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# Phosphoproteomic analysis reveals hyperactivation of mTOR/STAT3 and LCK/Calcineurin axes in pediatric early T-cell precursor ALL

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Early T-cell Precursor Acute Lymphoblastic Leukemia (ETP-ALL), first described in 2009, accounts for about the 10–15% of T-ALL in children.[1] Normal ETP cells, also known as double-negative 1 (DN1), the most primitive progenitor T cells in the thymus, retain the ability to differentiate in both T lymphocytes and myeloid cells but not in B lymphocytes.[2] ETP-ALL blasts display unique immunophenotype features with lack of T-lineage markers CD1a and CD8, weak or absent CD5, and expression of at least one myeloid or hematopoietic stem cell marker such as CD13, CD33, CD34, CD11b, CD65, HLADR or CD117.[1] This disease was initially associated with a very poor prognosis,[1,3] but recent studies report that with intensified protocols, as indicated after a poor early response to induction treatment, the ETP-ALL outcome is not significantly worse than in the other T-ALL subtypes.[4–6] The consolidation phase IB of contemporary AIEOP-BFM protocols, based on cyclophosphamide, 6-mercaptopurine and conventional doses of Ara-C, was effective in clearing disease in most patients,[6] supporting the hypothesis that ETP-ALL patients might benefit from some elements of acute myeloid leukemia therapy, as suggested by the similarity to acute myeloid leukemia stem cell subtype gene expression profiling.[2,6] Whole-genome sequencing studies revealed that ETP-ALL patients often display recurrent activating mutations, targeting genes involved in the regulation of cytokine receptors and RAS signaling, inactivating lesions of genes implicated in hematopoietic development, and mutations affecting epigenetic regulation.[2] On the other hand, although hyperactivation of the JAK/STAT pathway was observed[2,7,8] with particular high levels of phospho-STAT1, phospho-STAT3 and phospho-STAT5, proteomic aberrancies of ETP-ALL cells are still poorly understood.

To address this question, we analyzed bone marrow aspirates of 78 AIEOP (Italian Association of Pediatric Hematology and Oncology) T-ALL patients at diagnosis (16 ETP-ALL and 62 other T-ALLs of which 22 early-T, 31 thymic and 9 mature T-ALL defined as described in Supplementary Table S1, collected in the period September 2009–April 2014) through Reverse Phase Protein Arrays (RPPA). The whole blood blast percentage for all samples was between 78 and 97%. Protein lysates were freshly made from bone marrow mononuclear cells separated by Ficoll-Hypaque technique. RPPA analysis was performed as previously described,[9] and the activation and/or expression of 50 different proteins was studied (Supplementary Table S2) with a particular focus on AKT/mTOR, JAK/STAT, RAS/MAPK and TCR signaling pathways. Statistical analyses were performed using Prism (GraphPad Software, Inc., La Jolla, CA, USA) and identification of differentially activated or expressed proteins in ETP-ALL patients was obtained through Mann–Whitney test. The activation status of selected pathways was evaluated through Global test analysis[10] performed with R (www.r-project.org).

This latter analysis revealed that the AKT/mTOR and the JAK/STAT pathways are hyperactivated in ETP-ALL compared to both

