CASZ1 upregulates PI3K-AKT-mTOR signaling and promotes T-cell acute lymphoblastic leukemia

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Abstract

CASZ1 is a conserved transcription factor involved in neural development, blood vessel assembly and heart morphogenesis. CASZ1 has been implicated in cancer, either suppressing or promoting tumor development depending on the tissue. However, the impact of *CASZ1* on hematological tumors remains unknown. Here, we show that the T-cell oncogenic transcription factor TAL1 is a direct positive regulator of CASZ1, that T-cell acute lymphoblastic leukemia (T-ALL) samples at diagnosis overexpress *CASZ1b* isoform, and that *CASZ1b* expression in patient samples correlates with PI3K-AKT-mTOR signaling pathway activation. In agreement, overexpression of CASZ1b in both Ba/F3 and T-ALL cells leads to the activation of PI3K signaling pathway, which is required for CASZ1b-mediated transformation of Ba/F3 cells *in vitro* and malignant expansion *in vivo*. We further demonstrate that CASZ1b cooperates with activated NOTCH1 to promote T-ALL development in zebrafish, and that CASZ1b protects human T-ALL cells from serum deprivation and treatment with chemotherapeutic drugs. Taken together, our studies indicate that CASZ1b is a TAL1-regulated gene that promotes T-ALL development and resistance to chemotherapy.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological malignancy that results from transformation and clonal expansion of developmentally-arrested T-cell progenitors.[1,2] Conventional risk-adjusted multi-agent chemotherapy allows for high 5-year event-free survival

rates in children. However, a significant number of patients still relapse or do not respond to therapy (features that are striking in adults), and the intensive-treatment regimens are often associated with severe complications. Consequently, there have been considerable efforts to better understand the cell-intrinsic lesions and microenvironmental underpinnings of the disease, resulting in the identification of numerous key genetic,[3-7] epigenetic or posttranscriptional,[8-12] and cell-extrinsic[13-15] alterations involved in the development and resistance to treatment of T-ALL. Amongst these, several have revealed potential to translate into novel, hopefully less toxic and more efficient therapies,[2,16-19] which may contribute to circumventing resistance to chemotherapy. Thus, identification of new molecular regulators of T-ALL should contribute to a better understanding of the disease and, consequently, to the improvement of therapeutic approaches.

CASZ1 is a highly conserved[20] zinc finger transcription factor essential for blood vessel assembly,[21] cardiomyocyte differentiation and proliferation[22,23] and heart morphogenesis.[24,25] CASZ1 is also critical during neural development.[26,27] In accordance, loss of CASZ1 expression associates with high risk and poor prognosis in neuroblastoma.[28,29] In agreement with a tumor suppressor role for CASZ1, restoration of CASZ1 expression in neuroblastoma cell lines resulted in cell differentiation, increased adhesion and reduced migration, and inhibition of cell growth *in vitro* and *in vivo*.[28,29] CASZ1 displays an anti-oncogenic role also in hepatocellular carcinoma[30] and embryonal rhabdomyosarcoma.[31] Nevertheless, the involvement of CASZ1 in cancer may vary depending on the tissue. For instance, CASZ1 is upregulated in epithelial ovarian cancer cells and promotes epithelial-mesenchymal transition, cell migration, invasion and metastasis.[32]

There are two known CASZ1 isoforms in humans: *CASZ1a* (also known as CASZ11 or CASZ1 transcript variant 1), which has 21 exons, is roughly 8 Kb-long and encodes for a nuclear protein containing 11 zinc fingers; and *CASZ1b* (CASZ5 or CASZ1 transcript variant 2), which has 16 exons, is 4.4 kb-long and encodes for a mainly nuclear but also cytoplasmic protein with five zinc fingers. The sequence of CASZ1b is identical to that of the longer CASZ1 isoform until the end of exon 16, where a 5' splice donor located 1 bp before the stop codon of CASZ1b can be used with a 3' splice acceptor in exon 17 of CASZ1a to produce the latter by alternative splicing.[20] Interestingly, CASZ1a and CASZ1b are often co-expressed (although not always regulated in a similar fashion) and appear to exert similar functions in particular tissues.[29] For example, both isoforms were shown to have anti-tumoral activity in neuroblastoma,[28,29] while exerting protumoral effects in ovarian cancer.[32]

