Bayes classification, and support vector machine (Figure 1C; Figure S1G) (Banerji et al., 2012). A total of 16 compounds (Table S1) selected for validation based on these criteria were retested in a 2-fold dose-response series in DND41, MOLT4, and PF382 cell lines. Notably, multiple compounds reported to modulate calcium ion flux scored as dose-dependent Notch pathway inhibitors in all of the cell lines tested (Figures S1H–S1J).

(B) Notch1 *off* signature genes derived from the expression profiling of T-ALL cell lines treated with Cpd E. The columns represent individual cell lines treated in duplicate with Cpd E (0.5 μM) or vehicle for 24 hr. The rows represent the 28 genes selected for the Notch *off* signature. Dark red indicates high gene expression, and dark blue indicates low gene expression by Affymetrix microarray profiling.

(C) The x axis indicates the number of methods (summed score, weighted summed score, naive Bayes, K-nearest neighbor, and support vector machine), and the y axis the fraction of chemicals that scored for the number of methods. SERCA inhibitors are indicated. The number of compounds scoring by the indicated number of methods is noted above each column.

(D) Notch1 gain-of-function primary screen data for the negative (empty vector) and positive (MAML) controls versus SERCA-encoding cDNAs hits. Depicted is the fold induction of luciferase signal for each replicate normalized to the population median of reporter readout values of pcDNA3-L1601PΔP (n = 184).

(E) Retesting of cDNAs scoring in the Notch-sensitive luciferase reporter. Luciferase activity is expressed as relative activity compared to the pcDNA3 control. Errors bars denote the mean ± SD of 10 replicates. Statistical significance relative to pcDNA3 ($^{*}p < 0.05$) and to pcDNA3-L1601PΔP ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$) was determined by Student's t test.

See also Figure S1 and Table S1.

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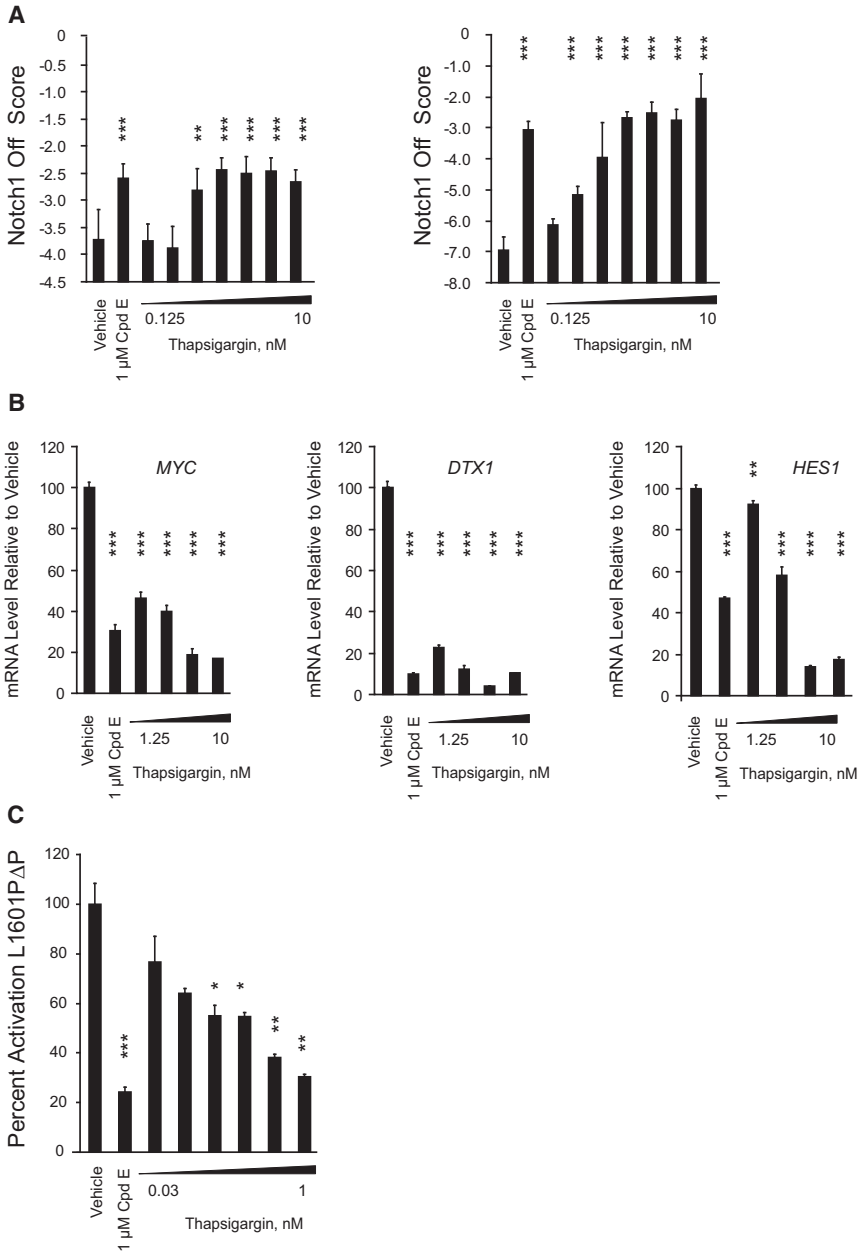

Figure 2. Validation of SERCA as a Notch1 Modulator

(A) Induction of the Notch1 *off* score (weighted summed score) measured by GE-HTS in DND41 (left) and MOLT4 (right) cells treated with thapsigargin in 2-fold dilution for 72 hr. Error bars denote the mean ± SD of 12 replicates for vehicle-treated (DMSO 0.06%) cells and six replicates for thapsigargin-treated cells.

(B) Expression of indicated Notch1 target genes in DND41 treated in 2-fold dliution for 24 hr was determined by quantitative RT-PCR. Error bars indicate the mean ± SD of three replicates. Data were analyzed using the ΔΔCT method and plotted as a percentage relative to the control gene *RPL13A*.

(C) Effects of thapsigargin on the activation of a Notch1 luciferase reporter by L1601PΔP in U2OS cells. Normalized luciferase activity relative to a Renilla control was expressed as a percentage of vehicle treatment. Error bars denote mean ± SD of four replicates. Statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) in all panels was determined by one-way analysis of variance (ANOVA) using Bonferroni's correction for multiple comparison testing.

A cDNA Library Screen Identifies SERCA as a Notch Signaling Enhancer

A complementary cDNA library screen for factors that enhance the signaling activity of the Notch1 mutant L1601PΔP was simultaneously conducted in the osteosarcoma cell line U2OS. L1601PΔP contains the same heterodimerization mutation that is present in the MOLT4 and KOPTK1 cell lines in *cis* with a PEST domain deletion (Chiang et al., 2008), a combination that is found in approximately 10%–15% of human T-ALL (Weng et al., 2004). U2OS cells were selected for the screen because they are readily transfected and have very low basal Notch signaling tone, a feature that produces favorable signal-to-noise ratios. A total of 18,000 open reading frames were scored for their ability to enhance L1601PΔP-dependent activation of a Notch luciferase reporter. Among the top hits were *ATP2A1*, *ATP2A2*, and *ATP2A3* (Figure 1D), which encode SERCA1, SERCA2, and SERCA3, respectively. Sarco/endoplasmic reticulum calcium ATPases (SERCAs) are closely related, inwardly directed, ATP-dependent calcium pumps that localize to the endoplasmic reticulum (ER). Retesting confirmed that *ATP2A2* and *ATP2A3* potentiate L1601PΔP-dependent signaling (Figure 1E). Of note, loss-of-function mutations in a *Drosophila* SERCA homolog, Ca-P60A, have been reported to produce Notch loss-of-function phenotypes in this model organism by altering Notch trafficking (Periz and Fortini, 1999). Thus, calcium modulators emerged at the nexus of two complementary screens.

