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Selective Blockade of Oncogenic NOTCH1 with the New SERCA Inhibitor CAD204520 in T-cell Acute Lymphoblastic Leukemia

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BACKGROUND

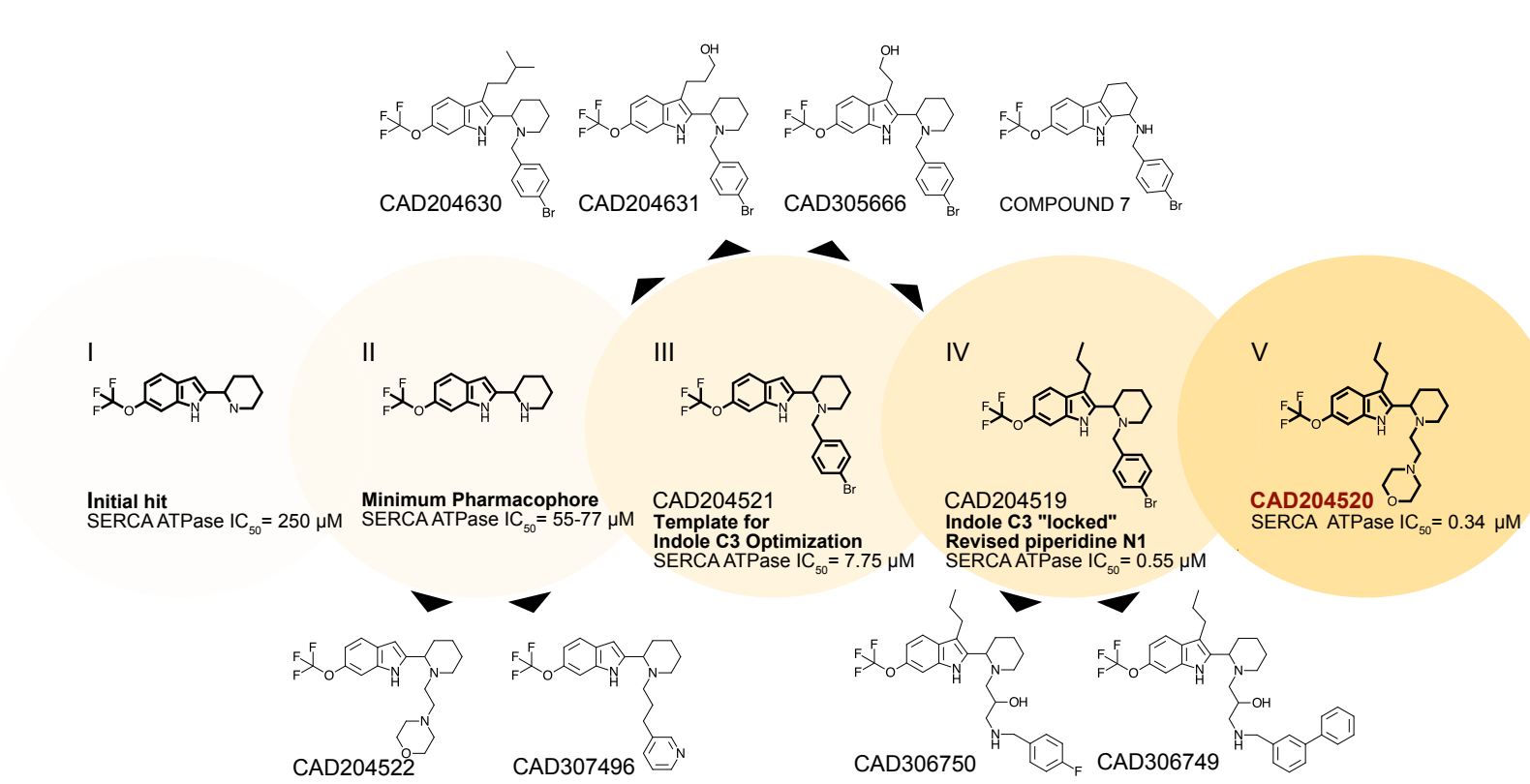
The identification of SERCA (sarco-endoplasmic reticulum Ca²⁺ ATPase) as a target for modulating gain-of-function *NOTCH1* mutations in relevant Notch-dependent disease models such as T-cell acute lymphoblastic leukemia (T-ALL) has spurred the development of new SERCA inhibitors. SERCA plays a critical role in Ca²⁺ regulation particularly in myocytes, thus inhibiting it may increase the risk of heart failure, consequently limiting the development of this compound class for cancer therapeutics. Considering this toxicity challenge, urgent discovery and early optimization of small molecules with better drug-like properties and reduced off-target toxicity is warranted.