In the current study, we aimed to define the role of CASZ1 in T-ALL. We demonstrate that CASZ1b, which is upregulated in T-ALL cells in part via transcriptional activity of TAL1, has an oncogenic role, transforming Ba/F3 cells and cooperating with NOTCH1 in promoting T-ALL in zebrafish. In human T-ALL cells, CASZ1b expression correlates with poor prognosis and activation of the PI3K-AKT-mTOR signaling pathway. Consistent with these observations, CASZ1b overexpression leads to PI3K-AKT-mTOR signaling activation, which is required for T-ALL cell viability. Overall, our findings establish CASZ1 as a putative novel T-cell oncogene and suggest that PI3K targeting drugs may offer clinical benefit for relapsed T-ALL cases exhibiting high CASZ1 levels.

Methods

Primary T-cell acute lymphoblastic leukemia samples, cell lines and culture conditions

Primary T-ALL cells collected from pediatric patients at diagnosis were isolated as described.[14] Informed consent was obtained in accordance with the Declaration of Helsinki after institutional ethical review board approval (authorization #13-105-1 obtained from the Institutional Review Board [IRB00003888] of the French Institute of Medical Research and Health). Upon isolation, primary samples were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Biowest), hereafter named RPMI-10. The human T-ALL cell lines CEM, DND-41, HPB-ALL, Jurkat, Loucy, MOLT4 and P12 were obtained commercially and cultured in RPMI-10. The IL-3-dependent murine pro-B cell line Ba/F3 was maintained in RPMI-10 with IL-3. Mouse IL-3 was produced by WEHI3B cells, whose supernatant was used to maintain Ba/F3 cells. The human embryonic fibroblast 293T cell line was maintained in DMEM medium (Life Technologies) supplemented with 10% FBS (DMEM-10). T-ALL primary cells, cell lines and Ba/F3 cells were cultured at 37°C with a 5% CO2 atmosphere in RPMI-10 alone (with the appropriate vehicle), RPMI (when indicated) or in RPMI-10 plus the following pharmacological agents: 4-hydroxy-tamoxifen (4OHT; Sigma); NVP-BEZ235 (Selleckchem); LY294002 (Cayman Chemical), daunorubicin (Selleckchem); dexamethasone (Sigma) and L-asparaginase (Sigma). At defined time points, cells were harvested and processed as indicated below for assessment of cell viability, RNA extraction and immunobloting.

Assessment of cell viability, activation and proliferation

Determination of cell viability was performed by flow cytometry analysis of forward scatter *versus* side scatter (FSC *vs*. SSC) distribution using a LSRFortessa cell analyzer (BD Biosciences). We have confirmed previously that this strategy measures lymphocyte viability as accurately as when using annexin V and propidium iodide staining.[8] Cell size, as a measure of cell activation, was assessed by FSC *versus* SSC gated on the live cell population. The samples were acquired using a LSRFortessa cell analyzer with a high-throughput-sampler (HTS) with a fixed volume of sample to be analyzed, which allowed us to extrapolate the number of cells that were analyzed and thus calculate proliferation.

Immunobloting

Cell lysates were prepared as described[16] and equal amounts of protein were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted with antibodies against p-mTOR (S2448), p-AKT (S473), AKT, p-S6 (S235/236) and S6 (all from Cell Signaling Technology), CASZ1 (Rockland), Lamin B (Santa Cruz Biotechnology), TAL1 (EMD Millipore), and $p27^{Kip1}$ (BD Transduction Labs). Immunodetection was performed by incubation with horseradish peroxidase-conjugated appropriate secondary antibodies and developed by chemiluminescence. Where indicated, densitometry analysis was performed using Adobe Photoshop CS3 software (version 10.0, Adobe Systems Incorporated, San Jose, CA, USA). Each band was analyzed with a constant frame and normalized to the respective loading control.

