Mutations in *LAT* lead to a novel form of severe combined immunodeficiency

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Abstract

Background: Signalling through the T cell receptor (TCR) is critical for T cell development and function. Linker for activation of T cells (LAT) is a transmembrane adaptor signalling molecule that is part of the TCR complex and is essential for T cell development as demonstrated by LAT-deficient mice which show a complete lack of peripheral T cells.

Objective: We describe a pedigree affected by a severe combined immunodeficiency (SCID) phenotype with absent T cells and normal B cells and natural killer (NK) cells. A novel homozygous frameshift mutation in the gene encoding for LAT was identified in this kindred.

Methods: Genetic, molecular and functional analyses were used to identify and characterise the LAT defect. Clinical and immunological analysis of patients was also performed and reported.

Results: Homozygosity mapping was used to identify potential defective genes. Sanger sequencing of the *LAT* gene showed a mutation that resulted in a premature stop codon and protein truncation leading to complete loss-of-function and loss-of-expression of LAT in the affected family members. We also demonstrate loss of LAT expression and lack of TCR signalling restoration in LAT-deficient cell lines reconstituted with a synthetic *LAT* gene bearing this SCID mutation.

Conclusion: The results of this study shows for the first time that inherited LAT deficiency should be considered in patients with combined immunodeficiency with T cell abnormalities.

Clinical implications:

Mutations in *LAT* can lead to severe combined immunodeficiency and LAT should be part of the genetic diagnostic work up in SCID patients.

Capsule Summary:

Severe combined immunodeficiency can arise from a number of different genetic causes. Here, we show that mutations in *LAT* can lead to SCID and highlight the critical role of LAT in TCR signalling in T cell development.

Key words: SCID, LAT, Immunodeficiency, T cell receptor signalling, Genetic defect, T lymphopenia

Abbreviations used

B: B lymphocyte

FACS: Fluorescence-activated cell sorting

GFP: Green fluorescent protein

LAT: Linker for activation of T cells

MOI: Multiplicity of infection

NK: Natural killer cell

OMIM: Online Mendelian inheritance in man

PBMCs: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PHA: Phytohaemagglutinin

SCID: Severe combined immunodeficiency

SNP: Single nucleotide polymorphism

STRs: Short tandem repeats

T: T lymphocyte

TCR: T cell receptor

Introduction

T cell receptor (TCR) signalling is an essential process, both for the development of T cells and also for their activation and function in the periphery. During thymic development, TCR engagement leads to the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic tails of TCR associated subunits, through the action of Src family kinases such as Lck and Fyn. This in turn leads to the recruitment and subsequent activation of the ZAP-70 (chain associated protein tyrosine kinase of 70 kDa) kinase which then phosphorylates specific residues on the TCR adaptor protein (TRAP), LAT (Linker for activation of T cells)[1]. Although it has no intrinsic enzymatic activity, LAT performs a critical function in TCR signal transduction by acting as a scaffold protein and coupling the TCR to downstream signalling pathways through enzyme activation and second messengers including calcium influx through recruitment of other factors and playing an essential role in intracellular Ca^{2+} mobilization, optimal tyrosine phosphorylation of PLC-γ1, Vav and SLP-76, Erk activation, CD69 up-regulation, AP- and NFAT-mediated gene transcription[2,3] and apoptosis[4,5].

Severe combined immunodeficiencies (SCIDs) are a group of immunological disorders that arise from a variety of monogenic defects that lead to an absence of lymphocyte development and function. Overall incidence is estimated to be approximately 1 per 50000 live births and patients with SCID do not survive beyond the first year of life unless immunity can be restored[6]. SCIDs are typically characterized by the abrogation of adaptive immunity and specifically by a low number of autologous T cells (< 300 cells/mm^3). The absence of mature T lymphocytes and B cell dysfunction commonly leads to lethal complications such as opportunistic and non-opportunistic infections, chronic diarrhoea and failure to thrive[7].

A number of genetic basis defects in the TCR signalling pathway have now been shown to lead to a SCID or a less severe CID (combined immunodeficiency – in which there is greater T cell development albeit often with abnormal function) phenotype. This includes mutations in the CD3 subunits (CD3δ, CD3ε, CD3ζ), CD45, and ZAP-70, interleukin-2 inducible T cell kinase (ITK), ORAI calcium release-activated calcium modulator 1 (ORAI-1), stromal interaction molecule 1 (STIM1), magnesium transporter 1 (MAGT1) and LCK[8].

LAT has not previously been associated with a human disease, although it has always been considered a strong candidate for SCID especially since LAT-deficient mice have severe defects in T cell development but normal B cell and NK cell populations[9]. Here we report for the first time 5 patients of a multi-generation consanguineous Pakistani family with a well defined SCID immunophenotype ($T^-B^+NK^+$ SCID) caused by a frameshift mutation in *LAT*.

Methods

Study approval

Genetics studies approved by the UCL Institute of Child Health Research Ethics Committee. Written informed consent was obtained from the patients' parents.

Genomic studies

Genomic DNA from patients and their relatives was extracted from blood according to standard methods (QIAamp DNA mini kit, QIAGEN). Genotypes from DNA of three affected children (V.1, V.12 and V.13), their parents (IV.4, IV.5, IV.11 and IV.12) and four unaffected siblings (V.9, V.10, V.11, V.14) was generated with the single nucleotide polymorphisms (SNP) chip arrays (GeneChip Human Mapping 250K Sty, Affymetrix) according to manufacturer's instructions. Haplotype analysis and homozygosity mapping was done using IBDFinder[10]. Oligonucleotide primers flanking the exons of *LAT* (CCDS 10647) and *CORO1A* encompassing splice sites were used for the polymerase chain reaction (PCR) experiments. Primer sequences are listed in supplementary Table I. PCR products were amplified with the use of Qiagen HotStar Taq DNA polymerase, purified using ethanol/EDTA precipitation and Sanger sequenced with Big Dye v1 (Applied Biosystems) using the forward and reverse primers used for PCR. Sequence alignments were performed and visualised using Sequencher DNA sequencing assembly and analysis software (Genecodes). Single nucleotide polymorphisms (SNP) searches were performed in the National Council for Biotechnology Information dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), the 1000 Genomes project (http://browser.1000genomes.org), the Exome Sequence Project's Exome Variant Server (http://evs.gs.washington.edu/EVS) and Exome Aggregation Consortium (ExAC), Cambridge, MA (http://exac.broadinstitute.org) (December 2015).

Cell culture

The human leukemic Jurkat T cell line (JE6.1)[11] and the derivative LAT-deficient cell lines, ANJ3[3] and J.CaM2[12,13], were maintained in RPMI 1640 (Gibco) supplemented with 10% FCS (Sigma) and 10µg/ml each of penicillin and streptomycin (Gibco), and cultured at 37℃, 5% CO_2.

Immunoprecipitation, Western blot and antibodies

For immunoprecipitation, Jurkat T cells were grown up to a density of $1x10^6$/ml, centrifuged, and serum-starved in RPMI 1640 with 0.1% FCS for 15-20h[14]. Then, about $1x10^8$ cells were either stimulated with anti-CD3ε antibody (OKT3, BioLegend, 50µg/10^8 cells/ml PBS) for 2min at 37℃, or left untreated. Cells were lysed in 2ml of ice-cold lysis buffer containing 1% Brij-97, 10mM Tris (pH7.6), 150mM NaCl, 2mM EDTA, and a cocktail of protease and phosphatase inhibitors (1X)[3]. Proteins of interest were then immunoprecipitated using the Dynabeads Protein G Immunoprecipitation kit (Novex, 10007D) according to the manufacturer's instructions.

For Western blot, protein samples were resolved on SDS-PAGE according to standard protocols, transferred to PVDF transfer membrane (Millipore) and immunoblotted with different antibodies. Protein loads were checked using rabbit anti-ACTIN antibody (Sigma, A2066). Immunoreactive proteins were detected with horseradish peroxidase-coupled secondary antibodies (GE Healthcare) followed by ECL (Thermo Scientific).

The antibodies used in the experiments were: mouse monoclonal anti-Phosphotyrosine (clone 4G10, Millipore, 05-321), mouse monoclonal anti-LAT (abcam, ab57204), rabbit monoclonal anti-Vav proteins (EP482Y, abcam, ab40875), mouse purified anti-SLP-76 (BioLegend, 625002).

Generation of c.44_45insT *LAT* gene-bearing lentiviral vector

The insertion of a T between base 44 and 45 of the *LAT* gene was performed using the GENEART site-directed mutagenesis system (Invitrogen) according to manufacturer's instruction. As a template for the mutagenesis we used a pMA plasmid bearing a synthetic copy of the human *LAT* gene (0957198_LAT_pMA, GENEART) (Suppl. Fig. 1 A). The mutagenesis reaction was performed using two overlapping primers containing the target mutation (Suppl. Fig. 1 C) and the correct insertion of a T between base 44 and 45 of *LAT* was verified by sequencing as shown in Suppl. Fig. 1 D and E. The mutant copy of *LAT* gene was then excised and cloned into a Lent_SF_Ires_GFP_C427 vector (0957199_LAT_Lent_SF_Ires_GFP, GENEART) (Suppl. Fig. 1 B) using BamHI cloning sites. The

Lent_SF_Ires_GFP_C427 vector bearing either the wild-type or the mutant copy of the *LAT* gene was then used to generate lentiviruses by transfecting 293T cells.