In this work, we describe a novel selective potent SERCA inhibitor identified from a series of yeast plasma membrane H⁺-ATPase modulators. Similar to the SERCA inhibitor thapsigargin, CAD204520 inhibits Notch signaling in T-ALL leukemia cells causing an arrest on proliferation and apoptosis. CAD204520 also preferentially targets mutated over wild type NOTCH1 T-ALL proteins thereby overcoming the therapeutic limitation associated with the use of pan-Notch inhibitors. Remarkably however, CAD204520 resulted in an effective treatment in a *NOTCH1* mutated T-ALL *in vivo* model without causing Ca²⁺-related cardiac toxicity.

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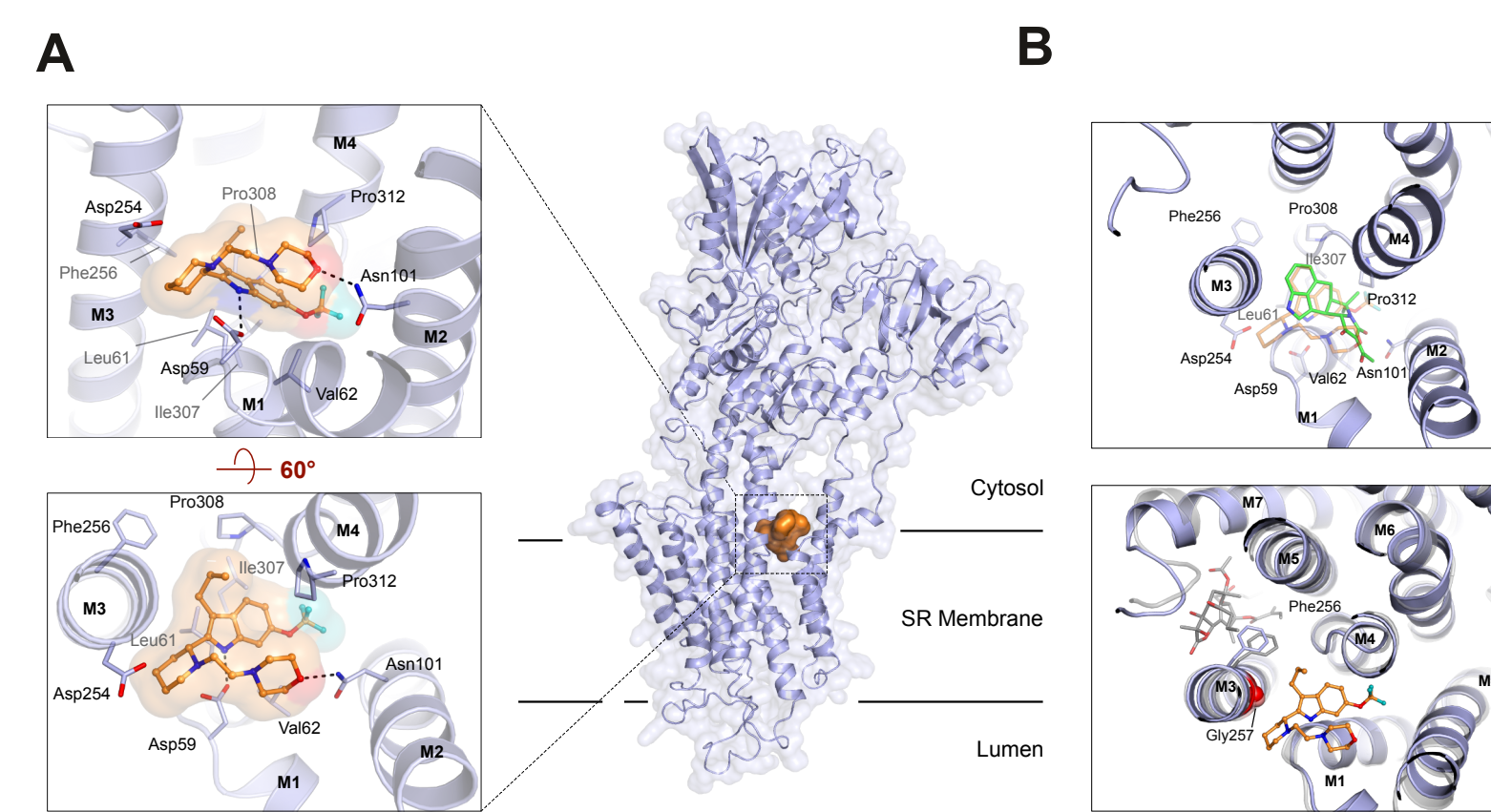
RESULTS