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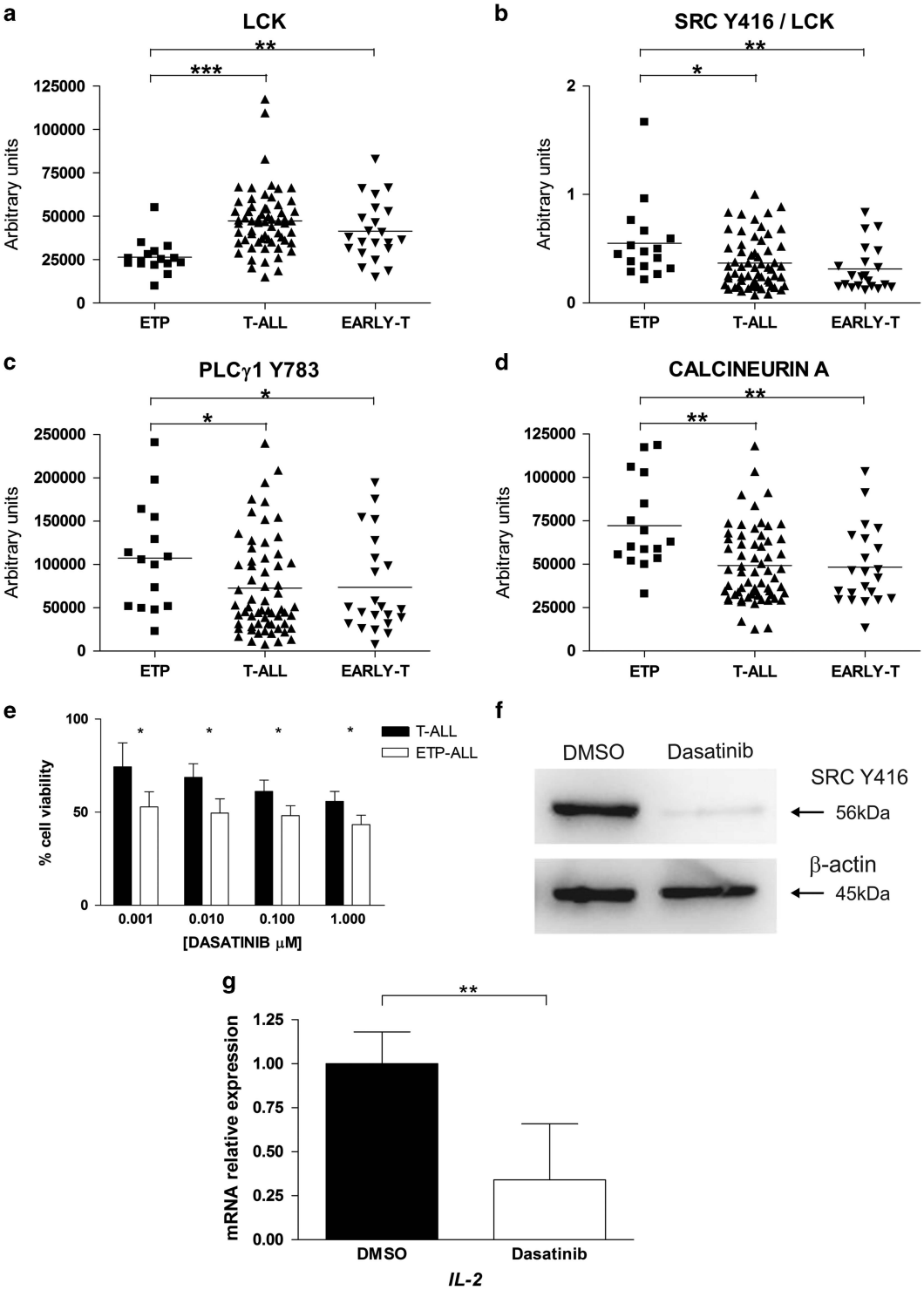