Lentivirus production

Both the lentiviral vector bearing a wild-type copy of *LAT* gene (0957199_LAT_Lent_SF_Ires_GFP, GENEART, Suppl. Fig. 1 B) hereafter called as wtLAT-GFP-lent, and the one bearing the mutated copy of *LAT* hereafter as mutLAT-GFP-lent, were used for producing lentivirus according to previously described protocol[15]. Briefly, lentiviral particles were produced by transient co-transfection of 293T cells in 16cm Petri dishes with 10ml Opti-MEM medium (Gibco) containing 50µg of the corresponding lentiviral construct, 17.5µg and 32.5µg of packaging plasmids pMD.G2 (VSVG envelope) and pCMVdR8.74 (gag-pol plasmid) (PlasmidFactory), respectively, using 1µl of 10mM polyethylenimine, for 4h at 37℃, 5% CO_2. Cells were then washed and incubated with 18ml DMEM medium (Gibco) at 37℃, 5% CO_2. The virus particles were harvested 48 and 72 hours after transfection, cleared of cell debris by low-speed centrifugation and filtered using 0.45mm Stericup filters (Millipore). The lentivirus supernatant was then ultracentrifuged at 50000g, 4℃ for 2h and the pellet resuspended in Opti-MEM and stored at -80℃. Virus titres (virus particles per ml concentrated aliquot) were determined in 293T cells transduced with serially diluted lentivirus aliquots by flow cytometry to measure the GFP expression.

Lentiviral transduction of LAT-deficient Jurkat T cell lines

Both ANJ3 and J.CaM2 cells ($2.5x10^5$ cells per well) were covered with 0.4ml medium (RPMI 1640, 10% FCS, 1X Pen/Strep) containing either wtLAT-GFP-lent or mutLAT-GFP-lent lentivirus at MOI 10 [lentivirus particles per cell]. After 24h incubation at 37℃ and 5% CO_2, the supernatant was exchanged to fresh medium and incubated for additional 24h. Cells were then kept in culture for expansion of cell number.

Cytofluorimetric analysis

Following lentiviral transduction, cells were harvested at interval of 6 days, washed, resuspended in PBS and checked for the expression of GFP using a FACSCalibur (BD Biosciences) to quantify the percentage of transduced cells.

Analysis of CD69 expression

Jurkat T cells were either left untreated or stimulated with immobilised anti-CD3ε antibody (OKT3, BioLegend) ON at 37℃. A 24-well-plate was firstly coated with 2µg/ml OKT3 for 1 hour at 37℃, washed and about $1x10^6$ of cells were plated per well for stimulation. Cells were then stained with APC-conjugated anti-CD69 antibody (Miltenyi), washed and analyzed by FACSCalibur (BD Biosciences).

Intracellular Ca^{2+} flux measurement

The measurement for intracellular free Ca^{2+} was performed as described previously[16]. Briefly, Jurkat T cells were harvested from growth medium and $1x10^6$ cells were resuspended in 1ml PBS, 0.5% BSA containing 5µg/ml Indo-1 (Invitrogen) and stained for 30min at 37℃. Cells were then washed and additionally incubated in PBS, 0.5% BSA containing 1mM Ca^{2+} and 1mM Mg^{2+}. Cells were kept on ice before equilibration at 37℃ for 5min directly befo re measurement. Changes in intracellular calcium were monitored using a flow cytometer LSRII (BD Biosciences). Indo-1 is excited by the UV-laser, its emission depends on whether it is bound to calcium (~420nm) or free (~510nm). The ratio of these two wavelengths indicates changes in intracellular calcium concentration. After monitoring the baseline activity for 30sec, the cells were stimulated either with 10µg/ml anti-CD3ε antibody (OKT3, BioLegend) or 10µg/ml ionomycin (Calbiochem) and calcium flux measured for additional 3.30 min. The kinetics of the data acquired were analysed using FlowJo 7.2.2 software (TreeStar).

Apoptosis assay

Jurkat T cells were stimulated with immobilized anti-CD3ε antibody (OKT3, BioLegend) ON at 37℃. A 24-well-plate was firstly coated with 10µg/ml OKT3 ON at 4℃, washed and $0.5\text{-}1x10^6$ cells were plated per well for stimulation. Cells were then stained with anti-human CD95 (APO-1/Fas, eBioscience), anti-

human CD178 (Fas Ligand, TONBO biosciences) and with Annexin V Apoptosis Detection Kit APC according to manufacturer instructions (eBioscience), and analysed by FACSCalibur (BD Biosciences).

Results

Clinical outcomes of the study patients

We describe a multi-generation consanguineous Pakistani family with five patients affected by an autosomal recessive $T^-B^+NK^+$ form of severe combined immunodeficiency (SCID) (Fig 1 A and Table I). Both male and female patients were born to two sets of first cousin parents within the same family. Patients V.1 and V.12 presented within the first three months of life with severe recurrent infections and failure to thrive and V.3, V.4 and V.13 were diagnosed at birth as a result of previous family history. T cell numbers were low (<300 cells/mm^3) with absent proliferation response to PHA as might be expected in patients with such low T cell numbers, but B and NK cell numbers were normal. No NK cell functional analysis was performed. In one patient, V.1, the total number of T, B and NK cells did not equate to the total lymphocyte count. One possible explanation is that the T cell analysis performed was specifically for T cells bearing α/β receptors only and it is possible that there was a significant γ/δ TCR population which can be seen in cases of dysregulation of T cell development, although this was not specifically looked for. Immunoglobulin levels were evaluated soon after birth in three individuals, V.3, V.4 and V.13. IgG was present most probably as a result of maternal transfer, IgA was below the level of detection and IgM levels were at the very low end of the normal range. IL7R alpha and CD3 subunits defects were considered unlikely due to haplotype analysis with microsatellite markers (STRs) and SNP arrays (data not shown). Also, no mutations were found in IL7R alpha by Sanger sequencing of all exons. Gamma chain expression was normal. All affected individuals underwent bone marrow transplant but three individuals V.1, V.3 and V.13 died due to transplant related complications.

Genetic studies

Genomic DNA was available for three affected individuals (V.1, V.12 and V.13), their unaffected parents (IV.4, IV.5, IV.11 and IV.12) and four unaffected siblings (V.9, V.10, V.11, V.14) for single nucleotide polymorphism (SNP) arrays. Haplotype analysis and homozygosity mapping in the family suggested that the disease gene localises to a 23.48 Mb region on chromosome 16 flanked by markers rs4787441 and rs10852513. Two genes within the region were prioritised based on information available in the literature and OMIM. Coronin Actin Binding Protein1A (*CORO1A*; MIM *605000) was selected as a strong

candidate for its causative involvement in immunodeficiency 8 (MIM #615401) characterised by recurrent infections and decreased number of lymphocytes but no causative variants were identified. Linker for activation of T cells (*LAT*; MIM *602354) also co-localized to this region and on sequencing of *LAT* coding regions in the family, we found all patients were homozygous for a recessive frameshift mutation in *LAT* exon 1 and both sets of parents were heterozygous for this variant (Fig 1 A and 1 B). As demonstrated in Fig 1 C, exon 1 is highly conserved across multiple vertebrate species. An insertion of a T between positions 44 and 45 in the coding sequence causes a frameshift and a putative stop codon 28 amino acids downstream (c.44_45insT; p.Leu16AlafsX28). The nucleotide insertion was not recorded in publically available databases NCBI dbSNP, 1000 Genomes Project, the Exome Sequence Project's Exome Variant Server or the Exome Aggregation Consortium (ExAC). To exclude the possibility of the insertion being a rare polymorphism present in the Pakistani population, genomic DNA from 150 ethnically matched controls representative of a total of 300 chromosomes were also sequenced and found to be wild-type for the insertion (data not shown). A further 15 SCID patients were sequenced but failed to reveal any pathogenic variants in *LAT*.

The variant results in a predicted truncation of the LAT protein. LAT is known to be expressed in peripheral blood lymphocytes, spleen and thymus, as well as in other blood cell types, notably platelets, megakaryocytes, mast cells and natural killer cells[17]. We therefore performed Western blot analysis for the expression of LAT in peripheral blood mononuclear cells (PBMCs) from patient V.4 and from a control blood sample (Fig 1 D). To control for the lack of T cells in the patient sample, control PBMCs were further processed for isolation of CD3 positive ($CD3^+$) and negative ($CD3^-$) cells. For each sample lysate from 5×10^6 cells was loaded in the gel. 293T cells and HeLa cells were also used as negative controls. As shown, even in control $CD3^-$ cells, LAT can be detected whereas it is not detectable in the patient PBMCs. It is likely that, because the premature termination codon is close to the 5'-end of the protein there is non-sense mediated decay and complete loss of protein expression.