1. Identification of CAD204520



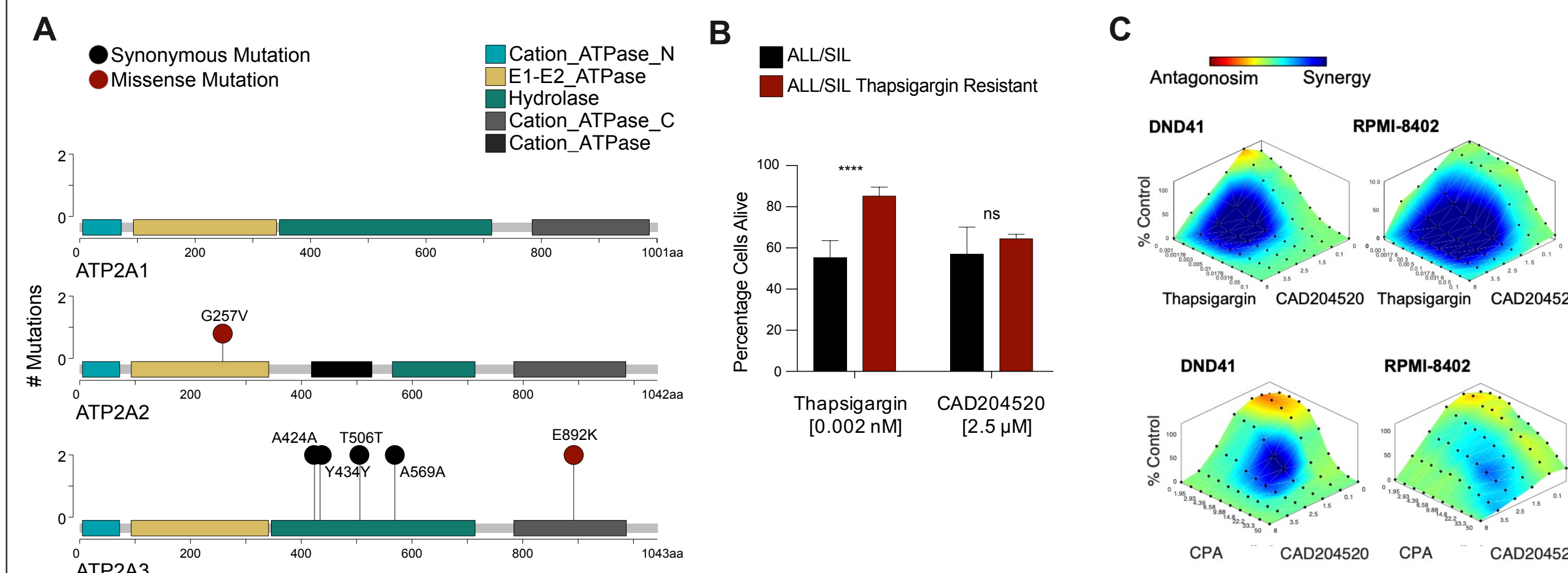
A total of 191,000 small molecules were screened for inhibition of the fungal H⁺-ATPase Pma1. Molecules with an enzymatic inhibition of the H⁺ ATPase that exceed 50% were retained for subsequent hit validation. The 407 molecules were counter screened for (Pma1 over SERCA or Na⁺/K⁺) ATPase selectivity and minimum growth inhibitory capacity (MIC) <150 mM. Hits were then characterized as described in Kjellerup et al. (2017). From a subsequent hit optimization process, indicated in this cartoon, CAD204520 was identified as the most promising candidate against mammalian Ca²⁺-ATPase (SERCA).