**Figure 1.** LCK/Calcineurin axis in pediatric ETP-ALL. (**a**) LCK is downregulated in ETP-ALL patients ($n = 16$, mean $26260 \pm 2418$) compared to the other T-ALL ($n = 62$, mean $47340 \pm 2309$; $P < 0.0001$) and Early-T ALL only ($n = 22$, mean $41430 \pm 3681$; $P = 0.002$). Values are expressed in Arbitrary units ± s.e.m. (**b**) The SRC Y416/LCK ratio is higher in ETP-ALL patients (mean $0.55 \pm 0.09$) compared to the other T-ALL (mean $0.37 \pm 0.03$; $P = 0.01$) and Early T-ALL only (mean $0.31 \pm 0.04$; $P = 0.005$). (**c**) PLCγ1 Y389 is hyperactivated in ETP-ALL patients (mean $107283 \pm 15934$) compared to the other T-ALL (mean $72770 \pm 7000$; $P = 0.02$) and early-T ALL only (mean $73546 \pm 11779$; $P = 0.04$). (**d**) Calcineurin A is overexpressed in ETP-ALL patients (mean $72280 \pm 6486$) compared to the other T-ALL (mean $49210 \pm 2762$; $P = 0.005$) and early-T ALL only (mean $48290 \pm 4776$; $P = 0.002$). (**e**) Cell viability after 48 h of treatment with Dasatinib, measured by MTT assay, of ETP-ALL patients' cells ($n = 3$) was strongly reduced and significantly different compared to the other T-ALL ($n = 4$) (Unpaired *t*-test: 1 μM $P = 0.02$, 0.1 μM $P = 0.03$, 0.01 μM $P = 0.019$, 0.001 μM $P = 0.05$). (**f**) Western blot of phosphorylated SRC at Y416 in DMSO and treated (1 h Dasatinib 1 nM) cells from one ETP-ALL patient. (**g**) *IL-2* mRNA levels in DMSO and treated (16 h Dasatinib 1 nM) ETP-ALL primary cells (one primary culture in triplicate) by Sybr Green Real-Time Quantitative PCR (Paired *t*-test, $P = 0.002$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the whole cohort of analyzed T-ALL (Supplementary Figure S1a) and the subgroup of early-T ALL only, not ETP (Supplementary Figure S1b). Besides, ETP-ALL patients showed also the RAS/MAPK pathway more activated when compared to the early-T ALL subgroup (Supplementary Figure S1b). This comprehensive analysis well supports the RAS/MAPK and JAK/STAT signaling deregulation reported by other groups.[2,7,8] Moreover, in ETP-ALL patients, we also found the hyperactivation of the RAS/MAPK pathway members B-RAF, the main ERK activator, phosphorylated in S445 (Supplementary Figure S1c) and ERK1/2 phosphorylated in