Thapsigargin Targets the Notch Pathway

One of the small molecules that scored in our GE-HTS screen across four scoring metrics was thapsigargicin, an analog of thapsigargin, a highly potent natural product inhibitor of SERCA. Low nanomolar concentrations of thapsigargin induced the Notch1 *off* signature in a dose-dependent fashion in *NOTCH1* mutant T-ALL cells (Figure 2A) and reduced the expression of the direct Notch1 target genes *MYC*, *HES1*, and *DTX1* (Figure 2B). Subnanomolar concentrations of thapsigargin also inhibited the expression of a Notch reporter by L1601PΔP in U2OS cells (Figure 2C).

Notch1 inhibition results in G_0/G_1 arrest in human T-ALL cells (Weng et al., 2004) and decreased T-ALL cell size (Palomero et al., 2006b; Weng et al., 2006). As expected, thapsigargin also induced a G_0/G_1 arrest (Figure 3A) and a decrease in cell size (Figure 3B) in *NOTCH1*-mutated T-ALL cell lines. We next studied the effect of thapsigargin in a panel of T-ALL cell lines that contain activating mutations in the heterodimerization domain (HD) of Notch1 and/or deletions in the degradation domain (PEST). Three T-ALL cell lines reported to be highly sensitive to GSI (ALL/SIL, DND41, and KOPTK1) were more sensitive to thapsigargin as measured by inhibition of cell growth and induction of apoptosis compared to two cell lines with intermediate sensitivity to GSI (MOLT4 and PF382) (Figure 3C). Furthermore, 24 hr of thapsigargin treatment decreased ICN1 levels in T-ALL cells (Figure 4A). As a further test of the idea that thapsigargin acts by preventing Notch1 activation, the Notch1-dependent T-ALL cell lines MOLT4 and DND41 were transduced with an empty MigR1 vector or with MigR1-ICN1 (Figure 4B). Transduction of ICN1, which lies downstream of the γ-secretase cleavage step in Notch activation, prevented induction of the Notch1 *off* signature (Figure 4C), growth inhibition (Figure 4D), G_0/G_1 cell cycle arrest (Figure 4E), and induction of apoptosis (Figure 4F) by thapsigargin. In contrast, empty MigR1 had no effect on these readouts of Notch inhibition. These results are consistent with a Notch pathway inhibitory effect of thapsigargin at low nanomolar concentrations.

Thapsigargin Interferes with Notch1 Maturation

Multiple compounds scoring in our screen, including thapsigargin, are predicted to alter intracellular calcium. For example, thapsigargin and cyclopiazonic acid are known Ca^{2+}ATPase inhibitors, impairing calcium entry into the ER. Of note, multiple EGF repeats and all three Lin12/Notch repeats in the extracellular domains of Notch receptors require calcium ions for proper folding (Aster et al., 1999; Gordon et al., 2007; Hambleton et al., 2004; Rand et al., 1997). We thus hypothesized that thapsigargin, by altering ER Ca^{2+} concentrations, would inhibit Notch1 maturation in T-ALL cells.

To test this hypothesis, we first determined if thapsigargin affected furin processing of Notch1, an event that occurs in the late Golgi compartment. Lysates from T-ALL cell lines treated with thapsigargin were immunoblotted with an antibody specific for the cytoplasmic portion of Notch1 that recognizes both unprocessed Notch1 ($\sim$270 kDa) and the furin-processed transmembrane subunit ($\sim$110 kDa). Thapsigargin reduced the levels of the furin-processed transmembrane Notch1 subunit, but not the unprocessed full-length Notch1 precursor, in multiple T-ALL cell lines (Figure 5A). Similar results were observed with the less potent SERCA inhibitor cyclopiazonic acid (Figure S2). Misfolded Notch1 receptors are expected to be retained in the ER/Golgi compartment. Immunostaining revealed that thapsigargin treatment reduced the levels of Notch1 on the cell surface (Figures 5B and 5C) and resulted in the colocalization of Notch1 and giantin, a Golgi membrane protein (Figure 5D). Thus, thapsigargin leads to defective Notch1 maturation in cultured T-ALL cells.

SERCA Antagonism Inhibits Notch Function and T-ALL Growth In Vivo

To confirm that chemical and genetic inhibition of SERCA lead to Notch inactivation in vivo, we evaluated a *Drosophila* intestinal stem cell model in which Notch inhibition perturbs differentiation. The adult midgut is maintained by pluripotent stem cells that give rise to two populations of terminally differentiated daughter cells: a large class of polyploid enterocyes (EC) and a smaller class of diploid enteroendocrine (ee) cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). The stem cells express escargot (esg), a transcription factor, and Delta, a membrane-bound ligand of the Notch receptor. High levels of Notch activation are required for daughter cells to adopt the EC cell fate, whereas lower levels of Notch activation specify daughters to adopt the ee fate. Thus, when Notch is completely inhibited, daughter cells fail to differentiate and remain as stem cells. By contrast, when Notch signaling is partially inhibited, daughter cells fail to differentiate into ECs and remain as stem cells or differentiate into ee cells.

To enhance the sensitivity of fly-based drug assays, we used transgenic flies that express a human *RAF* gain-of-function cDNA, *RAF*(gof), in their intestinal stem cells. Expression of the *RAF*(gof) cDNA results in rapid expansion of the esg+ population, which is composed of not only diploid stem cells but also polyploid EC daughter cells. When Notch is inhibited by feeding flies either DAPT or Cpd E, both GSIs, stem cell daughters fail to differentiate into EC cells and instead give rise mostly to additional stem cells, as well as some ee daughters (Figure 6A). Cyclopiazonic acid and thapsigargin treatment also expanded the stem cell and ee cell populations, thus phenocopying the effects of GSI (Figure 6B).

If thapsigargin inhibits Notch signaling through effects on calcium channels, then genetic modulation of SERCA should produce similar phenotypes. Indeed, knockdown of Ca-P60A, the only SERCA expressed in *Drosophila*, produced effects on stem cell and ee cell pools similar to those induced by GSI, thapsigargin, or cyclopiazonic acid treatment (Figure 6C). Thus, the results of chemical and genetic studies are consistent with a model in which SERCA inhibition by thapsigargin impairs Notch signaling in vivo.

We next tested thapsigargin in a human T-ALL xenograft model. Systemic administration of thapsigargin to SCID-beige mice bearing MOLT-4 tumors inhibited tumor growth compared to vehicle-treated controls (Figure 6D). In addition, ICN1 protein levels were diminished in the thapsigargin-treated tumors compared to vehicle-treated tumors (Figures 6E and S3A), linking growth inhibition to Notch inhibition.

To demonstrate further that thapsigargin impairs leukemic progression via Notch signaling inhibition, we established a second T-ALL xenograft model in which DND41 cells were transduced with MigR1 or MigR1-ICN1 and subsequently propagated in NSG mice. Thapsigargin treatment markedly decreased the growth of control tumors but had little effect on tumors expressing ICN1 (Figures 6F and 6G), indicating that tumor growth suppression by thapsigargin is mediated by inhibition of Notch1 signaling in the leukemic cells.