2. Crystal of CAD204520-SERCA Binding



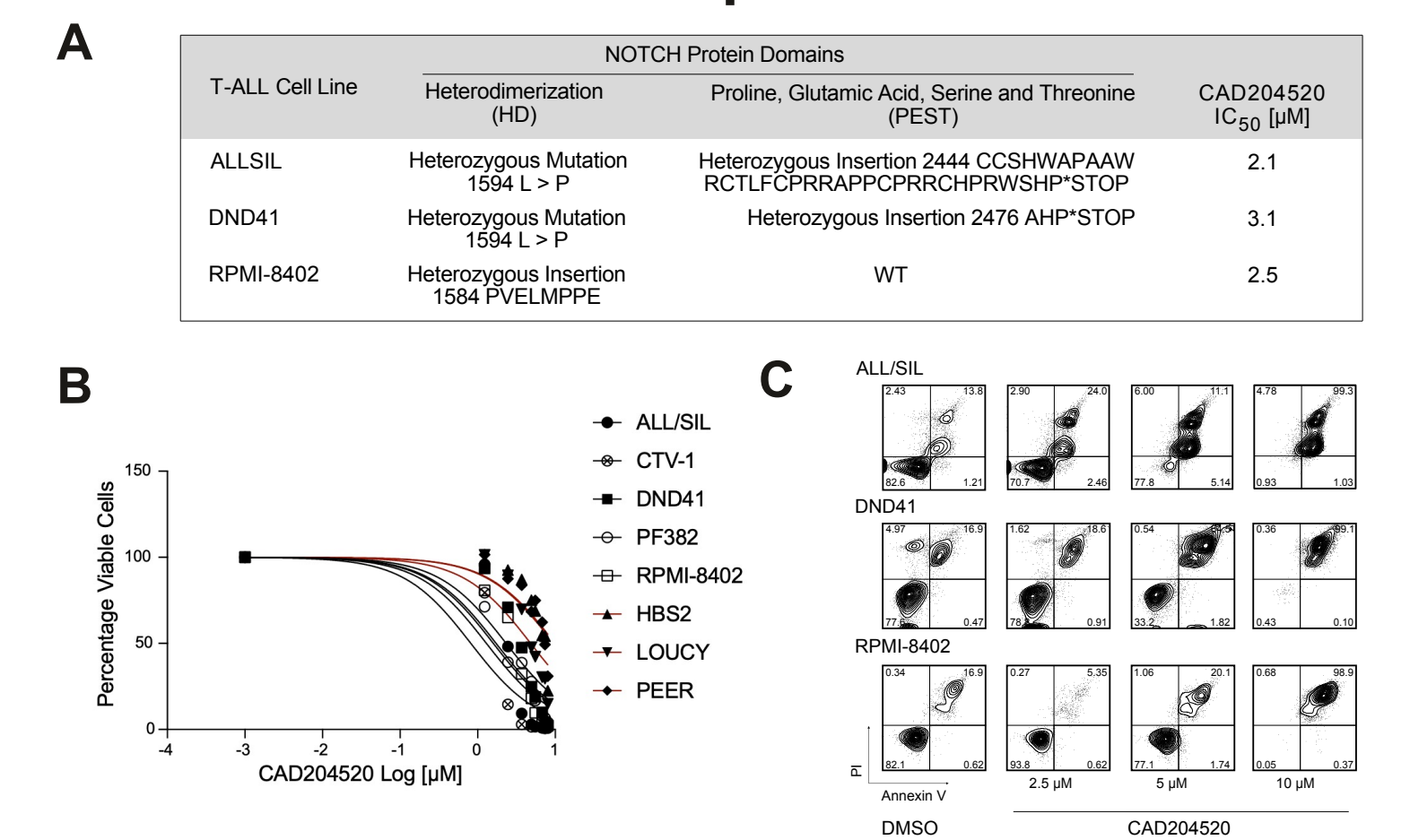
A) Right panel: representation of SERCA (light blue) with CAD204520 bound at the membrane interface (orange). Left panels: close-up of the CAD204520 binding sites **B)** Upper panel: superposition of SERCA-CAD204520 with SERCA-CPA (orange and green sticks, respectively). Lower panel: superposition of SERCA bound to CAD204520 (light blue and orange sticks, respectively) with SERCA bound to thapsigargin (grey cartoon and sticks, respectively).