Reconstitution of LAT-deficient Jurkat T cell lines

The absence of primary patient material led us to perform *in vitro* studies to demonstrate the functional effect of the observed LAT mutation. We generated a synthetic mutant copy of human *LAT* by site-directed mutagenesis and generated lentiviruses bearing wild-type (wtLAT-GFP-lent) or mutant (mutLAT-GFP-lent) *LAT*. We then used both LAT-encoding lentiviruses to transduce two different Jurkat-derived TCR-signalling mutants[2] both lacking expression of *LAT* gene, ANJ3[3] and J.CaM2[12,13,18] (Fig 2 A). The efficiency of transduction was evaluated by FACS analysis of the green fluorescent protein (GFP) expressed by the lentiviral vectors. One month after transduction, the percentage of GFP-positive cells were more than 85% in all the four cultured clones, ANJ3 and J.CaM2 transduced by either wtLAT-GFP-lent (hereafter as ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent) (Fig 2 B, upper panel) or mutLAT-GFP-lent (hereafter as ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent) (Fig 2 B, lower panel) lentivirus. As expected, both ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent clones also efficiently expressed LAT. On the contrary, the two clones transduced with the lentiviruses bearing the frameshift mutation in the *LAT* gene, ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent were not able to express LAT as demonstrated by the Western-blot (Fig 2 A). Taken together these data confirmed the lentiviral vector efficacy in mediating gene transfer and expression in Jurkat T cell lines and confirmed in an *in vitro* system that the insertion of a T between base 44 and 45 of the *LAT* gene prevents its expression.

CD69 up-regulation and intracellular Ca^{2+} mobilization after TCR stimulation

CD69 is a marker for T cell activation and its expression is rapidly induced in a Ras-dependent manner following TCR stimulation in Jurkat T cells and easily detectable by FACS analysis[19]. Stimulation with the anti-CD3ε antibody OKT3 induced CD69 up-regulation in JE6.1 control cells, but failed to induce CD69 expression in ANJ3 and J.CaM2 cells as shown in Fig 2 C (upper panel) and as previously reported[3,18]. To confirm that the failure of CD69 up-regulation was due to loss of LAT, both ANJ3 and J.CaM2 cells were transduced with a lentivirus containing a copy of the *LAT* gene (wtLAT-GFP-lent) as described above. Stably transduced cell lines reconstituted with wild-type *LAT*, ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent, showed restored expression of CD69 on the cell surface following anti-CD3ε antibody stimulation (Fig 2 C, middle panel). On the contrary, cell lines ANJ3/mutLAT-GFP-lent and

J.CaM2/mutLAT-GFP-lent reconstituted with 44_45insT *LAT* , failed to restore full CD69 expression following TCR signalling stimulation with OKT3 (Fig 2 C, lower panel).

Stimulation of TCR signalling with OKT3 also failed to induce Ca^{2+} mobilization in both LAT-deficient Jurkat T cell lines, ANJ3 (Fig 2 D, upper panel) and J.CaM2 (Suppl Fig 2 A, upper panel) as previously reported[3,18]. Similarly, LAT-deficient Jurkat T cell lines reconstituted with the mutant copy of *LAT*, ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent, failed to induce calcium flux following OKT3 stimulation (Fig 2 D and Suppl Fig 2 A, lower panels). On the contrary, control JE6.1 (Fig 2 D, upper panel) and wild-type *LAT* reconstituted cells, ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent (Fig 2 D and Suppl Fig 2 A, middle panels) showed a clear Ca^{2+} mobilization response to anti-CD3ε antibody stimulation. As expected, all Jurkat T cell lines, despite expression or not of LAT, were able to increase intracellular calcium levels following stimulation with ionomycin, a pharmacological agent which mobilizes intracellular calcium in a TCR-independent manner (Suppl Fig 2 B).

Altogether these results show that re-expression of LAT in ANJ3 and J.CaM2 following lentivirus transduction, restore TCR signalling, confirming the pivotal role of LAT in T cell activation. The mutant LAT in the described SCID patients is not able to restore these functions.

Mutant LAT reconstitution does not restore the tyrosine phosphorylation on downstream Vav and SLP-76.

A clear role for LAT in regulating tyrosine phosphorylation of intracellular proteins during TCR engagement has already been shown[3]. Thus, to confirm optimal restoration of TCR signalling in ANJ3 and J.CaM2 lines reconstituted with wild-type *LAT*, cells were stimulated with OKT3 and tyrosine phosphorylation was detected by probing protein samples with anti-Phosphotyrosine antibody 4G10 (Fig 3 and Suppl 3). The tyrosine phosphorylated LAT molecule was not detected in cellular lysates prepared from ANJ3 and ANJ3 cells reconstituted with mutant *LAT* (ANJ3/mutLAT-GFP-lent), but as expected was observed in ANJ3 reconstituted with wild-type *LAT* (ANJ3/wtLAT-GFP-lent) (Fig 3 A). Importantly, reconstitution of wild-type *LAT* in ANJ3 and J.CaM2 cells restored the tyrosine phosphorylation of two proteins downstream LAT, Vav and SLP-76, that are involved in the signal transduction cascade initiated

by engagement of the TCR[2]. Tyrosine phosphorylation of these proteins was absent in protein samples from stimulated ANJ3, J.CaM2 and cell lines reconstituted with mutant *LAT* (Fig 3 B and C, and Suppl 3).

Absence of LAT prevents TCR-induced apoptosis and is not restored by mutant LAT

Upon TCR cross-linking, a number of studies have shown that LAT can be a negative regulator of TCR signalling, including mutant LAT knock-in models which show a T lymphoproliferative disease[5,20-22]. We stimulated JE6.1 and LAT-deficient ANJ3 and J.CaM2 cell lines with immobilized OKT3 and showed that there was significantly reduced apoptosis in mutant lines than in JE6.1 control cells (Fig 4 A and B). In reconstituted lines, introduction of wild type *LAT* in ANJ3/wtLAT-GFP-lent and J.CaM2/wtLAT-GFP-lent led to a significant increase in the level of apoptosis after T cell receptor stimulation whereas LAT-deficient lines transduced with mutant LAT, ANJ3/mutLAT-GFP-lent and J.CaM2/mutLAT-GFP-lent, did not show any increase in levels of apoptosis (Fig 4 C and D). The expression levels of death factors such as Fas and Fas ligand[4,23-27] (FasL) in ANJ3 and J.CaM2 lines were similar to that observed in the wild type Jurkat line JE6.1 (Suppl Fig 4). These studies support suggestions that TCR stimulation leads to T cell apoptosis that is dependent on functional LAT expression and that expression of the mutant LAT in LAT-deficient lines does not restore apoptosis whereas wild type LAT is able to restore apoptosis.

Discussion

Genetic and molecular studies of SCIDs have led to the identification of mutations in many different genes that impair T lymphocyte differentiation and result in the clinical and immunological phenotype of SCID. A number of different mechanisms have been identified including: i) lymphocyte cell death triggered by purine metabolism abnormalities, ii) defective signalling through the common γ-chain-dependent cytokine receptors, iii) defective V(D)J recombination and iv) defective pre-TCR/TCR signalling[28]. Mutations in key proteins involved in pre-TCR/TCR signalling are indeed responsible for rare cases of SCID consisting of pure T-cell deficiencies. These defects account for about 2% of individuals with SCID. Patients with defects in the hematopoietic-specific transmembrane protein tyrosine phosphatase CD45, required for efficient lymphocyte signalling, are severely immunodeficient and have very few peripheral T lymphocytes unresponsive to mitogen stimulation[29,30]. Similarly, deficiency of CD3ε[31-34], CD3δ[33,35,36] and CD3ζ[37-40] subunits of the pre-TCR/TCR signalling complex cause near complete arrest of T-cell development and severe immunodeficiency. Further downstream, defects in ZAP-70, interleukin-2 inducible T cell kinase (ITK), ORAI calcium release-activated calcium modulator 1 (ORAI-1), stromal interaction molecule 1 (STIM1), magnesium transporter 1 (MAGT1) and LCK have been reported but the immunophenotype in these individuals shows evidence of T cell development although these T cells are abnormal and in some cases associated with severe immune-dysregulatory phenomena[8]. This report documents the first cases of LAT deficiency in a multi-generation consanguineous Pakistani family due to an insertion of a T between positions 44 and 45 in the coding sequence (c.44_45insT; p.Leu16AlafsX28) that causes a frameshift and a putative stop codon 28 amino acids downstream. The immunological phenotype in these individuals is characterized by a significant lack of T cell development and highlights the critical and non-redundant role of LAT in the regulation of T cell development.