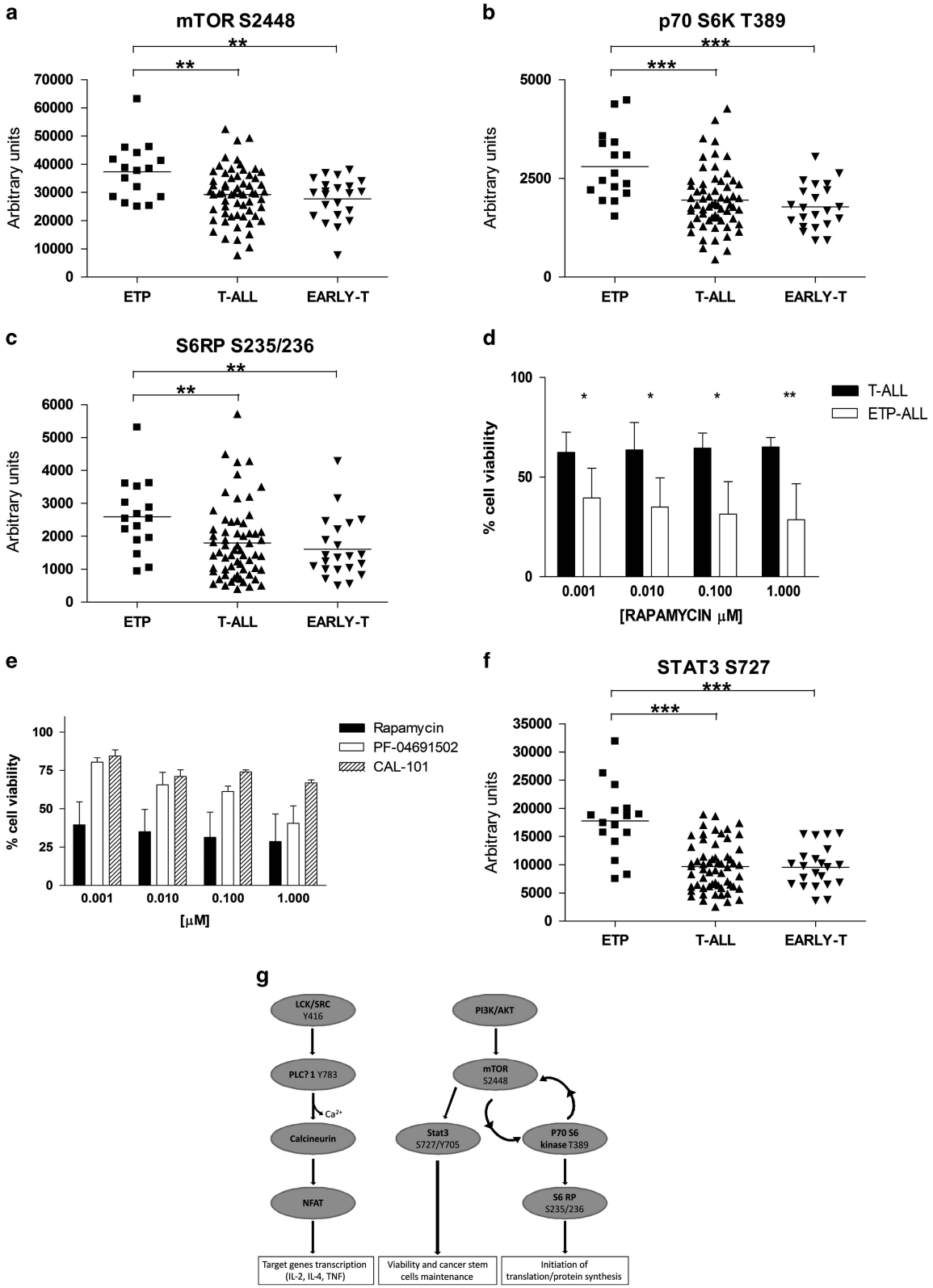


**Figure 2.** For Caption see next page.

T202/Y204 (Supplementary Figure S1d), while activated MEK1/2 in S217/221 is upregulated in ETP only when compared to the early-T subgroup (Supplementary Figure S1e). Their total forms do not differ between patients groups (Supplementary Figure S1f–h). Somatic mutations in the *NRAS* and *FLT3* genes were detected, respectively in 3 and 5 of the 16 ETP patients (Supplementary Table S3), a result which correlates very well with the observed hyperactivation of downstream signaling in the same patients. However, somewhat unexpectedly, we also found a similar hyperactivation in non mutated patients. Importantly, no differences were observed between *FLT3*-mutated and non-mutated patients at the level of AKT/mTOR signaling. Although this may be due to the limited number of patients analyzed, these data allow to hypothesize that the high signaling observed in ETP-ALL has grounds other than the solely somatic mutations, in keeping with the observation that the AKT/mTOR hyperactivation is neither ascribable to the presence of *PTEN* mutations, nor can the JAK/STAT pathway hyperactivation be associated to somatic mutations in *IL7R*, *JAK1*, *JAK2* or *JAK3* genes (Supplementary Table S3).

A striking finding, then, concerned the SRC family non-receptor tyrosine kinase LCK, which resulted dramatically under-expressed in ETP-ALL compared to non-ETP T-ALL or early-T ALL only (Figure 1a). This is in accordance with *LCK* mRNA downregulation observed in ETP-ALL patients by Zhang *et al.*[2] However, activated LCK (measured through SRC Y416) normalized on total LCK clearly indicated that LCK activity is significantly higher in ETP-ALL patients (Figure 1b). This observation is supported by results concerning the inhibited form of LCK (Supplementary Figure S2a), which does not differ between patients, suggesting a functionally leading role for the activating phosphorylation site. A well-known function of LCK, upon TCR engagement, is to trigger through the PLCγ1 phosphodiesterase activity the release of $Ca^{2+}$ from the endoplasmic reticulum, which in turn leads to the activation of the calcium- and calmodulin-activated serine/threonine phosphatase Calcineurin. We therefore analyzed the activation of PLCγ1 in Y783 and confirmed its upregulation in ETP-ALL patients (Figure 1c), along with the overexpression of Calcineurin A (Figure 1d). Among Calcineurin substrates are the NFAT transcription factors, which upon dephosphorylation redistribute to the nucleus and modulate the expression of target genes involved in the pathogenesis of cancer and in many aspects of the immune response. The activation of the Calcineurin-NFAT pathway was already demonstrated to be crucial for the maintenance of the T-cell leukemic phenotype *in vivo*,[11] and all these findings suggest that in ETP-ALL the upregulation of the LCK/Calcineurin pathway could contribute to leukemogenesis. To support our hypothesis, we treated primary cultures from three ETP-ALL and four non-ETP T-ALL pediatric patients with the LCK inhibitor Dasatinib. MTT test revealed a significative reduction of cell viability in ETP-ALL cells compared to non-ETP already at nanomolar concentrations of the drug (Figure 1e). Only one ETP-ALL specimen had enough cells available to perform additional experiments, thus we confirmed, after Dasatinib treatment, the dephosphorylation of SRC Y416 (Figure 1f) and the downmodulation of the whole LCK/Calcineurin axis through the decrease of mRNA levels of the NFAT target *IL-2* (Figure 1g).