Prior studies demonstrated that gastrointestinal toxicity and lack of sustained response were the major limitations of first-generation GSIs (DeAngelo et al., 2006). It was hypothesized that gastrointestinal toxicity was due to blockade of wild-type Notch1 and Notch2 in the gut leading to intestinal secretory metaplasia, increased number of goblet cells, and arrested proliferation in the crypts of the small intestine (Milano et al., 2004;

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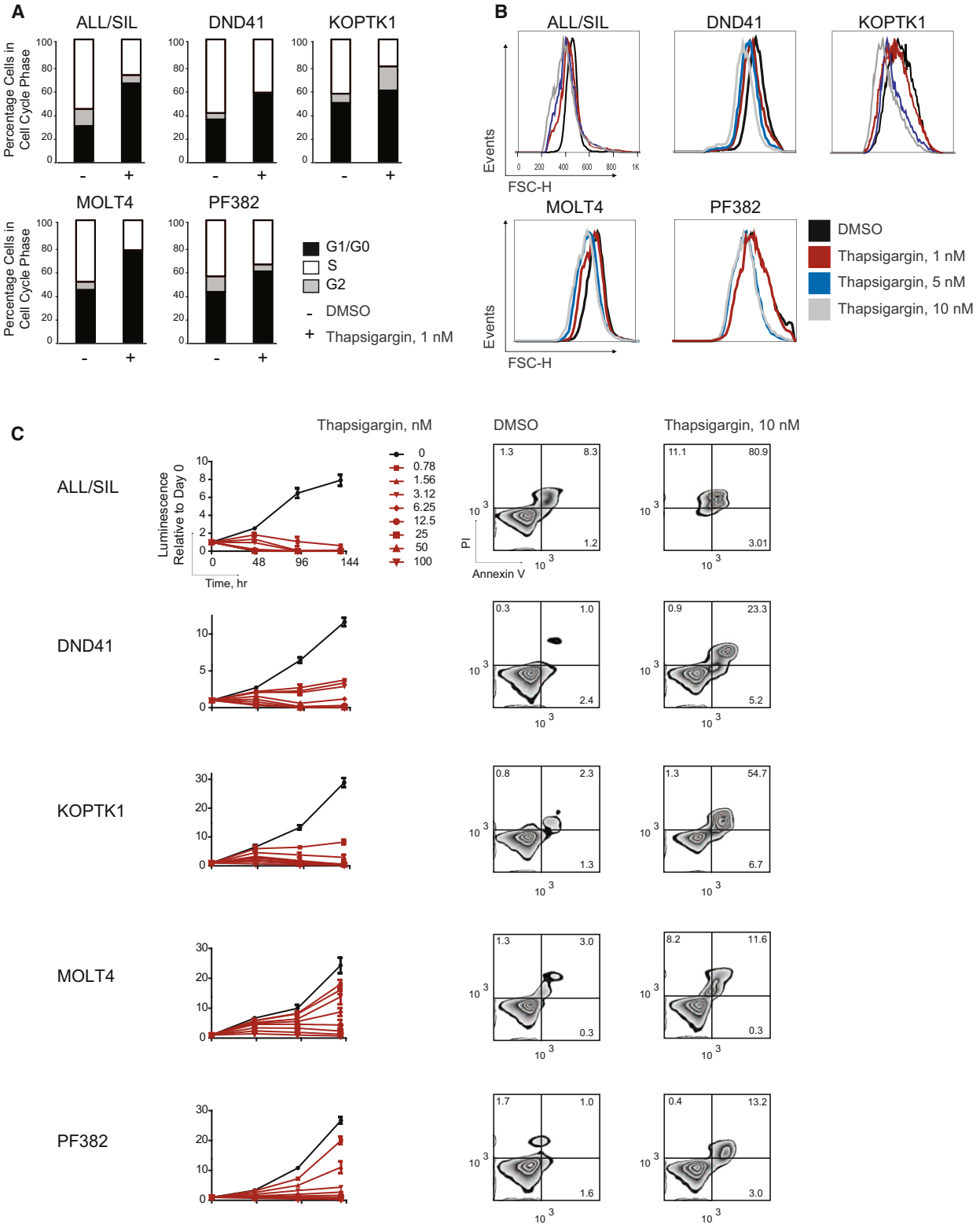


Figure 3. Thapsigargin Demonstrates Anti-Notch1 and Antileukemia Properties in T-ALL In Vitro

(A) Effect of thapsigargin treatment (6 days) on cell cycle of T-ALL cell lines, as assessed by measurement of DNA content on the viable fraction of cells.

(B) Effect of thapsigargin treatment for 24 hr on cell size as measured by forward-scatter flow cytometry.

(C) Effect of thapsigargin treatment on cell growth (left) and induction of apoptosis (right). Error bars denote mean ± SD of four replicates. Annexin V/PI staining of T-ALL cells following 72 hr of treatment with 10 nM thapsigargin.

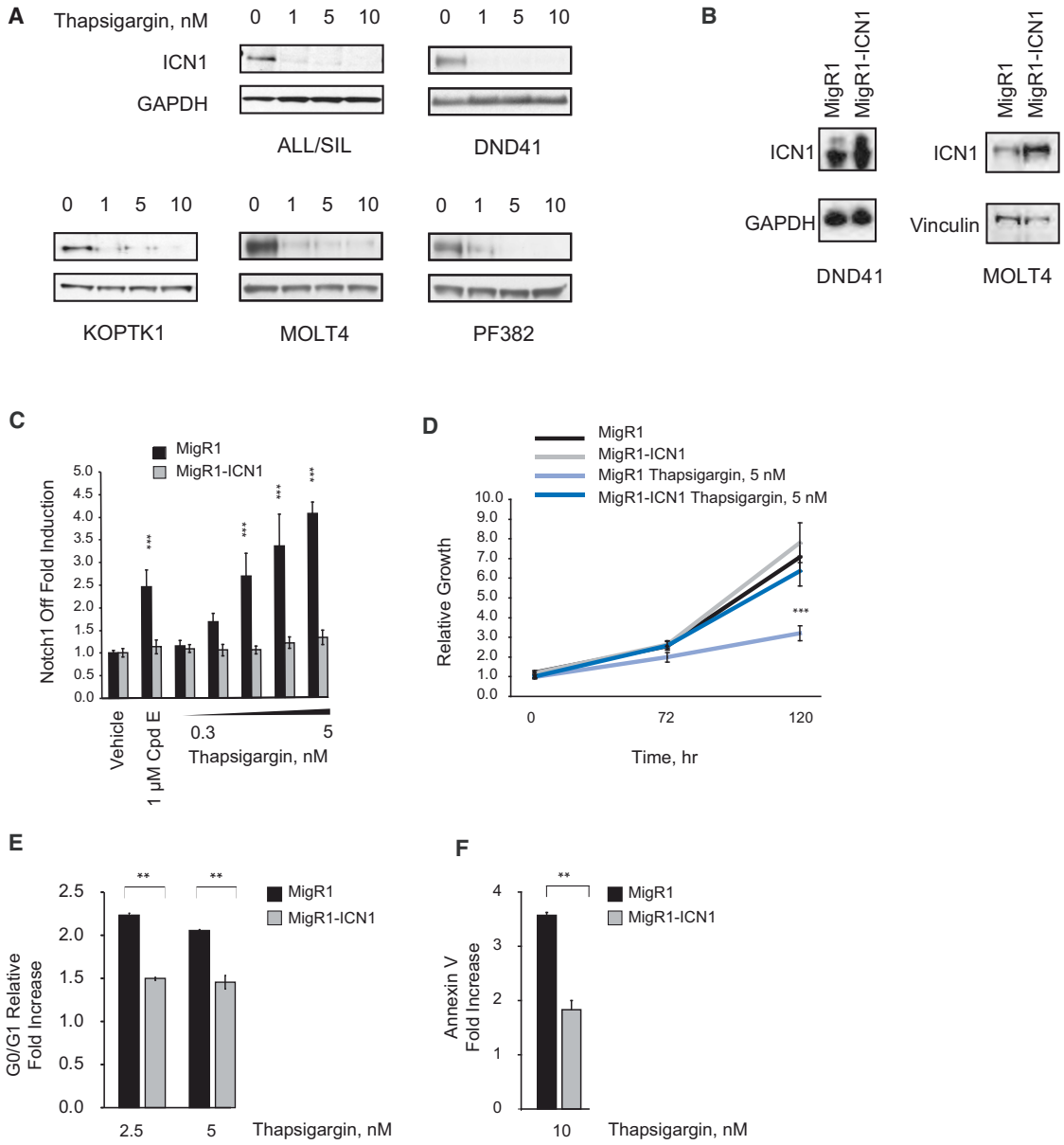


Figure 4. Thapsigargin Demonstrates Notch1 On-Target Activity In Vitro

(A) Effect of 24 hr of thapsigargin treatment on the ICN1 level in T-ALL cells. The immunoblot was stained with anti-ICN1 antibody.

(B) ICN1 level in MigR1- or MigR1-ICN1-transduced cells. ICN1 is detected using an anti-ICN1 antibody.