The human immunophenotype observed here is very similar to that observed in a murine knock-out model. In this model, there is a complete lack of peripheral T cells but LAT-deficient mice have normal B cell populations and no gross abnormality of NK numbers or function[9]. Further analysis of these mice shows that thymocyte development is arrested at the $CD4^-CD8^-$ double negative (DN) stage and

$CD4^+CD8^+$ double positive (DP) and $CD4^+$ or $CD8^+$ single positive (SP) thymocytes were undetectable. More specifically, the arrest is at the $CD25^+CD44^-$ DN3 stage and indicates the absolute requirement for LAT in the pre-TCR-mediated signal transduction that is required for further thymocyte development. The role of LAT beyond the DN3 stage has also been studied by the generation of LAT knock-in mice in which the *LAT* gene could be deleted upon expression of the Cre recombinase at later stages of thymocyte development[41]. These mice developed DP thymocytes but there was a significant block in the SP thymocytes and consequently a significant loss of mature peripheral T cells. In addition, there were abnormalities of Treg development and function which is also supported by other studies[42]. Together these murine studies highlight the essential role of LAT in early pre-TCR and later thymocyte development.

Mechanistically, it has already been shown that upon stimulation of the TCR, LAT becomes phosphorylated at several tyrosines residues on its cytoplasmic tail. This leads to the binding of SH2 domain-containing proteins and their associated molecules and the formation of large multiprotein complexes located at the plasma membrane[43,44]. These dynamic and highly regulated signalling complexes facilitate the production of second messengers, activate downstream pathways such as intracellular Ca^{2+} mobilization, induce actin cytoskeleton polymerization, and stimulate the activity of multiple transcription factors[1,2]. Thus, signalling pathways from the pre-TCR or TCR feed into LAT, which then integrates this information and selectively induces pathways critical for thymocyte development and T cell activation. In peripheral cells, there is evidence to suggest that LAT can also act as a negative regulator of TCR mediated signalling and that T cell apoptosis is dependent on a functional LAT protein. In the absence of LAT, there is reduced apoptosis, which in murine LAT mutant knock in models leads to lymphoproliferation[5,20-22].

In the absence of primary material (other than a limited source of PBMCs) from our patients who had either deceased or had undergone haematopoietic stem cell transplantation, we chose to demonstrate the non-functional effect of the patient mutation in specific cell lines. We used two LAT mutant Jurkat T cell lines, ANJ3[3] and J.CaM2[12,13], that are defective in TCR-mediated signal transduction. Although initial

TCR-mediated signalling events such as the inducible tyrosine phosphorylation of the CD3-ζ chain and ZAP-70 are intact, subsequent events, including phosphorylation of LAT downstream proteins such as SLP-76, Vav and PLCγ1, increases in intracellular Ca^{2+}, Ras activation, CD69 upregulation and IL-2 gene expression and apoptosis are defective in these mutant cell lines[3,18]. Through lentiviral gene transfer, we introduced either the wild-type or the observed mutant *LAT* gene into these mutant lines and determined the functional consequences. Both reconstituted cell lines that expressed wild-type *LAT* had levels comparable to that of Jurkat T cells (JE6.1) and all LAT downstream TCR-inducible signalling events were reconstituted. We also show failure to express LAT protein and loss of TCR-induced signalling and restoration of apoptosis in ANJ3 and J.CaM2 cells transduced by lentiviruses bearing a copy of *LAT* gene with the frameshift mutation. We thus confirmed in an *in vitro* system the loss of LAT expression as a consequence of an insertion of a T between positions 44 and 45 in the coding sequence. The frameshift and a putative stop codon 28 amino acids downstream resulted in a truncation of the LAT protein with complete loss of function. Needless to say, in cell lines in which the patient mutant LAT was expressed, there was no evidence of recovery of TCR downstream signalling events. We would therefore infer that there is a similar loss of pre-TCR and TCR signalling in these patients that would lead to and explain the lack of T cell development.

This is the first report of *LAT* mutations leading to SCID. The genetic data is highly significant in that it shows the presence of a highly deleterious variant that, in both primary patient cells and after *in vitro* expression, leads to the absence of LAT protein expression. The mutation co-segregates with disease and is found in heterozygous form in unaffected carriers and family members. No evidence of this mutation was identified in a number of different human publically available databases. Importantly, when we studied 300 chromosomes from an ethnically-matched cohort, the mutation was not identified and demonstrates that this variant is not a polymorphism but is a founder mutation that is carried in this consanguineous pedigree. The expression of this mutant in LAT-deficient cells showed the absence of protein expression and an inability to reconstitute LAT specific signalling events. Together these data argue strongly that the *LAT* mutation identified leads to the SCID immunophenotype in this pedigree.

Although, many gene defects have been identified as causing SCID, recent data from prospective SCID screening studies show that there are still >10% of SCID patients that remain genetically undiagnosed[6]. It is likely that, as in this family, mutations in other genes already known from murine and other studies to be critical for T cell development, will be soon be identified.

Acknowledgements

We are grateful to Prof. Weiguo Zhang for providing ANJ3 cell line and Dr. Marco Purbhoo for J.CaM2 cell line. HBG is supported by the Great Ormond Street Hospital Children's Charity. This study was supported by the National Institute of Health Research Biomedical Research Centre at Great Ormond Street Hospital and University College London.

Figure legend

Figure 1. A frameshift mutation in *LAT* leads to loss of LAT expression in patients' T cells. (**A**) Extended consanguineous pedigree. (**B**) Sequence analysis of genomic *LAT* in a control, unaffected heterozygous individual IV.5 and homozygous mutation in patient V.12 (**C**) LAT protein first 43 amino acids sequence showing frameshift and premature termination codon (*) in red letters in *LAT* exon 1 at position 43. Protein homology of LAT in various species (**D**) LAT expression in PBMCs from patient and control.

Figure 2. Reconstitution of LAT-deficient Jurkat T cell lines. (**A**) Analysis of LAT expression in Jurkat T cell lines. (**B**) Lentiviral vector gene expression in reconstituted Jurkat T cell lines by FACS analysis of GFP. (**C**) CD69 expression in Jurkat T cell lines unstimulated (red line) or stimulated (green line) with OKT3. (**D**) Ca^{2+} mobilization in JE6.1 and ANJ3 cell lines unstimulated (control) or stimulated with OKT3.

Figure 3. Tyrosine phosphorylation of LAT, Vav and SLP-76 in ANJ3 cell lines. (**A**) Cellular lysates from ANJ3 cell lines unstimulated (-) or OKT3-stimulated (+) were analysed by immunoblotting with anti-Phosphotyrosine (pY), anti-LAT and anti-ACTIN. (**B**) Vav and (**C**) SLP-76 were immunoprecipitated from unstimulated (-) or OKT3-stimulated (+) ANJ3 cell lines with antibodies against each individual protein. Immunoprecipitates were then analysed by immunoblotting with anti-pY and antibodies against each individual protein.

Figure 4. Apoptosis assay on Jurkat T cell lines. (**A**) Jurkat T cell lines were stimulated with OKT3, then stained with Annexin V and propidium iodate (PI) and analysed by FACS. Percentages of early stage apoptotic cells (Annexin V^+/PI^-) are plotted in (**B**), (**C**) and (**D**). P-values for statistical significance are indicated as (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (***) $p \leq 0.001$.

Table I. Clinical and Immunological features

Patient	P V.1	P V .3	P V.4	P V.12	P V.13
Gender	M	F	F	F	M
Year of birth	2004	2009	2012	2000	2005
Clinical presentation	Recurrent infections	Diagnosed at birth	Diagnosed at birth	Recurrent infection and failure to thrive	Diagnosed at birth
BMT donor and conditioning	Parental haploidentical 1st BMT – unconditioned 2nd BMT – Melphalan only	MUD Busulfan Fludarabine	MUD	MSD	MSD unconditioned
Outcome	Died – multi-organ failure post 2nd BMT	Died – cardiac arrest and multi-organ failure following conditioning	Alive and well	Alive and well	Died – severe acute and chronic GvHD and respiratory compromise
Immunological features at presentation					
Absolute lymphocyte count (cells/mm^3)	900	960	1190	N/A*	2610
CD3+ – T cells (cells/mm^3)	20	10	6	N/A*	300
CD19+ – B cells (cells/mm^3)	620	440	428	N/A*	1440
CD16+/CD56+ – NK cells (cells/mm^3)	140	450	666	N/A*	780
CD3+CD4+ (cells/mm^3)	10	0	4	N/A*	210
CD3+CD8+ (cells/mm^3)	0	0	0	N/A*	30
PHA response (SI)	1.9	4.8	N/A	N/A*	3.1
IgG (g/l) **(normal 1 – 3.3)	N/A	8.23	6.09	N/A*	6.8
IgA (g/l) (normal 0.07 – 0.37)	N/A	<0.06	<0.07	N/A*	<0.07
IgM (g/l) (normal 0.26 – 1.22)	N/A	0.28	0.20	N/A*	0.17

*Presentation immunology not available as patient referred from remote hospital after transplantation had already been undertaken

**normal ranges taken from the Mayo clinic reference range (http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/8156)

MUD – matched unrelated donor transplant

MSD – Matched sibling donor transplant

480 BMT – bone marrow transplantation
481 SI – stimulation index
482 N/A – not available
483

484

485

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Supplementary Figure 1. Generation of 44_45insT *LAT* gene-bearing lentiviral vector. Map of (**A**) pMA plasmid bearing a synthetic copy of human *LAT* gene and of (**B**) lentiviral vector bearing GFP and *LAT* genes (GENEART). (**C**) The overlapping primers designed for the side-directed mutagenesis. DNA sequences of wild-type (**D**) and mutant (**E**) *LAT* gene-bearing lentiviral vectors at the site of the insertional mutagenesis.