mTOR signaling was also investigated. Remarkably, while expression of total mTOR protein did not differ between patients groups (Supplementary Figure S2b), we noticed a dramatic increase of mTOR phosphorylation at S2448 in ETP-ALL patients (Figure 2a). Cellular mTOR can form two distinct complexes, called mTORC1 and mTORC2, which display substrate specificity. Phosphorylation at S2448 is known to be a marker of mTORC1, rather than mTORC2, integrity/activity.[12] mTORC1 keeps tight control of initiation of translation, protein synthesis and cell cycle progression through its specific target, the Ribosomal Protein S6 kinase (p70 S6K). Consistently with the above result, p70 S6K was also found hyperphosphorylated, and therefore activated, at the mTORC1 site T389 in ETP-ALL patients (Figure 2b). In turn, phosphorylation by p70 S6K of the 40S ribosomal protein S6 at S235/236 was boosted in ETP-ALL patients (Figure 2c). Since p70 S6K has been identified as the mTOR S2448 kinase, it is tempting to speculate that in ETP-ALL patients a permanent circuit is at work, where p70 S6K may mediate phosphorylation of mTOR at S2448 once it has been activated by mTOR phosphorylation at T389 in a possible feedback loop. To test our hypothesis, PI3K/mTOR signaling was modulated with the mTORC1 inhibitor Rapamycin. Its effect on cell viability was assessed by MTT (Figure 2d) in three ETP-ALL and four non-ETP T-ALL primary samples. Results show that direct mTOR inhibition by Rapamycin drops cell viability significantly at all concentrations tested only in ETP-ALL cells, while exerting only a very weak effect in T-ALL samples. To dissect what step of the cascade is boosted in ETP-ALL samples, vertical targeting was performed by means of the PI3K delta inhibitor CAL-101, which has already been shown to be cytotoxic to ETP-ALL-like cell models,[13] as well as of the dual pan-PI3K and mTOR inhibitor PF-04691502. Compared to mTOR single targeting by Rapamycin, neither CAL-101 nor PF-04691502 displayed as much efficacy (Figure 2e). However, it is worth noting that while CAL-101 was completely ineffective, dual inhibition by PF-04691502 showed some efficacy, likely due to mTOR inhibition. This strongly supports our hypothesis that mTOR S2448 and p70 S6K T389 phosphorylation may constitute a self-sustaining loop that contributes to the higher signaling phenotype of ETP-ALL.

In keeping with the observed constitutive activation of mTOR signaling is also the striking phosphorylation of STAT3 at two well-known mTOR phosphorylation sites, namely S727 (Figure 2g) and, to a lesser extent, S705 (Supplementary Figure S2c), whereas total STAT3 protein level was unaffected (Supplementary Figure S2d). Interestingly, mTOR-driven phosphorylation of STAT3 at both sites has been reported in human cancer cells and monocytes,[14] and has been claimed to promote viability and maintenance of cancer stem cells while counteracting differentiation.[15–17] It is therefore conceivable that mTOR and STAT3 could work in tandem to