(C) The weighted summed score fold induction is shown for the Notch1 *off* signature after treatment of cells with thapsigargin or Cpd E for 48 hr. Error bars indicate the mean ± SD of 12 replicates for vehicle-treated and six replicates for GSI- or thapsigargin-treated cells. Statistical significance (***$p < 0.001$) was determined by one-way ANOVA using Bonferroni's correction for multiple comparison testing.

(D) Effect of thapsigargin on the growth of MigR1- or MigR1-ICN1-transduced DND41 cells. Normalized data are plotted relative to time 0. Error bars indicate mean ± SD of four replicates. Statistical significance (***$p < 0.001$) was determined by two-way ANOVA with Bonferroni's correction for multiple comparison testing.

(E) Effect of 3 days of thapsigargin treatment on DNA content of MigR1- or MigR1-ICN1-transduced MOLT4 cells. Error bars indicate mean ± SD of two replicates with results expressed relative to vehicle treatment. Statistical significance was determined by Student's t test (**$p < 0.01$).

(F) Effect of 3 days of thapsigargin treatment on apoptosis of MigR1- or MigR1-ICN1-transduced DND41 cells. Error bars indicate mean ± SD of two replicates with results expressed relative to vehicle. Statistical significance was determined by Student's t test (**$p < 0.01$).

Real et al., 2009). Mice treated with thapsigargin did not develop gastrointestinal toxicity (Figures S3B and S3C), suggesting that HD-mutated Notch1 receptors were more sensitive to the effects of thapsigargin than wild-type Notch1/Notch2 receptors expressed in normal cells. These preclinical studies support SERCA as a possible therapeutic target in T-ALL.

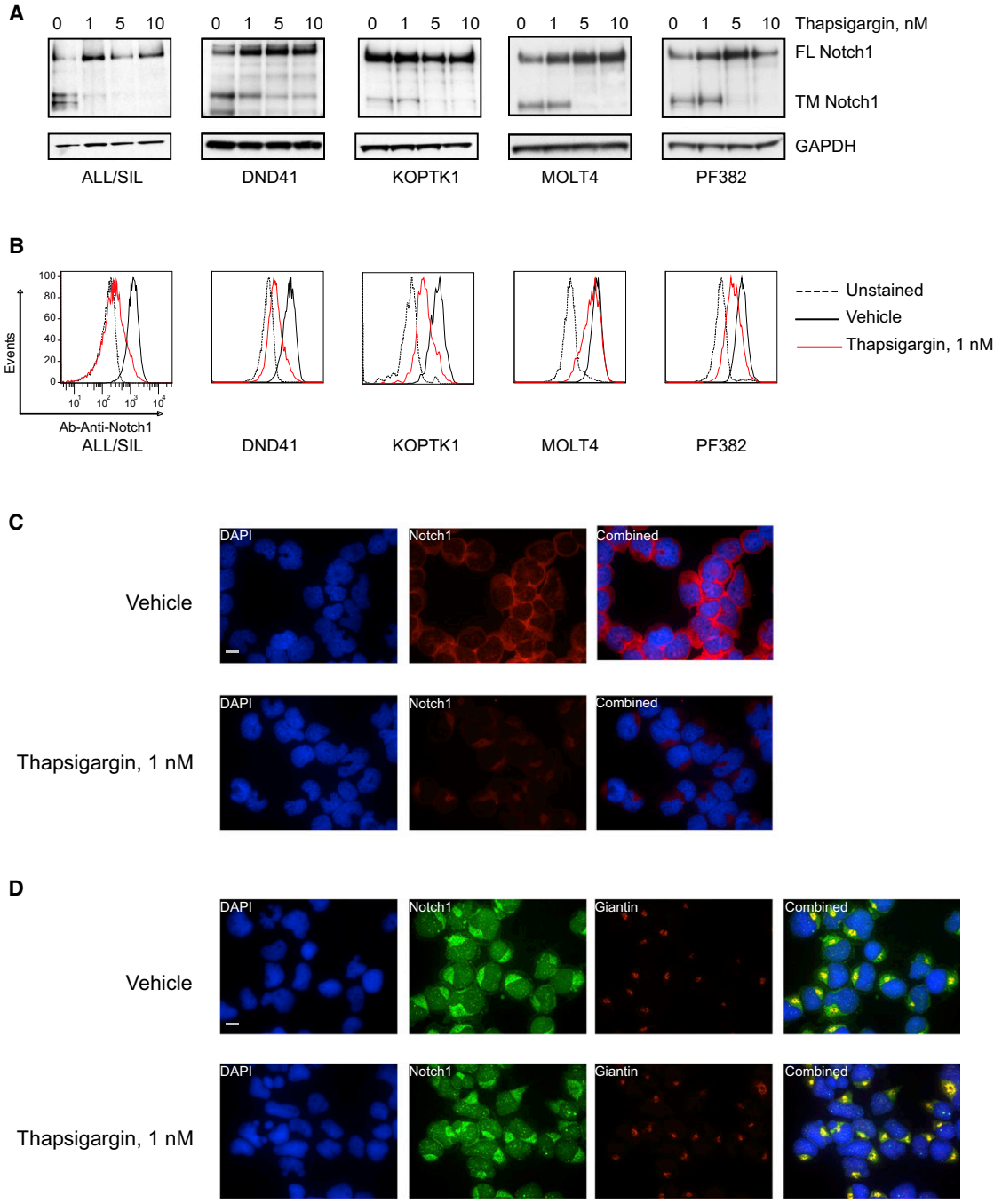


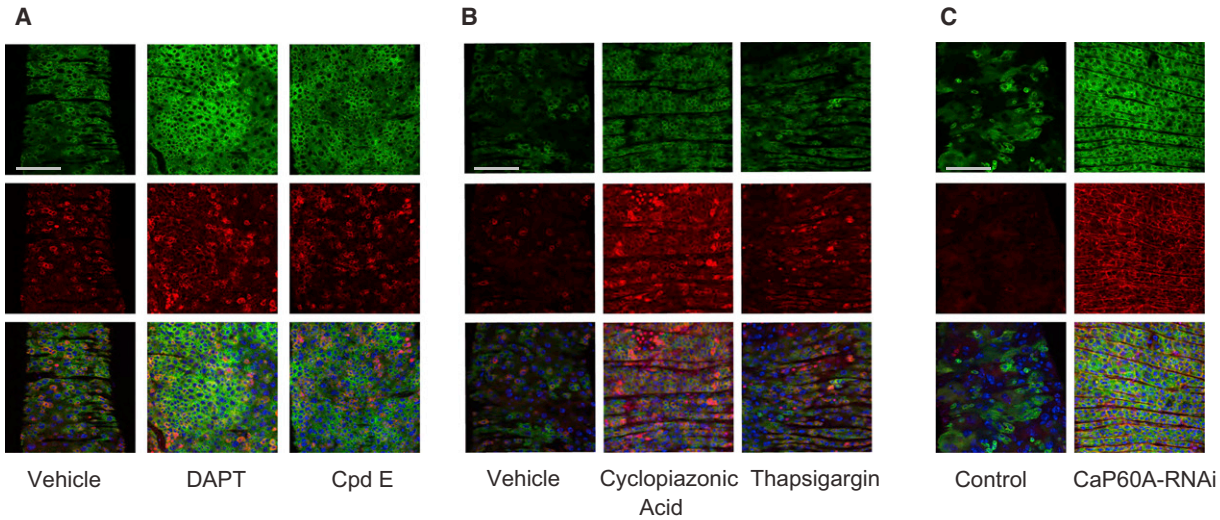
Figure 5. Thapsigargin Impairs Notch1 Maturation in T-ALL Cell Lines