Supplementary Figure 2. Restoration of calcium flux in J.CaM2 cells and ionomycin-induced mobilization of calcium. Ca^{2+} mobilization in (**A**) J.Cam2 cell lines loaded with Indo-1 and stimulated with OKT3 or kept unstimulated (control) and in (**B**) Jurkat T cell lines loaded with Indo-1 and stimulated with ionomycin or kept unstimulated (control). The ratio of the fluorescence emission of Ca^{2+}-bound and Ca^{2+}-free Indo-1 was plotted as a function of time after stimulation.

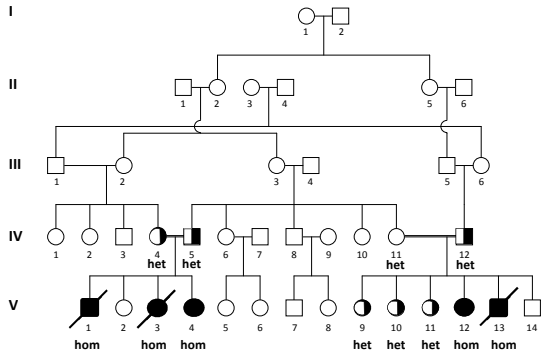
Supplementary Figure 3. Tyrosine phosphorylation of SLP-76 and Vav in J.CaM2 T cell lines. (**A**) Cellular lysates from JE6.1 and J.CaM2 cell lines unstimulated (-) or OKT3-stimulated (+) were analysed by immunoblotting with anti-Phosphotyrosine (pY), anti-SLP-76 and anti-ACTIN. (**B**) Vav was immunoprecipitated from unstimulated (-) or OKT3-stimulated (+) J.CaM2 cell lines with an antibody against Vav protein. Immunoprecipitates were then analysed by immunoblotting with anti-Phosphotyrosine (pY) and anti-Vav antibodies.

Supplementary Figure 4. Expression analysis of death-factors Fas and FasL in Jurkat T cell lines. JE6.1 and LAT-deficient ANJ3 and J.CaM2 cell lines were unstimulated (green line) or stimulated with OKT3 (blue line), then stained with

antibodies against CD95/Fas (**A**) or CD178/Fas Ligand (**B**) and with isotype controls (shaded areas).

Fig. 1

A



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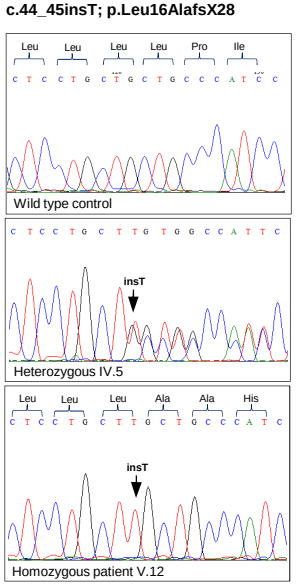

C

Protein sequence

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p.Leu16AlafsX28    1 MEEAILVPCVLGLLLAAHPGHVDGTVCALPQTARLLRQHILR* 43

Human              MEEA-ILVPCVLGLLLLPILAML-MALCVHCHRLPGSYDSTSSDS
Mouse              MEAD-ALSPVGLGLLLLPFLVTLLAALCVRCRELPVSYDSTSTES
Pan                MEEA-ILVPCMLGLLLLPILAML-MALCVHCHRLPGSYDSTSSDS
Gorilla            MEEA-ILVPCVLGLLLLPILAML-MALCVHCHRLPGSYDSTSSDS
Rattus             MEAD-ALSPVELGLLLLPFVVMLLAALCVRCRELPASYDSASTES
Felis              MEAV-VLIPYVLGLLLLPLLA---VVLCVRCRKLPGFYDHTASDS
Canis              MEAV-VLIPYMLGLLLLPLLA---VVLCVRCRELPGSYDSTASDS
Cavia              MEENSVVLSEPLGLLLLPLIAMLLMVLCVRCRELRGSYGSASENR
Zebrafish          MDSI-ELLSMVGGLLLLSIIFAT--GLCTYCWGHKQPTSIPQRPS
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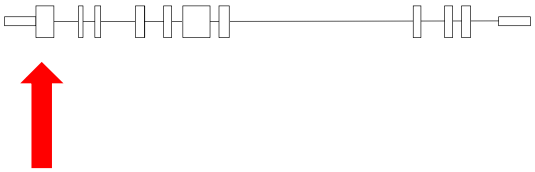