Statistical analysis

The GraphPad Prism software was used for statistical analysis. Differences between mean values were calculated using two-tailed Student's *t* test and one-way analysis of variance, as appropriate. Correlation in gene expression levels was determined using the Pearson correlation coefficient. Differences in survival curves were analyzed using the log-rank (Mantel-Cox) test. Differences were considered significant at $P<0.05$.

Information on plasmid generation, electroporation, viral transduction, mouse and zebrafish *in vivo* models, quantitative PCR (including primer sequences: *Online Supplementary Table S1*), chromatin immunoprecipitation, RNA sequencing and bioinformatics analyses are available in the *Online Supplementary Appendix*.

Results

CASZ1b is upregulated in T-cell acute lymphoblastic leukemia, especially in TAL1-positive cases

We first analyzed the pattern of *Casz1* expression during normal mouse hematopoiesis using the BloodSpot database.[33] *Casz1* was expressed at low levels in long-term hematopoietic stem cells (HSC) and upregulated in short-term HSC (*Online Supplementary Figure S1*). *Casz1* expression appeared to be highest in common lymphoid progenitors and it was downregulated upon commitment to either T or B lymphoid lineage, with both B- and T-cell precursors displaying low *Casz1* levels (*Online Supplementary Figure S1*). Because T-ALL arises from clonal expansion of T-cell precursors arrested at different stages of differentiation, we next used a publicly available dataset to assess CASZ1 expression in leukemia samples collected from T-ALL patients at presentation, as compared to normal thymocyte subsets representative of the main T-cell developmental stages.[34] We found that, in contrast to *CASZ1a* (Figure 1A), the short isoform of CASZ1 (*CASZ1b*) was significantly upregulated in T-ALL cells (Figure 1B). These observations, together with the fact that *CASZ1b* is the most conserved of the two CASZ1 isoforms,[20] drove us to focus on *CASZ1b* (hereafter referred to simply as CASZ1) for the remainder of our studies.

Although *CASZ1* levels were generally increased in the T-ALL patients (Figure 1A, B) and T-ALL cell lines (*Online Supplementary Figure S2*) as compared to normal T-cell precursors, we noticed that TAL1 high cases expressed higher levels of CASZ1 than TAL1 low samples in three different T-ALL patient cohorts (Figure 1C; *Online Supplementary Figure S3*).[35,36]

TAL1 upregulates *CASZ1* in T-cell acute lymphoblastic leukemia cells

The association between TAL1 and CASZ1 expression in T-ALL patient samples prompted us to evaluate whether TAL1 might transcriptionally regulate *CASZ1*. Forced expression of TAL1 in two TAL1-negative T-ALL cell lines, P12 (*data not shown*) and MOLT4 (*Online Supplementary Figure S4A*), led to the upregulation of *CASZ1* expression (Figure 2A), whereas *TAL1* inactivation in TAL1-positive Jurkat and CEM cells, using CRISPR/cas9 technology (Figure 2B; *Online Supplementary Figure S4B*), downregulated *CASZ1* (Figure 2B). Importantly, TAL1 silencing in two primary T-ALL patient samples also resulted in CASZ1 downregulation (Figure 2C), further reinforcing the positive link between TAL1 and CASZ1 in T-ALL.

CASZ1 transforms Ba/F3 cells through the activation of PI3K-AKT-mTOR signaling pathway

Given that CASZ1 is overexpressed in T-ALL cells, including downstream of the major T-cell oncogene TAL1, we postulated that CASZ1 should have an oncogenic role in lymphoid cells. To test this possibility, we ectopically expressed CASZ1 in a stable manner in IL-3-dependent Ba/F3 cells[7] (*Online Supplementary Figure S5A*). CASZ1 rescued viability, cell size and proliferation of Ba/F3 cells under IL-3 deprivation (*Online Supplementary Figure S5B, C*), which allowed Ba/F3 cells expressing CASZ1 in the absence of IL-3 to maintain their viability (Figure 3A) and proliferate (Figure 3B) throughout time to similar levels as mock-transduced control cells cultured with the growth factor. These findings indicate that CASZ1 has the capacity to transform Ba/F3 cells, rendering them growth factor-independent.