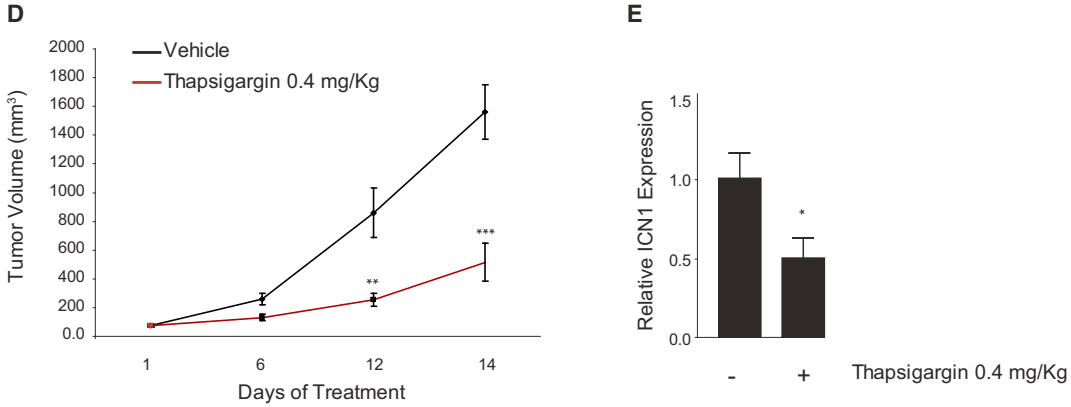
(A) Effect of thapsigargin treatment (24 hr) on Notch1 processing in T-ALL cell lines all with HD mutations, DND41 and ALL/SIL (L1594PΔPEST), KOPTK1 and MOLT4 (L1601PΔPEST), and PF382 (L1575PΔPEST). The blot was stained with an antibody against the C terminus of Notch1 that recognizes both the furin-processed Notch1 transmembrane subunit (TM) and the unprocessed Notch1 precursor (FL).

(B and C) Effect of thapsigargin treatment (24 hr) on Notch1 cell surface staining, as assessed by flow cytometry (B) and staining of nonpermeabilized cells (C). Scale bar, 10 μm.

(D) Effect of thapsigargin treatment (24 hr) on the subcellular localization of Notch1. Double-immunofluorescence staining of permeabilized DND41 cells stained with anti-Notch1 (green) and Giantin (red) is shown. Colocalization is indicated by yellow signal. Scale bar, 10 μm.

See also Figure S2.





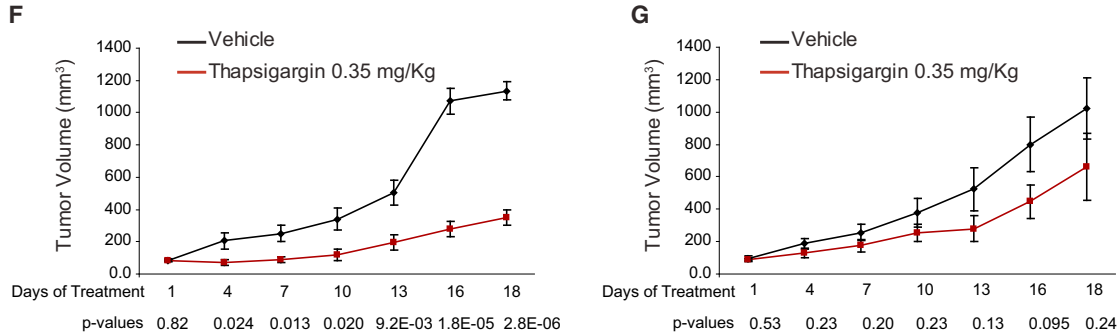


Figure 6. SERCA Inhibition Causes Notch Loss-of-Function In Vivo

(A–C) Immunofluorescence staining of *Drosophila* midguts expressing GFP (green) and stained with anti-Delta (membrane red), antiprospero (nuclear red), and DAPI (blue) is depicted. Treatment was with DAPT (400 μM) or Cpd E (100 μM) for 7 days in (A) and with cyclopiazonic acid (1 mM) or thapsigargin (100 μM) for 7 days in (B). In (C), effects of knockdown of Ca-P60A are shown. Scale bars, 75 μm.

(D) Effect of thapsigargin on the growth of xenografted MOLT4 tumors. Error bars indicate mean ± SD of six replicates for the thapsigargin-treated and nine replicates for the vehicle-treated mice. Statistical significance (**$p < 0.01$; ***$p < 0.001$) was determined by two-way ANOVA using Bonferroni's correction for multiple comparison testing.

(E) Effect of thapsigargin treatment on ICN1 levels in xenografted MOLT4 tumors was measured by western blotting, and statistical significance was determined by Student's t test (*$p < 0.05$). Error bars represent the mean ± SD of six replicates for each group.

(F and G) Effect of thapsigargin on the growth of DND41 cells transduced with MigR1 (F) or MigR1-ICN1 (G) xenografted in NSG mice. Error bars indicate mean ± SD of replicates for each cohort. Statistical significance was determined by Student's t test as indicated.

See also Figure S3.

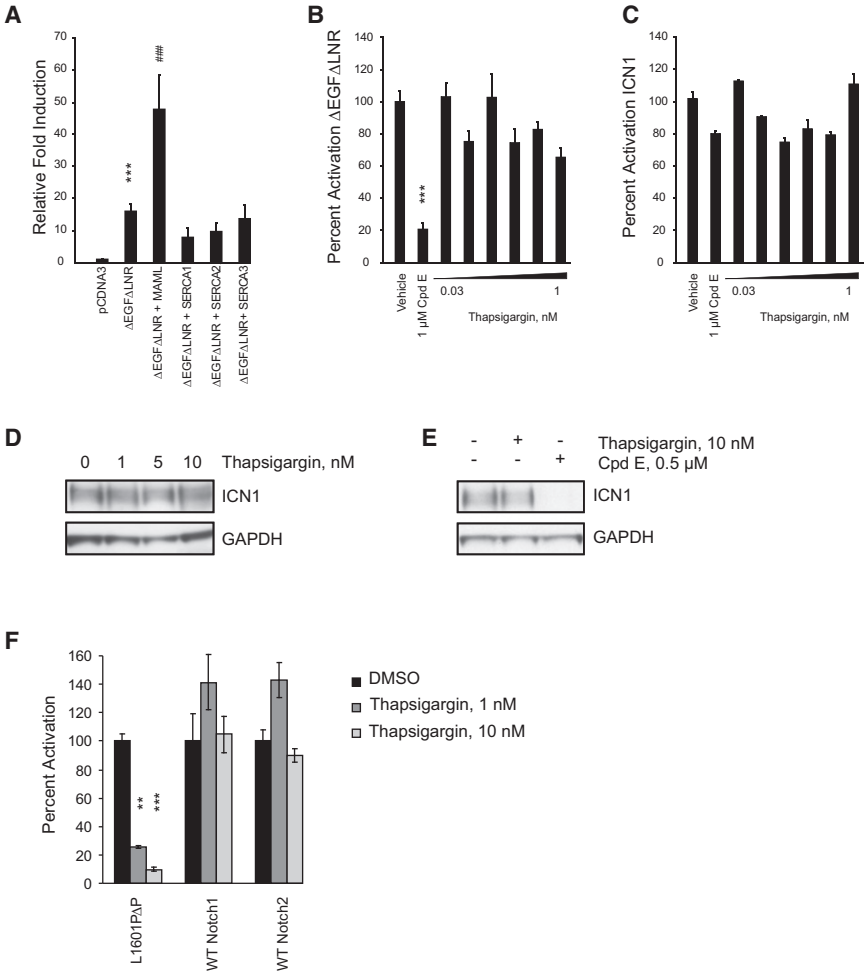


Figure 7. Notch1 Ca^{2+} Binding Modules Are Required for the Anti-Notch1 Activity of Thapsigargin

(A) Effect of SERCA coexpression on the activity of ΔEGFΔLNR in a Notch reporter assay. Normalized firefly luciferase activity was expressed as fold induction relative to the empty plasmid. Error bars denote the mean ± SD of 10 replicates. Statistical significance relative to pcDNA3 (***$p < 0.001$) and to pcDNA3-ΔEGFΔLNR ($^{\#\#\#}p < 0.001$) was determined by one-way ANOVA using Bonferroni's correction for multiple comparison testing.