3. CAD204520 Rescues Thapsigargin Resistance



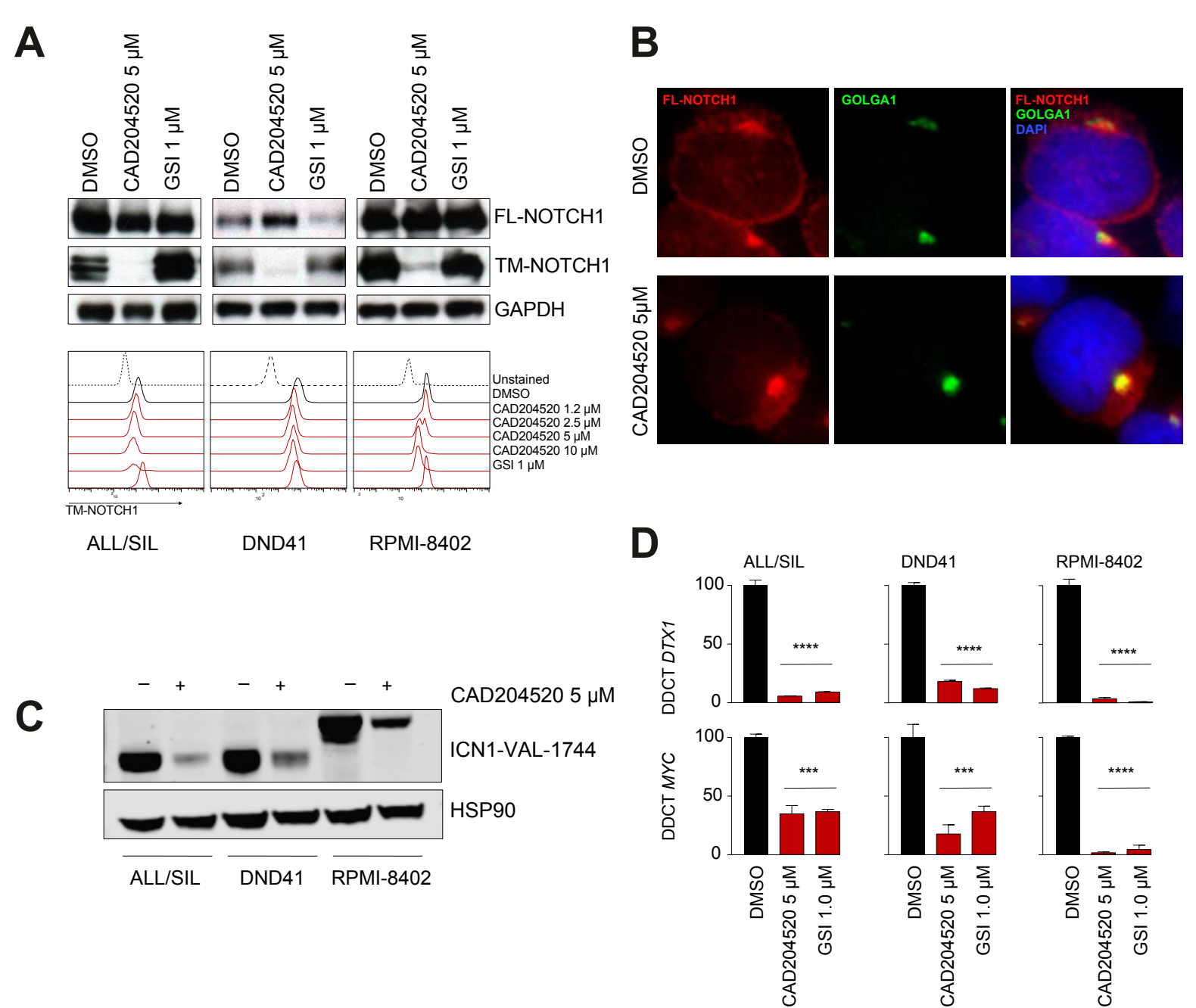
A) Lollipop graphs showing sequenced mutations in the exonic region of ATP2A1, ATP2A2, and ATP2A3 genes of ALL/SIL naive and Thapsigargin resistant. Allelic variants are depicted with a circle (black, synonymous; red, missense). The length of the lollipop (number of mutations) indicates: 0, no mutations; 1 mutations of Thapsigargin resistant ALL/SIL; 2 common mutations. **B)** Histograms show percentage of cells alive after 72 h of treatment at the indicated concentrations (IC₅₀). Statistical significance among groups (****p < 0.0001) was determined by one-way ANOVA. **C)** Surface plots analysis of DND41 and RPMI-8402 T-ALL cell lines treated with CAD204520 plus thapsigargin (top) or CAD204520 plus CPA (bottom).