To identify potential mechanisms by which CASZ1 exerted its oncogenic effects, we analyzed the transcriptional program engaged by CASZ1 in Ba/F3 cells. RNA-sequencing analysis showed that CASZ1 affected the expression of 1,207 genes (*Online Supplementary Figure S6*; *Online Supplementary Table S2*), and subsequent KEGG pathway overrepresentation analysis indicated that PI3K-AKT signaling was highly enriched in CASZ1 overexpressing cells (Figure 3C). In agreement, immunoblot analysis showed that CASZ1 overexpression in Ba/F3 cells (*Online Supple-*

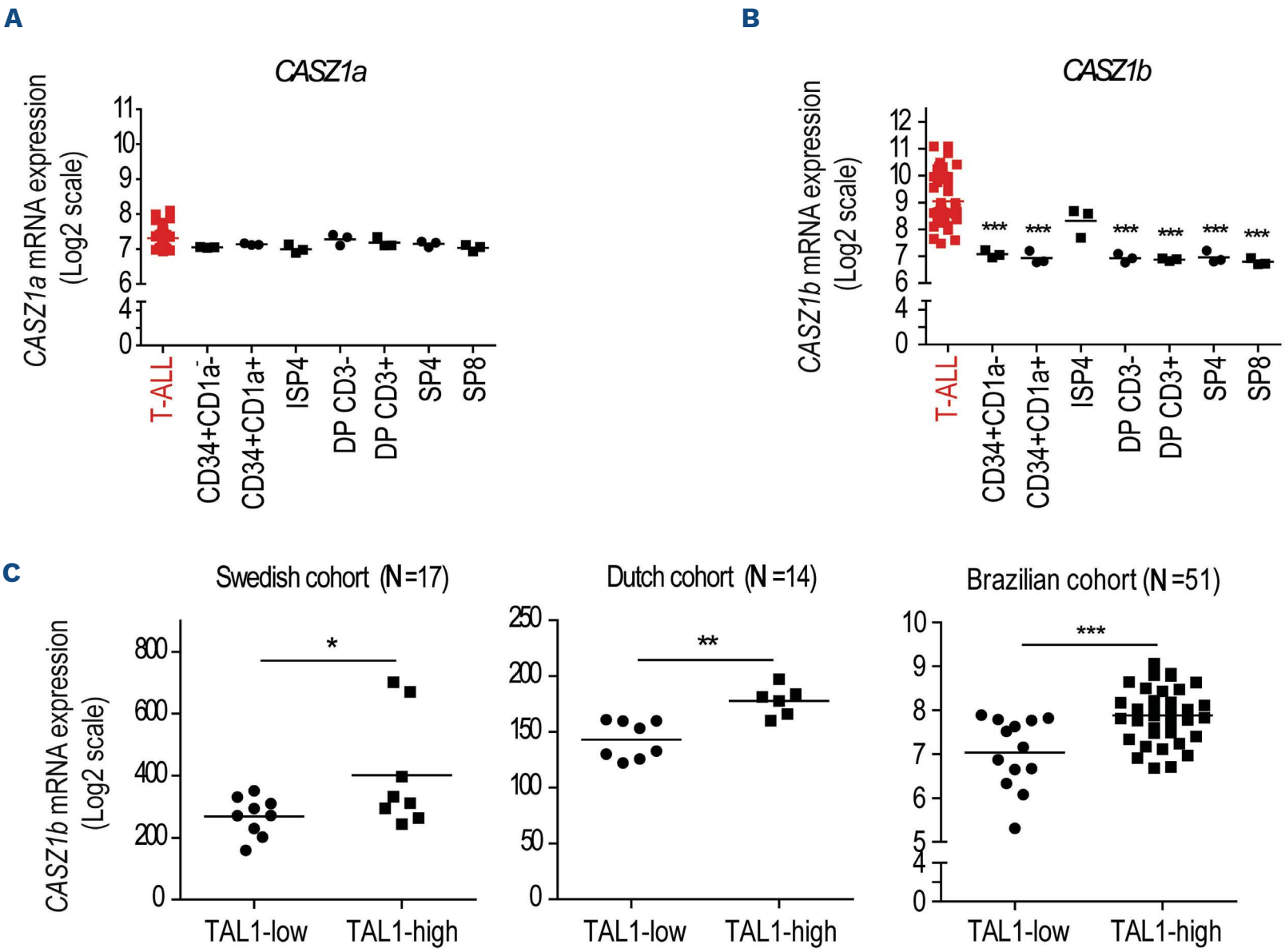

Figure 1. *CASZ1b* is overexpressed in T-cell acute lymphoblastic leukemia. (A, B) *CASZ1a* (A) and *CASZ1b* (B) transcript levels in T-cell acute lymphoblastic leukemia (T-ALL) patients *versus* normal thymocyte subpopulations. Gene expression data set GSE33469-33470. ISP4 - immature single positive CD4; DPCD3$^-$ - CD4 CD8 double positive, without CD3 expression; DPCD3$^+$ - CD4 CD8 double positive, with CD3 expression; SP4 - single positive CD4; SP8 - single positive CD8. (C) *CASZ1b* transcript levels in 3 different T-ALL patients cohorts (Swedish - GSE41621, Dutch - GSE18497 and Brazilian - GSE51001 and GSE66638) analyzed according to the expression levels of the TAL1 transcript (TAL1-high *vs*. TAL1-low). *P<0.05, **P<0.01; ***P<0.001, student's t test.

mentary Figure S7A) upregulated the phosphorylation levels of members of the PI3K-AKT-mTOR signaling pathway, such as AKT, mTOR and S6 (Figure 3D). In addition, CASZ1 promoted the downregulation of p27^{Kip1}, a readout of cell cycle progression consistent with PI3K-AKT activation.