(B and C) Effects of thapsigargin or Cpd E on the activity of ΔEGFΔLNR (B) and ICN1 (C) in a Notch reporter assay. Assay conditions and interpretations were as in (A). Errors bars denote mean ± SD of four replicates. Statistical significance (***$p < 0.001$) was determined by one-way ANOVA using Bonferroni's correction for multiple comparison testing.

(D and E) The ICN1 protein level in SUPT1 cells treated for 24 hr as indicated was determined using immunoblots stained with anti-ICN1 antibody.

(F) Ligand-mediated activation of Notch activity was determined using U2OS cells stably (Notch1 and Notch2) or transiently (L1601PΔP) expressing Notch-Gal4 fusion receptors and treated as indicated. Normalized Gal4 firefly luciferase activity was expressed relative to vehicle treated control. Error bars denote mean ± SD of four replicates. Statistical significance (**$p < 0.01$; ***$p < 0.001$) was determined by Student's t test.

NOTCH1 Mutational Status Influences Thapsigargin Sensitivity

The aforementioned results suggest that thapsigargin inhibits signaling through wild-type and mutated Notch receptors but may have stronger effect for mutated Notch1. Prior work has shown that many activating HD mutations found in T-ALL result in destabilization of the Notch negative regulatory region and have deleterious effects on Notch1 folding and maturation (Malecki et al., 2006). Because the Lin12/Notch repeats (LNRs) of the Notch negative regulatory region rely on calcium for folding and function (Aster et al., 1999), mutated Notch1 might be more sensitive to reduced calcium availability than wild-type Notch1, providing a therapeutic window for SERCA inhibitors.

One simple prediction of the aforementioned model is that constitutively active forms of Notch1 lacking Ca^{2+} binding modules should be insensitive to SERCA inhibitors. To test this prediction, we performed Notch1 reporter assays in U2OS cells transfected with a plasmid encoding ΔEGFΔLNR, a membrane-tethered form of Notch1 lacking the extracellular epidermal growth factor (EGF) repeats and LNRs, or ICN1. As anticipated, coexpression of SERCA did not enhance reporter gene activation by ΔEGFΔLNR (Figure 7A), nor was reporter gene activation by ΔEGFΔLNR (Figure 7B) or ICN1 (Figure 7C) affected by thapsigargin. In contrast, ΔEGFΔLNR was highly sensitive to GSI (Figure 7B). Taken together, these results indicate that, at low nanomolar concentrations, thapsigargin inhibits Notch1 through a mechanism that requires the Ca^{2+}-binding modules of the Notch1 extracellular domain.

To determine if the Notch1 extracellular domain is important for the ability of thapsigargin to inhibit leukemia cell growth, we studied the human T-ALL cell line SUPT1, which has two copies of a t(7;9)(q34,q34) fusing the 3′ end of *NOTCH1* with enhancer/promoter elements of the T cell receptor β locus (*TCRB*) and has no normal *NOTCH1* allele (Ellisen et al., 1991). The rearranged *NOTCH1* alleles in SUPT1 cells drive the expression of a series of truncated mRNAs encoding N-terminally deleted polypeptides lacking the Notch1 extracellular domain, some of which are inserted into membranes and require γ-secretase cleavage for activation (Das et al., 2004). As anticipated, thapsigargin had no effect on ICN1 levels in SUPT1 cells (Figure 7D), whereas Cpd E eliminates the generation of ICN1 (Figure 7E). In line with this idea that protein structure affects drug response, thapsigargin failed to inhibit wild-type Notch1 or Notch2 at concentrations that impaired signaling of Notch1-bearing leukemogenic HD mutations (Figure 7F).

To further test the effect of thapsigargin on wild-type Notch1 maturation, we tested two Notch1 wild-type T-ALL and one chronic myelogenous leukemia (CML) cell lines in which high expression of Notch1 was previously reported (Palomero et al.,

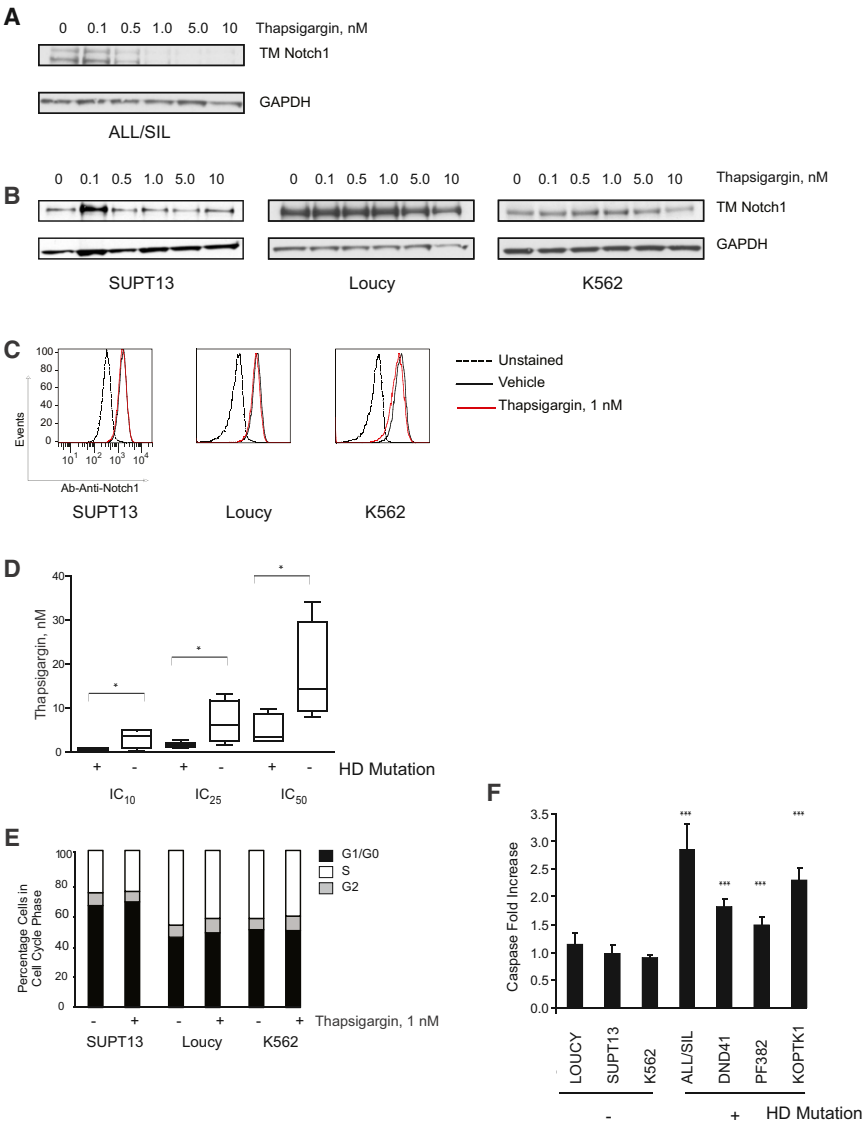


Figure 8. *NOTCH1* Mutational Status Influences the Sensitivity to Thapsigargin

(A and B) Effect of thapsigargin treatment (6 hr) on processing of HD mutant (A) or wild-type (B) Notch1. Notch1 was detected with an antibody against the C terminus of Notch1 that recognizes the furin-processed Notch1 transmembrane subunit (TM).

(C) Effect of thapsigargin treatment (24 hr) on Notch1 cell surface staining, as assessed by flow cytometry.

(D) The relative growth of thapsigargin-treated T-ALL cell lines with wild-type (LOUCY, MOLT16, and SUPT13) or rearranged alleles (SUPT1) of *NOTCH1* versus those with HD mutations (ALL/SIL, DND41, KOPTK1, MOLT4, and PF382). The line in the box plots represents the median. The upper edge (hinge) of the box indicates the 75th percentile of the data set, and the lower hinge indicates the 25th percentile. The ends of the vertical line indicate the minimum and the maximum data values. Statistical significance was determined by Student's t test (*$p < 0.05$).

(E) Effect of thapsigargin treatment on cell cycle progression in Notch1 wild-type cell lines, as assessed by measurement of DNA content on the viable fraction of cells.