4. CAD204520 Impairs T-ALL Growth



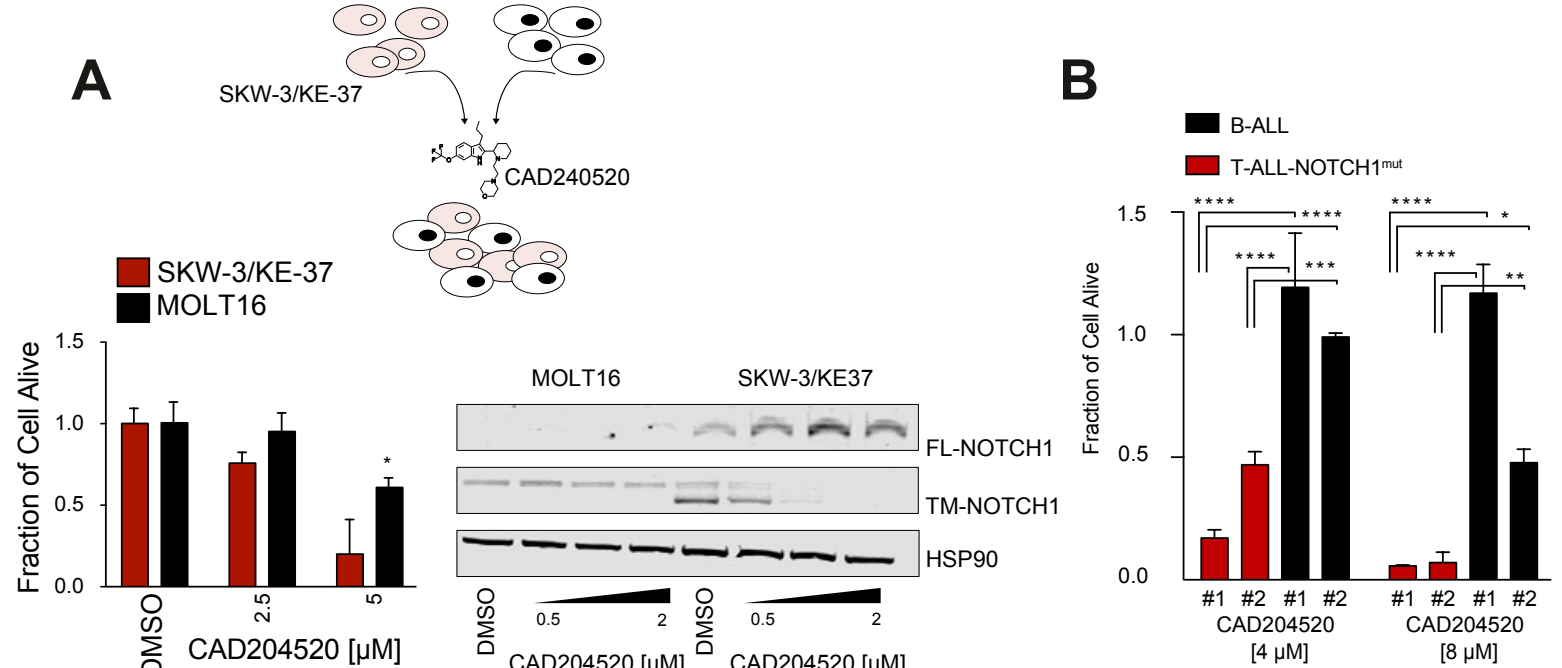
A) NOTCH1 mutational status and IC₅₀ of T-ALL cell lines. **B)** Effect of CAD204520 on cell viability after 72 hours of treatment in the indicated T-ALL cell lines. **C)** Cytofluorimetric assay for the detection of apoptosis of ALL/SIL, DND42, RPMI-8402 treated with CAD204520 at the indicated concentrations.