[37] Notably, CASZ1 upregulated *Pik3cd* (the gene that encodes PI3Kδ) transcript levels (*Online Supplementary Figure S6B*) and PI3Kδ protein expression (*Online Supplementary Figure S8A*), suggesting that CASZ1 may promote PI3K-AKT-mTOR pathway activation, at least in part, via upregulation of *PI3KCD*/PI3Kδ.

In order to test the involvement of PI3K-AKT-mTOR signaling in CASZ1-mediated transformation, we treated CASZ1-expressing Ba/F3 cells with two distinct specific small molecule PI3K/mTOR inhibitors. As expected, NVP-BEZ235 (dactolisib)[38] and LY294002 dampened PI3K-AKT-mTOR signaling (Figure 3E; *Online Supplementary Figure S9A*), and abrogated the effects of CASZ1 on viability (Figure 3F; *Online Supplementary Figure S9B*) and proliferation (Figure 3G; *Online Supplementary Figure S9C*) of Ba/F3 cells. These results demonstrate the importance of maintaining the integrity of the PI3K-AKT-mTOR signaling cascade for CASZ1-mediated transformation.

CASZ1 expression activates the PI3K-AKT-mTOR signaling pathway in T-cell acute lymphoblastic leukemia

We next sought to evaluate whether, similar to Ba/F3 cells, CASZ1-mediated effects in T-ALL cells involved upregulation of PI3K-AKT-mTOR signaling. We compared expression levels of all the transcripts to those of *CASZ1* in the T-ALL GSE18497 dataset[36] and calculated their Pearson correlation coefficient. Genes whose correlation coefficient was ≥0.7 (indicating a strong positive correlation with CASZ1) were subsequently employed to perform KEGG pathway overrepresentation analysis. Our analysis shows that genes whose expression correlates with that of *CASZ1* were enriched in different KEGG pathways, including PI3K-AKT signaling