p.Leu16fs

D

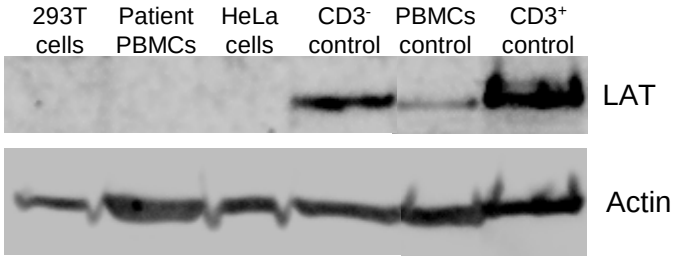


Fig. 2

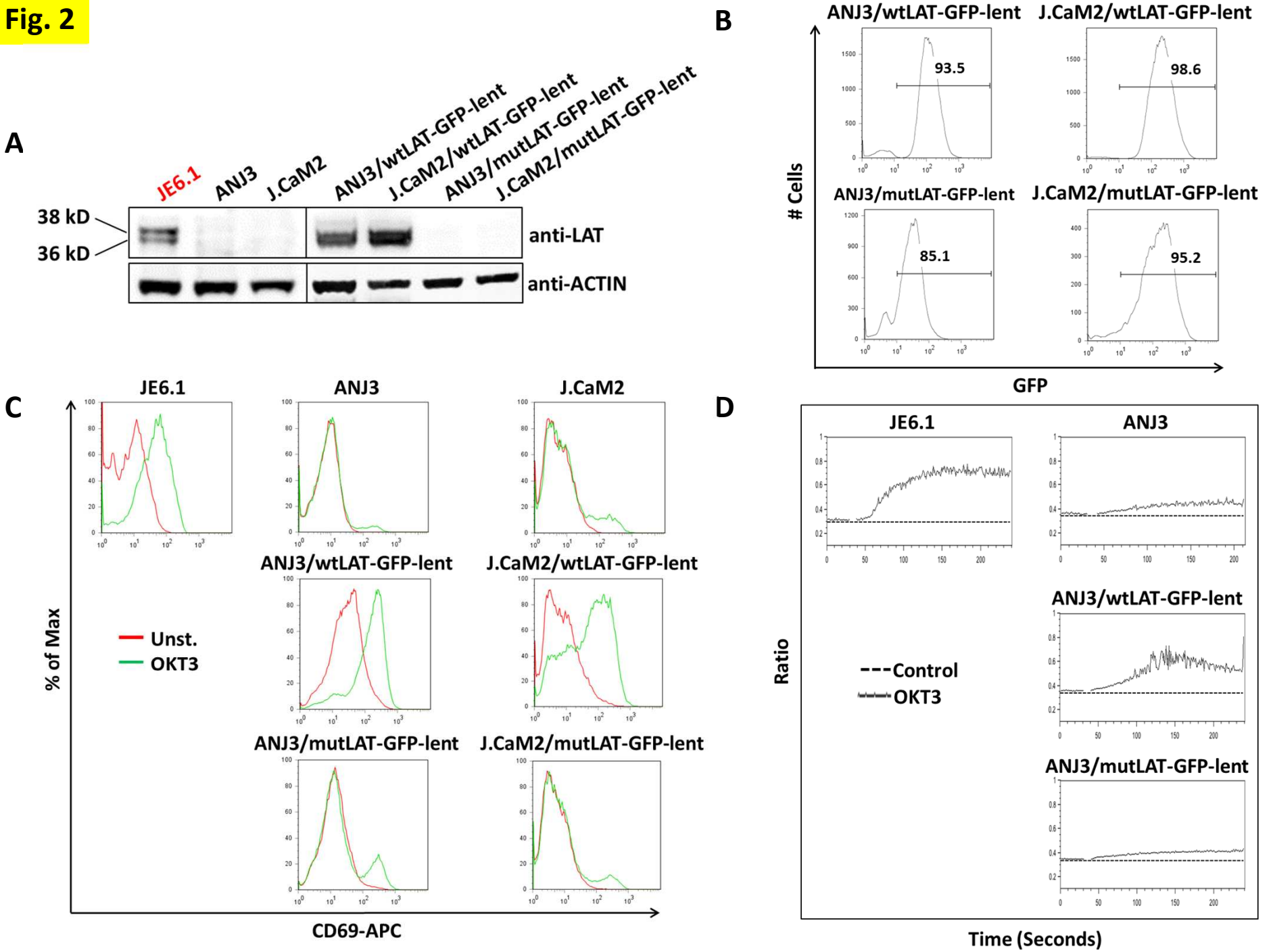
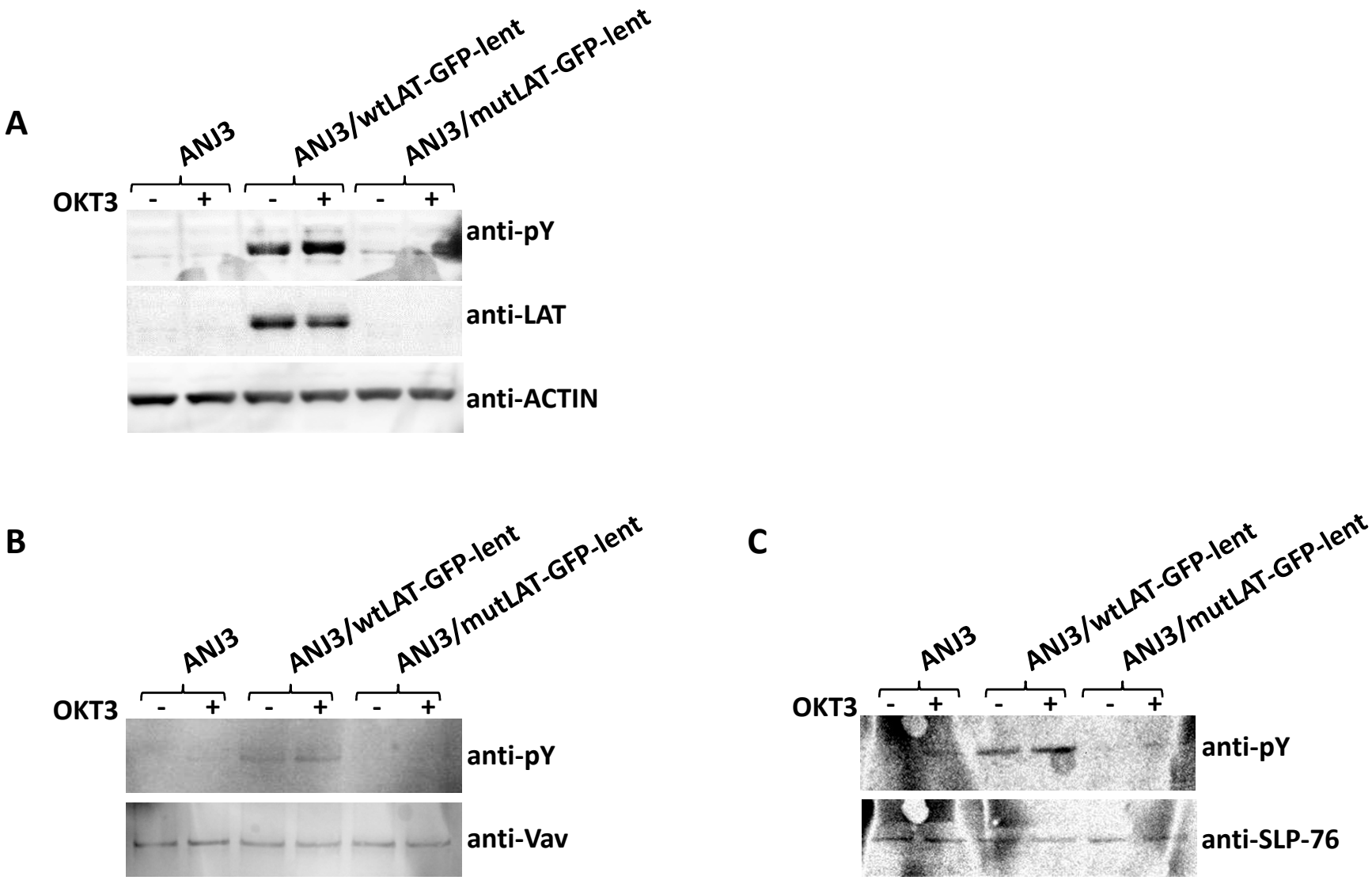

A
JE6.1
ANJ3
J.CaM2
ANJ3/wtLAT-GFP-lent
J.CaM2/wtLAT-GFP-lent
ANJ3/mutLAT-GFP-lent
J.CaM2/mutLAT-GFP-lent
38 kD
36 kD
anti-LAT
anti-ACTIN
B
ANJ3/wtLAT-GFP-lent
J.CaM2/wtLAT-GFP-lent
93.5
98.6
ANJ3/mutLAT-GFP-lent
J.CaM2/mutLAT-GFP-lent
85.1
95.2
Cells
GFP
C
JE6.1
ANJ3
J.CaM2
ANJ3/wtLAT-GFP-lent
J.CaM2/wtLAT-GFP-lent
ANJ3/mutLAT-GFP-lent
J.CaM2/mutLAT-GFP-lent
Unst.
OKT3
% of Max
CD69-APC
D
JE6.1
ANJ3
ANJ3/wtLAT-GFP-lent
ANJ3/mutLAT-GFP-lent
Control
OKT3
Ratio
Time (Seconds)