5. CAD204520 Blocks NOTCH1 Trafficking



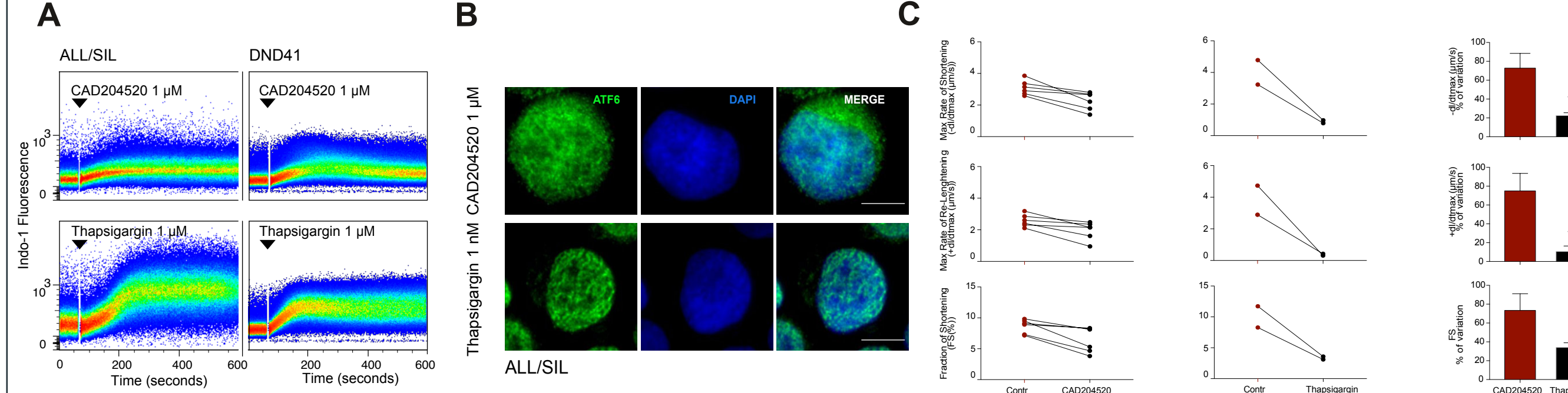
A) Effect of CAD204520 treatment on NOTCH1 (ICN1) processing and activation in T-ALL cell lines by western blot (top) or cytofluorimetry (bottom) or **B)** immunofluorescence. FL = Full length. TM = Trans membrane. **C)** Western blot showing the expression of cleaved NOTCH1 (ICN1) in T-ALL cells treated at the indicated concentration of CAD204520. **D)** qRT-PCR of NOTCH1 target genes in T-ALL cells of T-ALL cells after drugs treatments.