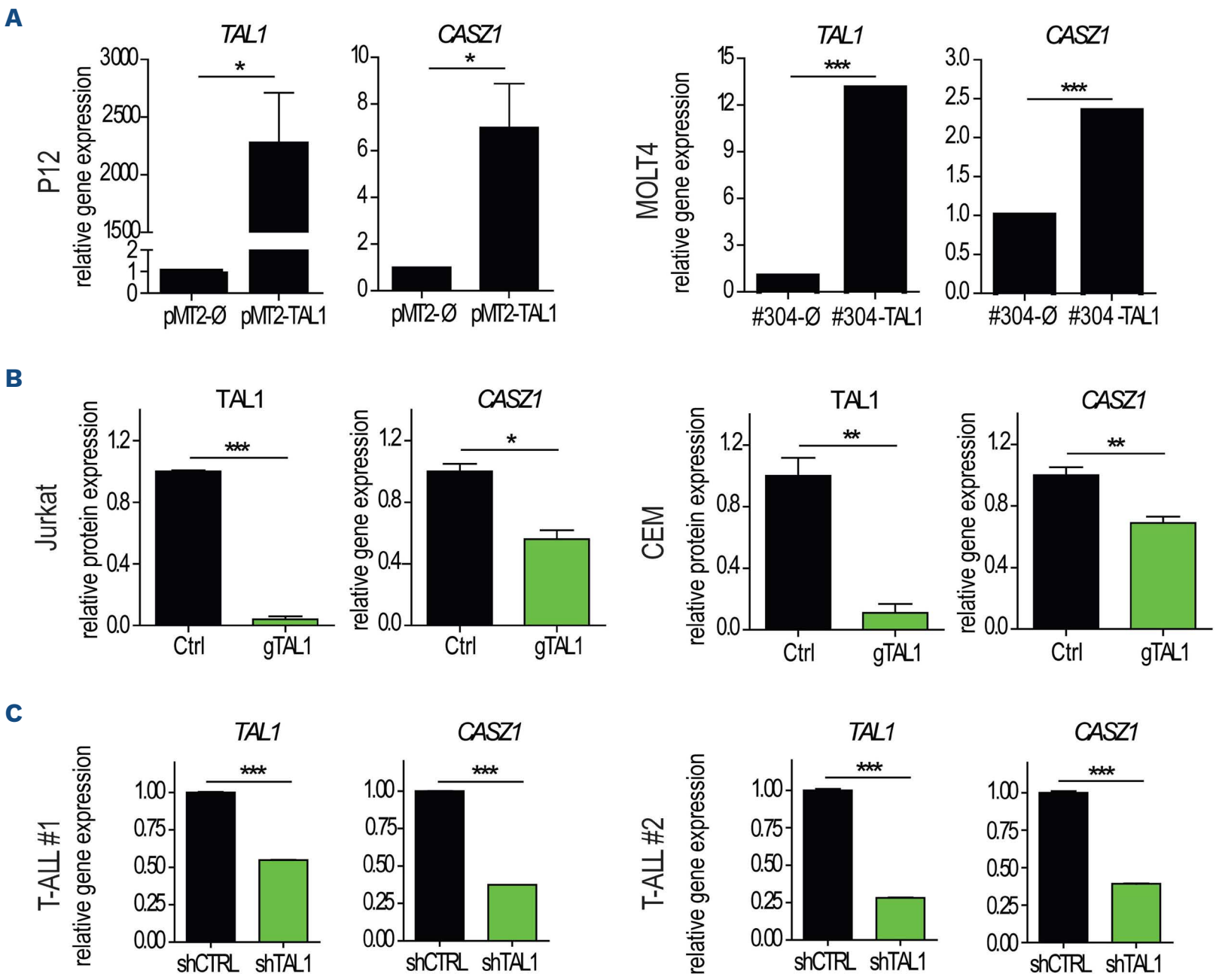

Figure 2. *CASZ1b* is regulated by TAL1 in T-cell acute lymphoblastic leukemia cells. (A) P12 and MOLT4 cells ectopically expressing the TAL1 protein were evaluated for *TAL1* and *CASZ1* expression by quantitative polymerase chain reaction (qPCR). Values were normalized to the respective control condition (empty vector). (B) TAL1 expression was inactivated in Jurkat and CEM cells by CRISPR/cas9. Three control clones (Ctrl) and 3 clones with inactivated TAL1 (gTAL1) from each of the cell lines were analyzed for TAL1 protein expression and *CASZ1* mRNA levels. Values were normalized to the average of the control clones. (C) Two primary T-cell acute lymphoblastic leukemia (T-ALL) patient samples collected at diagnosis were transduced with short hairpin RNA (shRNA) against TAL1. Cells were expanded for 72 hours, and *TAL1* (left) and *CASZ1* (right) transcript levels were detected by qPCR. Values were normalized to the control condition (shCTRL). Values represent the mean ± standard deviation of experimental triplicates of a representative experiment (N=3). **P*<0.05, ***P*<0.01; ****P*<0.001, student's *t* test.