Fig. 3


A
ANJ3
ANJ3/wtLAT-GFP-lent
ANJ3/mutLAT-GFP-lent
OKT3
- + - + - +
anti-pY
anti-LAT
anti-ACTIN
B
ANJ3
ANJ3/wtLAT-GFP-lent
ANJ3/mutLAT-GFP-lent
OKT3
- + - + - +
anti-pY
anti-Vav
C
ANJ3
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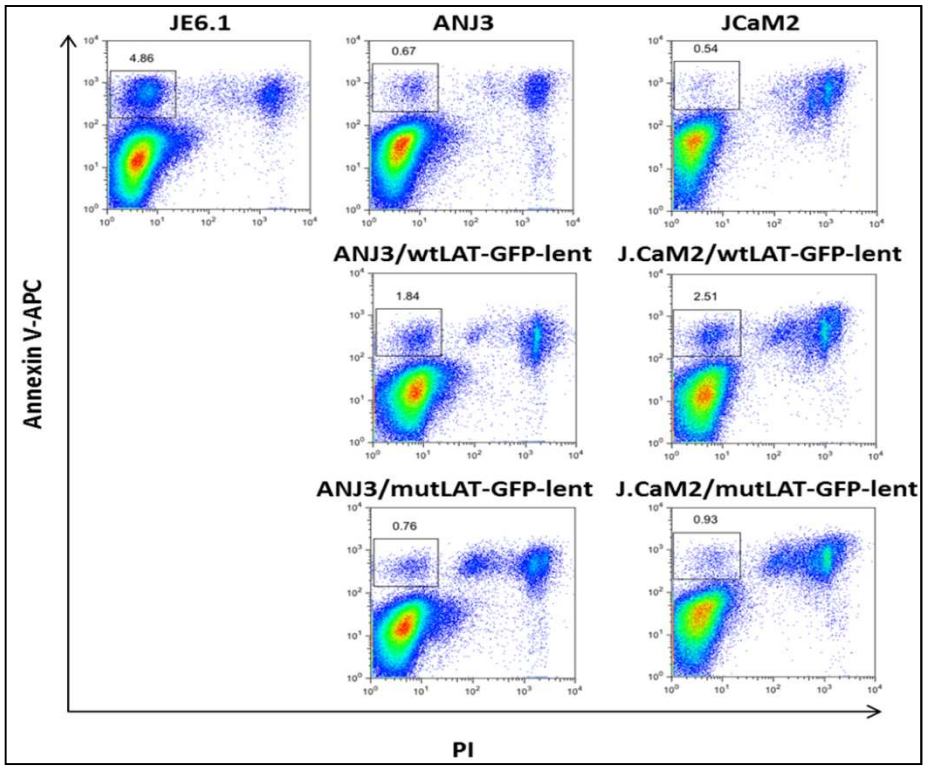
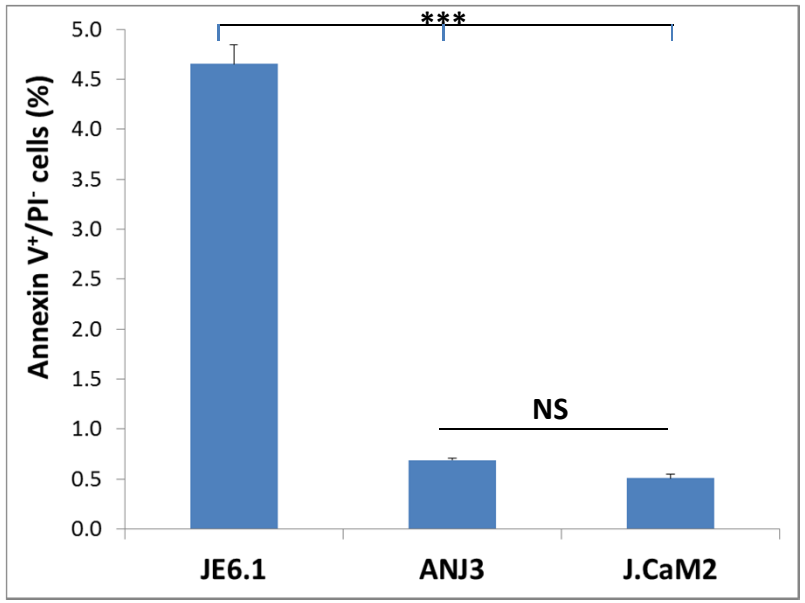
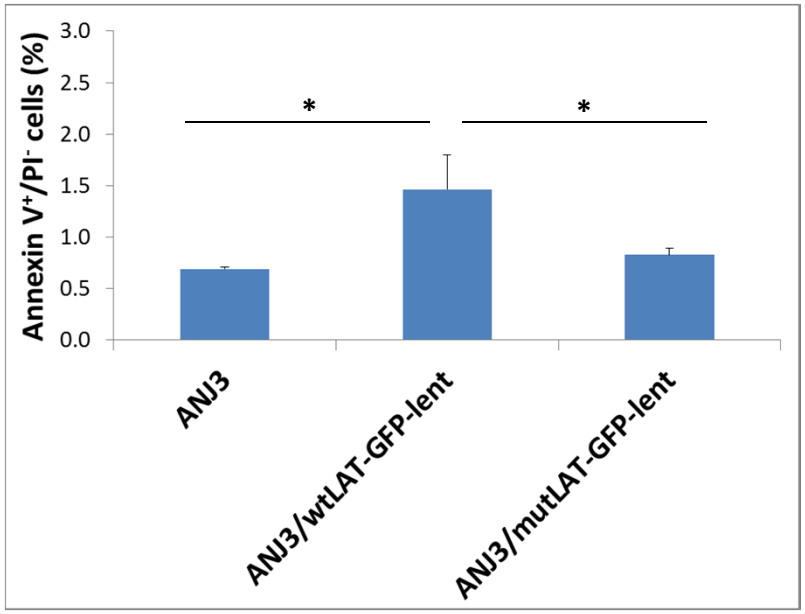
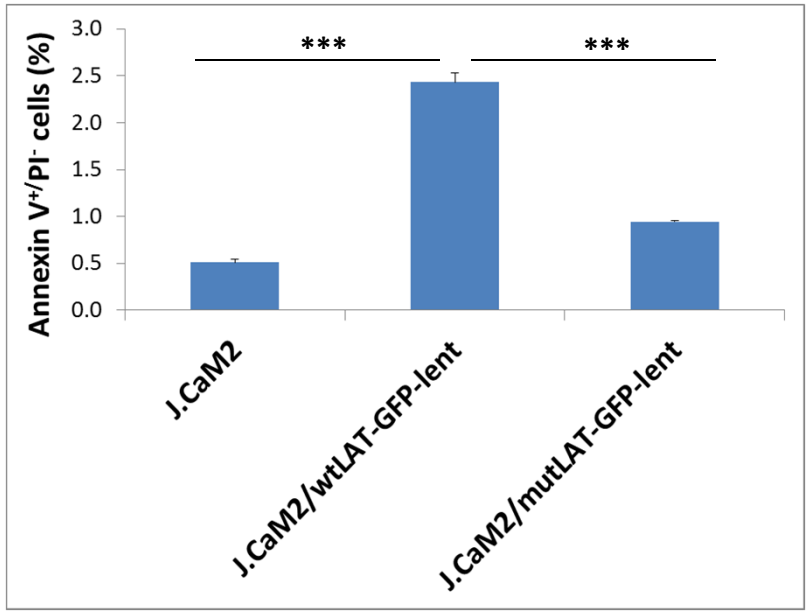
- + - + - +
anti-pY
anti-SLP-76

g. 4


A
JE6.1
ANJ3
JCaM2
4.86
0.67
0.54
ANJ3/wtLAT-GFP-lent
J.CaM2/wtLAT-GFP-lent
1.84
2.51
ANJ3/mutLAT-GFP-lent
J.CaM2/mutLAT-GFP-lent
0.76
0.93
Annexin V-APC
PI


B
***
NS
Annexin V+/PI- cells (%)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
JE6.1
ANJ3
J.CaM2


C
*
*
Annexin V+/PI- cells (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ANJ3
ANJ3/wtLAT-GFP-lent
ANJ3/mutLAT-GFP-lent

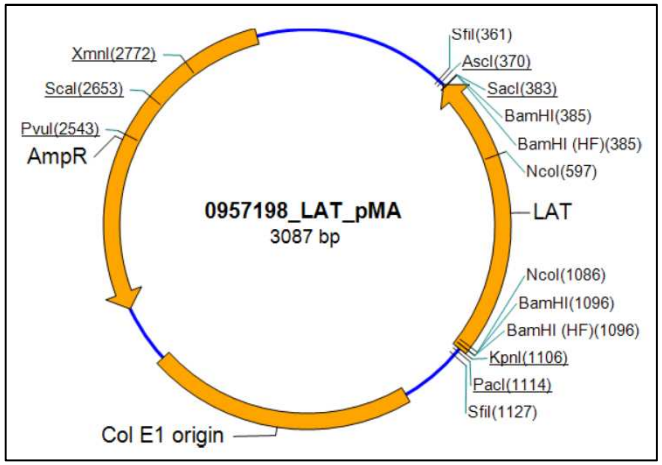

D
***
***
Annexin V+/PI- cells (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
J.CaM2
J.CaM2/wtLAT-GFP-lent
J.CaM2/mutLAT-GFP-lent

Supplementary Table I. Primers used for PCR amplification and Sanger sequencing of genomic DNA of human *CORO1A* and *LAT* genes.

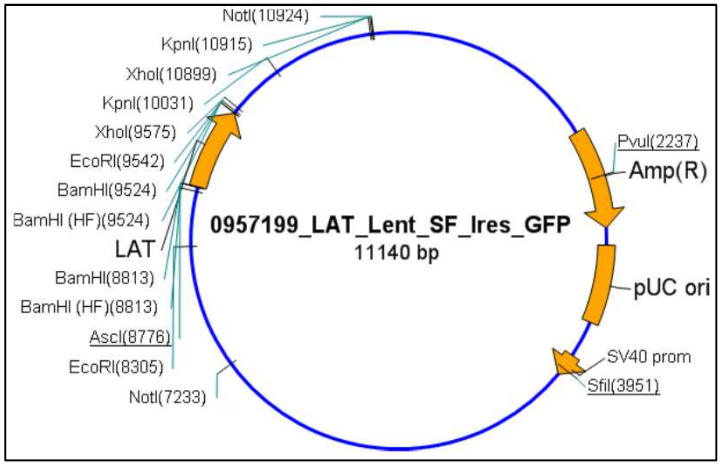
CORO1A			
Exon 2F	AGACTGAGGGGTGTCCTGG	362bp	60
Exon 2R	CACCTAATCAGGACCTGCAC		
Exon 3-4F	GCCTCTCTGAAGGAGGTGTG	448bp	60
Exon 3-4R	AGTGTCAGAAGCCATGAGCC		
Exon 5-6F	TCAGTGCAGGTGCTGCG	563bp	60
Exon 5-6R	AGCAGGGACGAAACCTCC		
Exon 7-8F	GTTGTTCCCACTGGTTGGTC	463bp	60
Exon 7-8R	CTGGTTGGGTGGGCTTG		
Exon 9-10F	ATGCTCCTTGGGCAGTGG	485bp	62
Exon 9-10R	GATTCCCAATGTTGTGAGTTTG		
Exon 11F	AGGGCTCTAGGGATGGGG	205bp	62
Exon 11R	CATGTGGCTGGGAATGGG		
LAT			
Exon 1-2-3F	TTCATCTGGCCTTGACTCTG	635bp	60
Exon 1-2-3R	AAGGTGGCAGGGGAAGTC		
Exon 4-5-6F	GCTTTCAGGGGCTTAGTCTG	596bp	60
Exon 4-5-6R	GGTAACGACACAGGGGTCAG		
Exon 6-7F	AGAACGAGGGTGCGTCTG	403bp	59
Exon 6-7R	TACAATGGCACCACTGCAC		
Exon 8-9-10F	CTCTGCATGGCTGAGGTTG	648bp	60
Exon 8-9-10R	GGTCCTGGAGGGAAGGAG		