(F) Effect of thapsigargin treatment (1 nM) on apoptosis induction as assessed by the luminescence Caspase 3/7 assay. Errors bars denote mean ± SD of four replicates. Statistical significance (***$p < 0.001$) was determined by one-way ANOVA using Bonferroni's correction for multiple comparison testing.

2006a). Compared to HD mutant, wild-type proteins appear to be less affected by thapsigargin treatment (Figures 8A–8C). The effects of thapsigargin on cell viability were then determined in a larger panel of human T-ALL cell lines of known *NOTCH1* mutational status (Weng et al., 2004). Cell lines carrying *NOTCH1* alleles with HD domain mutations were more sensitive to thapsigargin than cells with wild-type *NOTCH1* alleles or lacking the Notch1 extracellular domain (Figures 8D–8F).

In summary, these data suggest that Notch1 receptors bearing leukemogenic HD domain mutations are more sensitive to SERCA inhibitors, such as thapsigargin, than normal receptors.

DISCUSSION

Integrating Cell-Based Screens for Small Molecule and Protein Target Discovery

While there is a strong rationale for target-based therapies for cancer, with the exception of the nuclear hormone receptors, transcription factors have largely been refractory to conventional small-molecule screening approaches due to the challenges in developing high-throughput, robust screening assays. By definition, any therapeutic agent that modulates a transcription factor must alter the expression of its target genes. While there have been significant advances in our ability to assess global gene expression changes, almost all existing approaches cannot yet be applied to large-scale screening efforts due to cost and throughput limitations. Recognizing these shortcomings, we developed an approach that allows measurement of the expression of hundreds of endogenous genes in 384-well format and applied it to identify antagonists of leukemogenic increases in Notch signaling in T-ALL.

A limitation of both phenotypic and expression-based screening, however, is that identification of the relevant target of lead compounds can be difficult. The development of alternative genomic and chemical proteomic approaches for identifying protein targets holds the promise of accelerating the elucidation of underlying mechanism. Integrating results of a cDNA screen with GE-HTS data allowed us to identify SERCAs as Notch1 modulators and potential therapeutic targets in Notch1-associated leukemias.

Altering Maturation of Mutant Notch1 by SERCA Inhibition

We show here that SERCA inhibitors such as thapsigargin cause a Notch1 maturation defect marked by the accumulation of unprocessed Notch1 in the ER/Golgi compartment. The resulting effects on Notch1 signaling and leukemia cell growth depend on the nature of the *NOTCH1* mutations. The most common activating *NOTCH1* mutations in human T-ALL, the so-called class I *NOTCH1* mutations, consist of point substitutions or small in-frame deletions or insertions in the extracellular heterodimerization domain, which disrupt heterodimerization domain structure and permit ligand-independent ADAM-type metalloprotease cleavages (Gordon et al., 2009; Malecki et al., 2006). Folding and maturation of Notch1 are partially impaired by these mutations (Malecki et al., 2006), and it appears that Notch1 receptors bearing such mutations are more sensitive to the inhibitory effects of thapsigargin than wild-type Notch1 receptors. Another possible contributing factor to the greater sensitivity to thapsigargin in cells with mutated Notch1 receptors is that the presence of these mutated polypeptides may itself engender ER stress and thus render these cells more susceptible to the ER stress induced by thapsigargin. Indeed, this may account for the inability to rescue fully the effects of thapsigargin with ICN1 in some of our experiments. Taken together, these data suggest the potential for a therapeutic window for thapsigargin in T-ALLs bearing this type of mutation.

Other types of activating *NOTCH1* mutations also exist. Rarely, juxtamembrane in-frame tandem duplications create new "deprotected" ADAM-metalloprotease cleavage sites (class II mutations) (Malecki et al., 2006), or translocations create *NOTCH1* alleles encoding polypeptides that lack the Notch1 extracellular domain (Ellisen et al., 1991; Palomero et al., 2006a). As anticipated, we observed that a T-ALL cell line with two translocated *NOTCH1* alleles is relatively resistant to thapsigargin. Our proposed mechanism of action also predicts that murine T-ALLs, which often have Notch1 deletions that remove the Notch1 ectodomain coding sequence (Ashworth et al., 2010), as well as uncommon human breast cancers with *NOTCH1* rearrangements (Robinson et al., 2011), will be more resistant to thapsigargin and other SERCA inhibitors. It will be of importance to determine if CLLs, which have recently been reported to have frequent Notch1 PEST domain deletions (Di Ianni et al., 2009; Puente et al., 2011) but lack heterodimerization domain mutations, are sensitive to SERCA inhibitors.

Connecting *NOTCH1* and *SERCA* Mutations in Human Disease

Germline mutations in *ATP2A1* are reported in the congenital disorder Brody syndrome, characterized by impaired muscle relaxation and myopathy (Odermatt et al., 1996). *ATP2A2* mutations are reported in Darier's disease (Sakuntabhai et al., 1999), an autosomal dominant skin disorder characterized by loss of adhesion between keratinocytes, scaling due to hyperkeratosis, and thickening of the epidermis due to keratinocyte hyperproliferation. Skin cancers have been reported in patients with Darier's disease (Robertson and Sauder, 2012). Moreover, in aged *Atp2a2*$^{+/-}$ mice, tumors develop from the keratinized squamous epithelia (Liu et al., 2001) while the wild-type *Atp2a2* allele is retained and expressed, supporting a role for SERCA2 haploinsufficiency in tumor development (Prasad et al., 2005). In addition, thapsigargin acts as a tumor promoter in a skin carcinogenesis mouse model (Hakii et al., 1986).

There is also mounting evidence that Notch signaling suppresses the transformation of squamous epithelial cells. Genetically engineered mice with decrements in Notch signaling in the skin have a high incidence of skin cancers (Nicolas et al., 2003; Proweller et al., 2006). Recent human clinical trial data revealed that semagacestat, a GSI, is associated with an increased risk of skin cancer (discussed in Crump et al., 2011), possibly due to inhibition of Notch in the skin by chronic GSI administration. Furthermore, Notch pathway loss-of-function mutations have been reported in squamous cell carcinomas of the head and neck (Agrawal et al., 2011; Stransky et al., 2011) and of the skin and lung (Wang et al., 2011). Of note, at least one *NOTCH1* point substitution in human cutaneous squamous cell carcinoma impairs Ca^{2+} binding and folding of EGF repeat 12 (Hambleton et al., 2004; Wang et al., 2011) and a second (R1549Q) impacts folding of the LNRs (Wang et al., 2011), thus recapitulating the proposed pharmacologic effect of thapsigargin. Additional murine studies indicate that Notch signaling may have a tumor suppressive function in other cell lineages as well, including myeloid progenitors (Klinakis et al., 2011) and endothelial cells (Liu et al., 2011; Yan et al., 2010). It is intriguing that several recent studies report SERCA mutations in head and neck squamous cell carcinoma (Korosec et al., 2008; Stransky et al., 2011), in acute myeloid leukemia (Yan et al., 2011), and in other malignancies (Korosec et al., 2009), implicating loss-of-function mutations of SERCA as an additional possible mechanism for Notch inactivation in these diseases. Indeed, while *NOTCH1* mutations enhance sensitivity to SERCA inhibitors, providing a potential therapeutic window for application of this compound class, wild-type Notch1 is also sensitive to SERCA inhibition but at higher concentrations of the compound. One hypothesis to explain a Notch1 and SERCA functional dependency is by a physical interaction. It has been previously shown that presenilin and SERCA2b colocalize in the ER (Green et al., 2008). Since presenilin immunoprecipitation is also reported to preferentially recover the full-length Notch1 precursor prior to Notch1 cleavage in the Golgi (Ray et al., 1999), it is possible that Notch1, SERCA, and presenilin are part of a macromolecular complex.