pathway (Figure 4A). Immunoblot analysis confirmed that T-ALL cell lines overexpressing CASZ1 (*Online Supplementary Figure S7B*) displayed higher levels of PI3K-AKT-mTOR signaling activation than mock-transduced controls (Figure 4B). In agreement with our transcriptomics data indicating elevated levels of *Pik3cd* (the gene that encodes PI3Kδ) in CASZ1-overexpressing Ba/F3 cells (*Online Supplementary Figure S6B*), we found that CASZ1 upregulates PI3Kδ protein levels in DND-41 cells (*Online Supplementary Figure S8B*). Interestingly, PI3Kβ was the isoform that was upregulated by CASZ1 in MOLT4 cells (*Online Supplementary Figure S8C*).

CASZ1 promotes tumorigenesis *in vivo*

Next, we evaluated whether CASZ1 was able to promote tumorigenesis *in vivo*. We subcutaneously transplanted empty vector- and CASZ1-transduced Ba/F3 cells into opposite flanks of each recipient NOD/SCID mouse (Figure 5A). Whereas none of the control transplants originated any detectable tumors, ten of ten CASZ1-expressing transplants originated large tumor masses within 20 days (Figure 5B). Then, to ascertain whether PI3K-AKT-mTOR signaling pathway is essential for the maintenance of CASZ1-triggered tumors *in vivo*, we let tumors from subcutaneously transplanted Ba/F3 cells overexpressing CASZ1 grow until 500 mm^3, at which point the mice were randomly divided into two groups that either received vehicle or NVP-BEZ235/dactolisib (Figure 5C). Treatment with NVP-BEZ235/dactolisib clearly delayed tumor growth (Figure 5D) and the effect