**Figure 2.** mTOR/STAT3 axis in pediatric ETP-ALL. (**a**) mTOR is hyperphosphorylated at S2448 in ETP-ALL patients (mean $37320 \pm 2512$) compared to the other T-ALL (mean $29240 \pm 1188$; $P = 0.009$) and early-T ALL only (mean $27690 \pm 1594$; $P = 0.005$). (**b**) p70 S6K is hyperactivated at T389 in ETP-ALL patients (mean $2799 \pm 217.2$) compared to the other T-ALL (mean $1949 \pm 96.9$) ($P = 0.0005$) and early-T ALL only (mean $1775 \pm 120.7$; $P = 0.0003$). (**c**) S6 ribosomal protein is hyperactivated at S235/236 in ETP-ALL patients (mean $2595 \pm 275.3$) compared to the other T-ALL (mean $1799 \pm 143.6$; $P = 0.004$) and early-T ALL only (mean $1607 \pm 196.4$; $P = 0.003$). (**d**) Cell viability after 48 h of Rapamycin treatment, measured by MTT assay, of ETP-ALL patients' cells ($n = 3$) was strongly reduced and significantly different compared to the other T-ALL ($n = 4$; Unpaired *t*-test: 1 μM $P = 0.007$, 0.1 μM $P = 0.01$, 0.01 μM $P = 0.04$, 0.001 μM $P = 0.05$). (**e**) Cell viability after 48 h of Rapamycin, PF-04691502 or CAL-101 treatment, measured by MTT assay, of ETP-ALL patients' cells ($n = 3$). (**f**) STAT3 is hyperactivated at S727 in ETP-ALL patients (mean $17790 \pm 1570$) compared to the other T-ALL (mean $10620 \pm 761.1$; $P < 0.0001$) and Early-T ALL only (mean $9555 \pm 776.8$; $P < 0.0001$). (**g**) Schematic figure of the mostly activated pathways in ETP-ALL patients. On the left, the LCK/Calcineurin pathway in which SRC activation at Y416 is followed by PLCγ1 activation at Y783, promoting $Ca^{2+}$ release from the endoplasmic reticulum, which in turn leads to the activation of the calcium- and calmodulin-activated serine/threonine phosphatase Calcineurin. Calcineurin promotes *IL-2* gene transcription through the activation of the NFAT transcription factors family. On the right, the activated mTOR pathway leads to phosphorylation of STAT3 at S727 and Y705, and to the activation of p70 S6 kinase at T389 and downstream S6 ribosomal protein at S235/236. Activation of mTORC1 is boosted by a positive feedback loop with p70 S6 kinase. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

maintain a markedly undifferentiated phenotype of ETP-ALL patient cells, while sustaining cell proliferation and survival.

In conclusion, our study demonstrates that ETP-ALL patients display a peculiar proteomic activation pattern, which differs from that of the other pediatric T-ALL patients. Indeed, not only we confirmed that the JAK/STAT and the RAS/MAPK signaling pathways are upregulated, but we also demonstrated for the first time that the LCK/Calcineurin and the mTOR/STAT3 axes are boosted (Figure 2g). In particular, the hyperactivation of LCK triggers the Calcineurin/NFAT-mediated transcription, which is critical for T-cell leukemogenesis. Moreover, the hyperactivation of mTOR, probably not a consequence of *FLT3* mutations but of an aberrant feedback loop through p70 S6K, increases STAT3 and 40S ribosomal protein S6 phosphorylation promoting cancer cells viability. The abnormal signaling activation observed in ETP-ALL will be extremely helpful to elucidate the mechanism behind the poor response to induction treatment of these patients, and might also suggest new druggable targets for personalized and less toxic therapies (that is, LCK[18,19] or mTOR[20–22] inhibitors).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

VS, VL and BA conceived the experiments; VS and VL performed the experiments; VS and BA analyzed the data; BA and SB performed statistical analyses; BB provided the patients data and classification; MP, FG, NA, CP and GC performed mutational screening; BA, VS, VC and SM interpreted the data; BA and SM wrote the manuscript; VC, GB and BA supervised the research.

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