Toward Translating SERCA Inhibitors to the Clinic

Our studies and other recent work bring to light a challenge in targeting Notch1 in cancer: its pleiotropic roles. *NOTCH1* is an oncogene in some human cancers, such as T-ALL and CLL, whereas it is a tumor suppressor in others, most notably, squamous cell carcinomas. Several strategies have been explored to inhibit Notch1 including the use of GSIs, Notch1-directed antibodies, and direct inhibition of the Notch complex with a hydrocarbon stapling approach (reviewed in Roti and Stegmaier, 2011). Each of these, however, is anticipated to also inhibit wild-type Notch1. One strategy to mitigate potential cancer-promoting effects in nondiseased cells is intermittent dosing of Notch inhibitors. A second approach is the selective targeting of the oncogenic protein. Our results suggest that common heterodimerization domain mutations in Notch1 render the protein more susceptible to the thapsigargin-induced maturation defect,

allowing for a therapeutic window in targeting mutant versus wild-type Notch1 (we observed an antileukemia effect with no measureable gut toxicity). The selective targeting of BRAF kinase bearing an activating V600E mutation by vemurafenib in melanoma is an example of successful application of this strategy (Bollag et al., 2010), although acquired resistance with RAS pathway lesions is common (Nazarian et al., 2010; Su et al., 2012). Similarly, reactivation of Notch1 signaling, for example, by altered EGF/LNR repeats, may pose a resistance mechanism in SERCA-targeted therapy in T-ALL.

Given the pervasive role of calcium signaling in normal physiology, it is unlikely that pan-SERCA inhibitors such as thapsigargin will have an immediate clinical application unless additional development is pursued. One strategy might be the development of isoform-specific small-molecule inhibitors of SERCA. A second is to derivatize thapsigargin for specific delivery to T-ALL cells. This tactic has already been used for a derivative of thapsigargin that is designed to treat prostate cancer, which is currently in clinical trials (NCT01056029 and NCT01734681) (Denmeade et al., 2003). Successful "targeted" delivery of SERCA inhibitors to T-ALL cells would further improve the therapeutic window with this class of drugs and enhance the likelihood of clinical translation.

In summary, this study demonstrates the power of an integrative screening strategy to identify alternative ways to target aberrant transcription factors, identify the modulation of SERCA as a tractable approach for inhibiting Notch1 in Notch-driven cancers, and implicate SERCA mutation as another potential pathogenic mechanism for Notch downregulation in human cancers in which the Notch pathway has a tumor suppressive role.

EXPERIMENTAL PROCEDURES

Full experimental details are in the Supplemental Experimental Procedures.

Cell Culture, Compounds, and Antibodies

Cells were cultured in RPMI 1640 (Cellgro) with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin. Cpd E, thapsigargin, and cyclopiazonic acid were obtained from ENZO Life Sciences; and bepridil hydrochloride, ionomycin, salinomycin, methotrexate, dexamethasone, vincristine and DAPT were obtained from Sigma-Aldrich. We obtained western blot antibodies against Notch1 from Cell Signaling and Santa Cruz Biotechnology, Actin from Thermo Scientific, Vinculin from AbCAM, and GAPDH from Santa Cruz Biotechnology. Antibodies for immunofluorescence include Notch1 [A6] and Giantin (AbCAM). Cell surface Notch1 was evaluated by staining nonpermeabilized cells with monoclonal antihuman Notch1 antibody (R&D).

Notch1 *Off* Signature Detection

Marker and control genes for the Notch1 *on* versus *off* signature were chosen using publicly available Affymetrix microarray expression profiling data sets (GEO ID GSE5716) (Palomero et al., 2006b). Probes are shown in Supplemental Experimental Procedures. The signature was adapted to GE-HTS as described elsewhere (Peck et al., 2006). Signature performance was evaluated by calculating the summed score and weighted summed score (Hahn et al., 2008).

Small-Molecule Library Screening

DND41 cells were incubated with compounds at approximately 20 μM in dimethyl sulfoxide (DMSO) for 72 hr. We screened 3,801 compounds in triplicate, including the BSPBio collection (Prestwick, Biomol, and Spectrum libraries) and the HSCI1 collection (Broad Institute). The GE-HTS assay was performed as described elsewhere (Peck et al., 2006; Stegmaier et al., 2004). Compounds that induce the Notch1 *off* signature were identified using five discrete analytic metrics: summed score, weighted summed score, K-nearest neighbor analysis, naive Bayes classification, and support vector machine as described (Hahn et al., 2009).

cDNA Library Screen and Validation

The cDNA screening strategy involved the use of three key components: (1) a pcDNA3 plasmid encoding a modestly strong *NOTCH1* gain-of-function mutant, L1601PΔP, driven from a CMV promoter; (2) a Notch firefly luciferase reporter; and (3) a preplated cDNA library cloned into the Sport6 plasmid. Luminescence was measured 48 hr postplating.

Viral Transduction

Sequences targeted by each shRNA are listed in Supplemental Experimental Procedures. Viral supernatant production and MigR1 retroviral infections were performed as described (Aster et al., 2000).

Cell Growth, Apoptosis, and DNA Content Assays

Cell growth was assessed using the Promega Cell-Titer Glo ATP-based assay (Promega), apoptosis using a Caspase-Glo 3/7 assay (Promega) or Annexin V and propidium iodide (PI) staining by flow cytometry (eBioscience), and cellular DNA content by staining with PI. Values for IC_{10}, IC_{25}, and IC_{50} (the concentration of an inhibitor where the response is reduced by 10%, 25%, and 50%, respectively) were calculated using Prism 5 Software (Version 5.03).

Reporter Gene Assays

Expression plasmids for L1601PΔP (Weng et al., 2004), L1601PΔP-GAL4 (Malecki et al., 2006), ΔEGFΔLNR (Chiang et al., 2008), and ICN1 (Aster et al., 2000) have been described. Cotransfection of U2OS cells with expression plasmids, a Notch firefly luciferase reporter gene, and an internal Renilla luciferase control gene, was performed as described elsewhere (Aster et al., 2000). A second approach used a Notch1-Gal4 receptor ligand stimulation assay (Malecki et al., 2006).

RT-PCR

Primers and probes for real-time (RT)-PCR were obtained from Applied Biosystems. The data were analyzed using the ΔΔCT method and plotted as percentage of transcript compared to vehicle.

Drosophila Experiments

To generate RAF(gof) tumors in the adult *Drosophila* midgut, we created a stock containing the UAS-RAF(gof) transgene on the X chromosome (Brand and Perrimon, 1994) and the esg-Gal4, UAS-GFP, Tubulin, Gal80(ts) transgenes on the second chromosome (Micchelli and Perrimon, 2006). Drugs were prepared in DMSO, mixed with fly food 1:100, and fed to flies for 7 days. Flies were given freshly prepared drug every 2–3 days. Drug effects were evaluated by immunofluorescence microscopy.

T-ALL Xenograft Studies

MOLT4 xenografts were established by injecting 1.7×10^6 cells subcutaneously into 6-week-old female severe combined immunodeficiency (SCID)-beige mice (Charles River Laboratories). Tumor volume was determined by caliper measurements using this formula: volume = 0.5 × length × weight squared. When tumors reached a mean volume of 75 mm^3, mice were divided into two groups: 0.4 mg/kg thapsigargin or vehicle by intraperitoneal injection daily. Three mice that died prematurely due to drug toxicity were excluded from the study, leaving six evaluable mice in the thapsigargin-treated arm and nine in the vehicle arm. DND41 MigR1 and DND41 MigR1-ICN1 xenografts were established by injecting 10×10^6 cells subcutaneously into NSG mice (n = 20 per line). When tumor volume reached over 50 mm^3, mice were divided into two groups: 0.35 mg/kg thapsigargin or 10 ml/kg vehicle by intraperitoneal injection daily. Mice that were not ready at start of treatment were subsequently added to treatment groups when their tumors reached appropriate sizes. Mice were treated daily through the course of the study, and tumors were measured every 3 days. Five thapsigargin-treated mice were found dead during the course of the study with no prior weight loss or clinical signs of illness. All animal studies were performed under a protocol

approved by the Dana-Farber Cancer Institute Institutional Care and Use Committee.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.01.015.

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