Supplementary Fig. 1

A

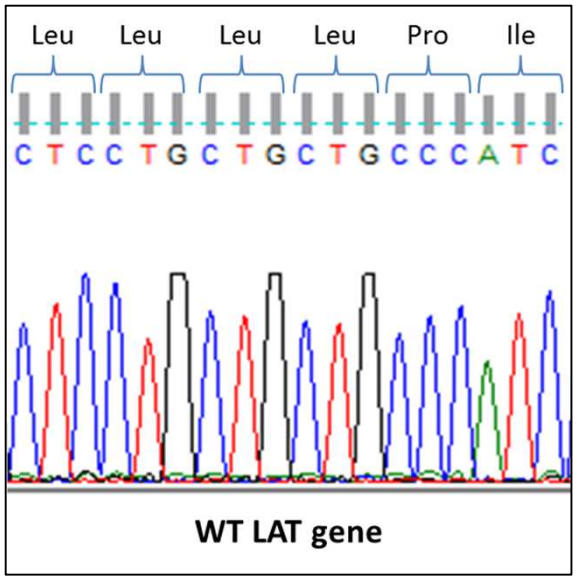


B

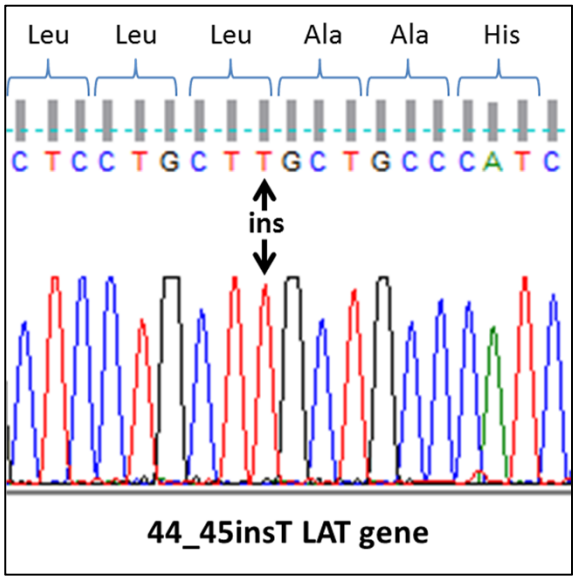


C

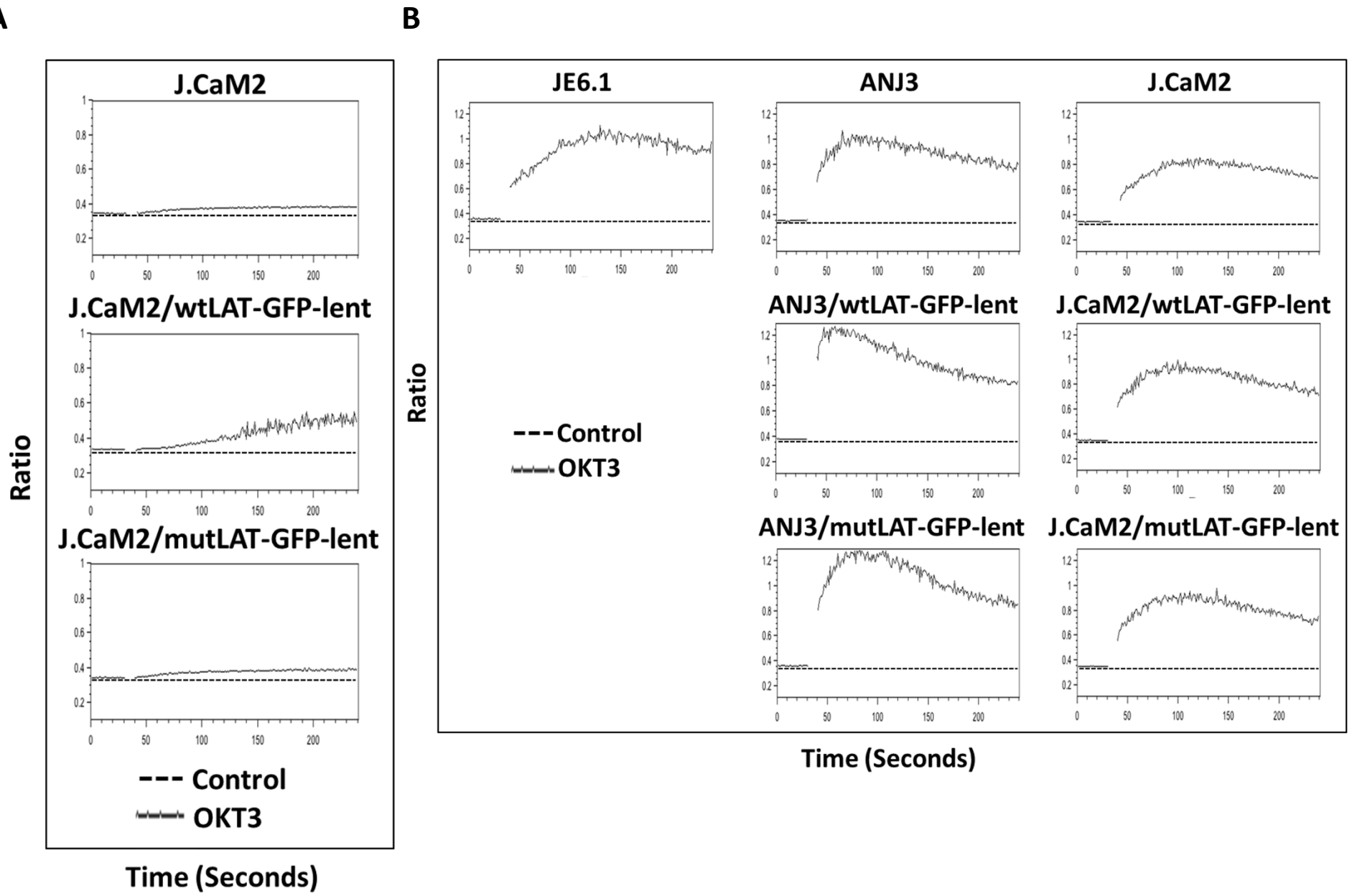
D



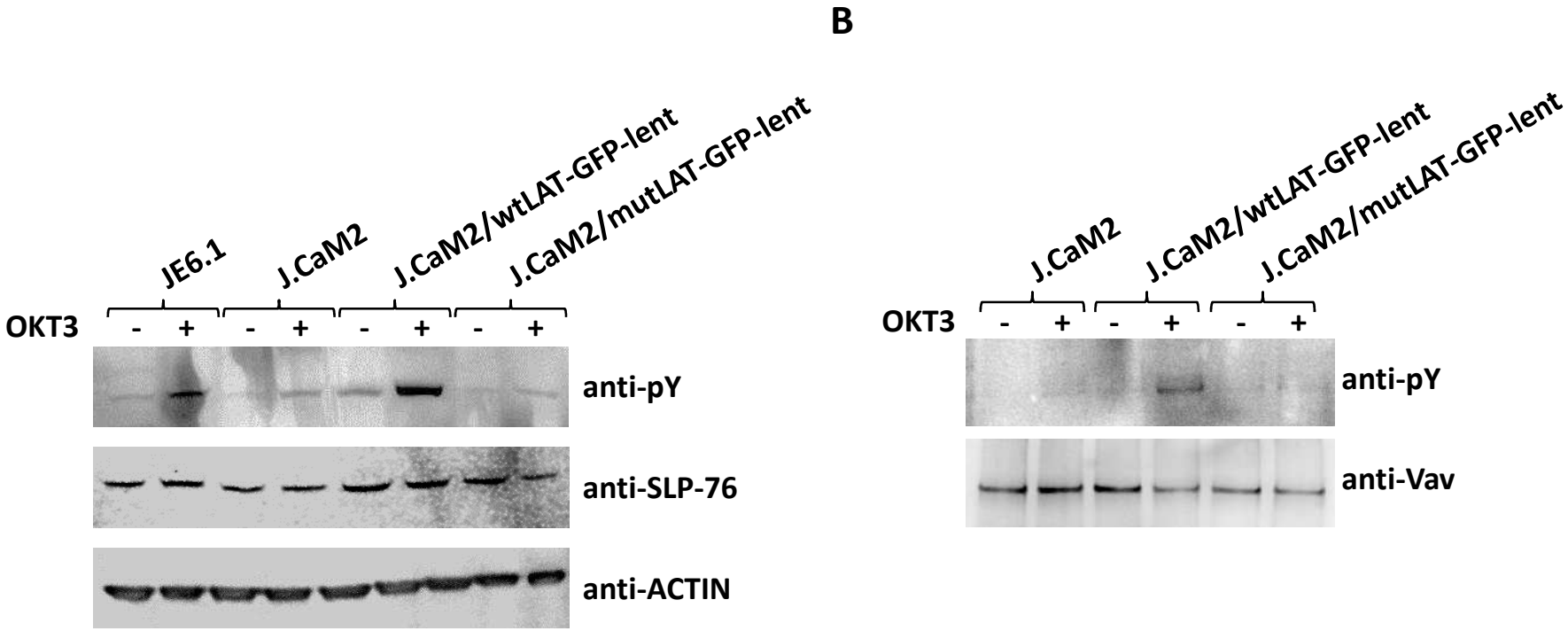
E



Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4