6. CAD204520 Biasedly Inhibits NOTCH1^{mut} T-ALL



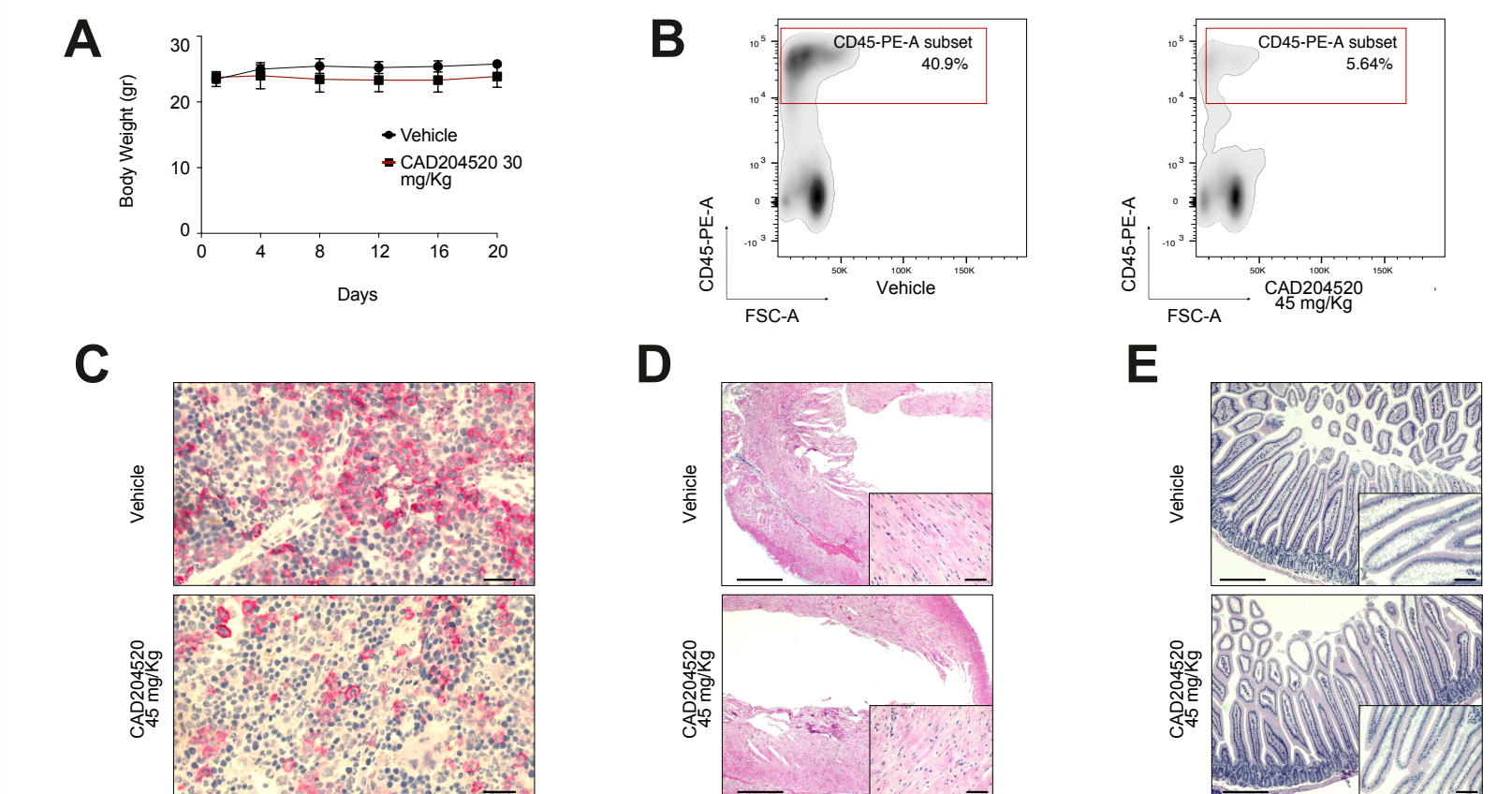
A) Left: effects of CAD204520 on cell viability in MOLT16 (NOTCH1 wt) and SKW-3/KE-37 (NOTCH1^{mut}) T-ALL cell lines. Right: effect of CAD204520 treatment on NOTCH1 (ICN1) processing and activation **B)** Normalized effect of the CAD204520 in primary NOTCH1-mutated T ALL cells (n = 2) or primary B-ALL cells (n = 2) on cellular viability. (***p < 0.001, ****p < 0.0001).

7. CAD204520 does not activate Ca²⁺- mediated UPR



A) Cytoplasmic Ca²⁺ mobilization of T-ALL cells treated with CAD204520 or thapsigargin. Baseline and post-treatment fluorescence is indicated by a black arrow. **B)** ATF6 nuclear migration after CAD204520 or thapsigargin treatment on ALL/SIL cell line (Scale bar, 10 μm). **C)** Left : effect of CAD204520 on rat cardiomyocytes : maximal rate of shortening (dI/dtmax), re-lengthening (+dI/dtmax), and fraction of shortening (FS) Right : mean percentage effect (**p < 0.001, *p < 0.05).

8. Efficacy and Tolerability of CAD204520



(A) Body weight of NSG mice treated CAD204520. **(B)** Effect of CAD204520 on T-ALL leukemia burden in an SKW-3/KE-37-xenograft treated for 4 days. **(C)** HIC analysis of the spleen stained with hCD45. Scale bars, 20 mm. **(D)** H&E of the left ventricle and small intestines **(E)**. Scale bars, 0.2 mm and 0.05 mm (insets).

CONCLUSIONS

Given its oncogenic role in several human cancers Notch signaling has garnered increased attention as a potential therapeutic target. To date, several NOTCH1 modulators have shown therapeutic efficacy in preclinical models. However, despite this promise, few of these candidates have shown a meaningful clinical benefit for patients, in part due to tissue-dependent on-target toxicities from the simultaneous repression of both mutant and wild-type Notch proteins.

The discovery of SERCAs as actionable modulators of Notch1 suggested a new targeted approach to treat NOTCH1- dependent cancer, including T-ALL, MCL, and CLL. Uniquely among Notch modulators, SERCA inhibitors preferentially impair the clinically relevant class of oncogenic NOTCH1 mutants compared with wild type. Thus, the development of tolerated SERCA modulators may uncover a therapeutic avenue for one of the most frequently mutated genes in cancer.



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