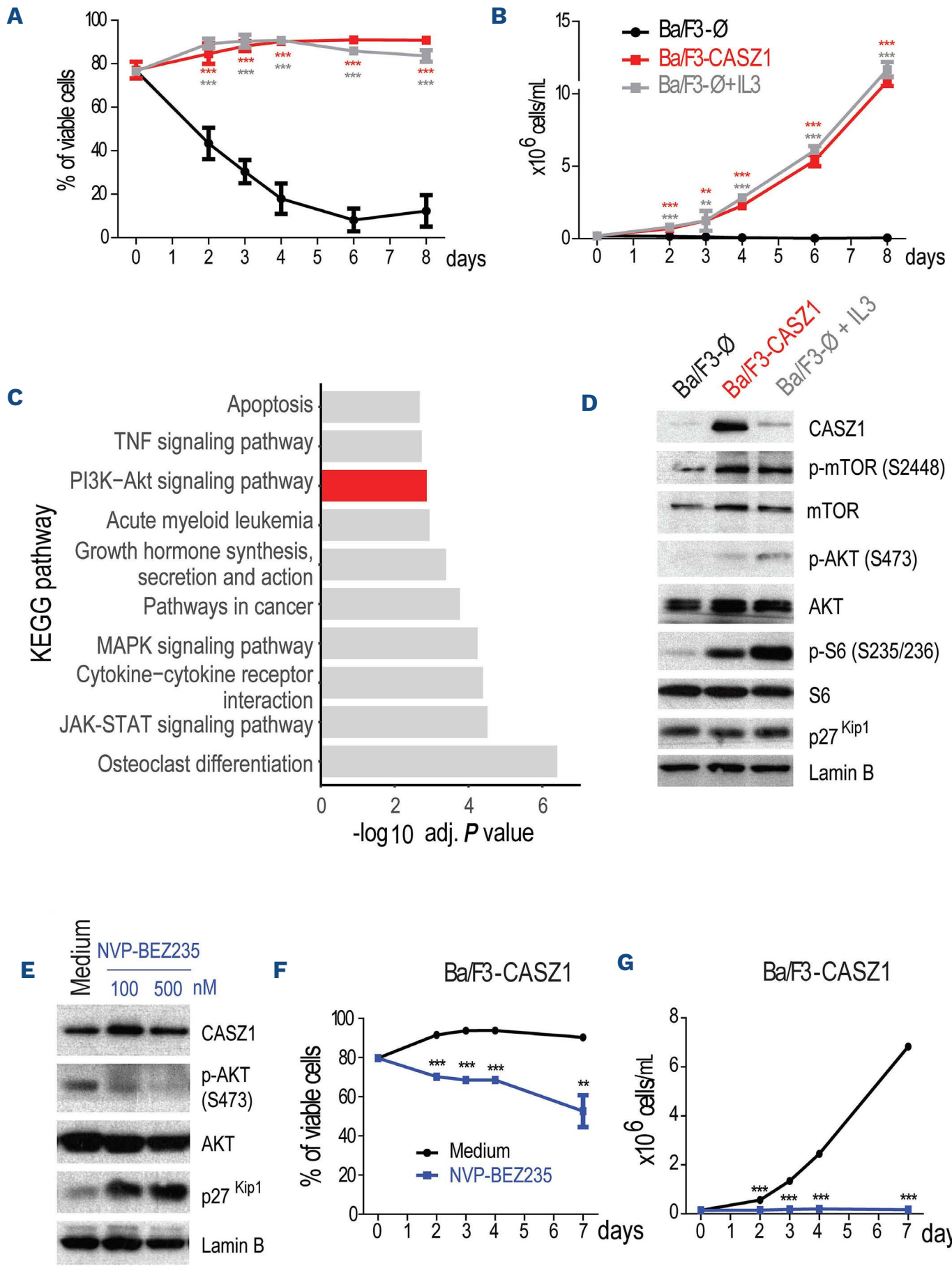

Figure 3. CASZ1 overexpression transforms Ba/F3 cells by activating the PI3K-AKT-mTOR signaling pathway. (A-B) Ba/F3 cells stably transduced with empty vector (Ba/F3-Ø) or CASZ1 (Ba/F3-CASZ1) were cultured in medium without growth factors. As a positive control, Ba/F3-Ø cells were also cultured in the presence of IL-3 (Ba/F3-Ø + IL-3). Viability (A) and proliferation (B) were determined at the indicated time points. Values represent the mean ± standard deviation of at least 3 independent experiments. ***P*<0.01; ****P*<0.001 (C) Top 10 most significantly enriched KEGG pathways (adj. *P*<0.05) from upregulated genes (adj. *P*<0.05, log2 fold change>1) in the RNA-sequencing analysis of Ba/F3-CASZ1 *versus* Ba/F3-Ø cells. (D) Ba/F3-Ø and Ba/F3-CASZ1 cells were cultured in the indicated conditions for 24 hours. PI3K signaling activation was evaluated by immunoblot detection of the phosphorylation levels of AKT and S6. Total levels of CASZ1 and p27^{Kip1} were also analyzed. Lamin B was used as loading control. Data are representative of 2 independent experiments. (E) Ba/F3-CASZ1 cells were cultured for 24 hours in the presence of 100 or 500 nM of NVP-BEZ235 or vehicle (medium). PI3K signaling activation was evaluated by immunoblot detection of the phosphorylation levels of AKT. Total levels of CASZ1 and p27^{Kip1} were also analyzed. Lamin B was used as loading control. Data are representative of 2 independent experiments. (F, G) Ba/F3-CASZ1 cells were cultured in the presence or absence of 500 nM of NVP-BEZ235. Viability (F) and proliferation (G) were determined at the indicated time points. Values represent the mean ± standard deviation of experimental triplicates of a representative experiment (N=3). ***P*<0.01; ****P*<0.001, one-way analysis of variance.

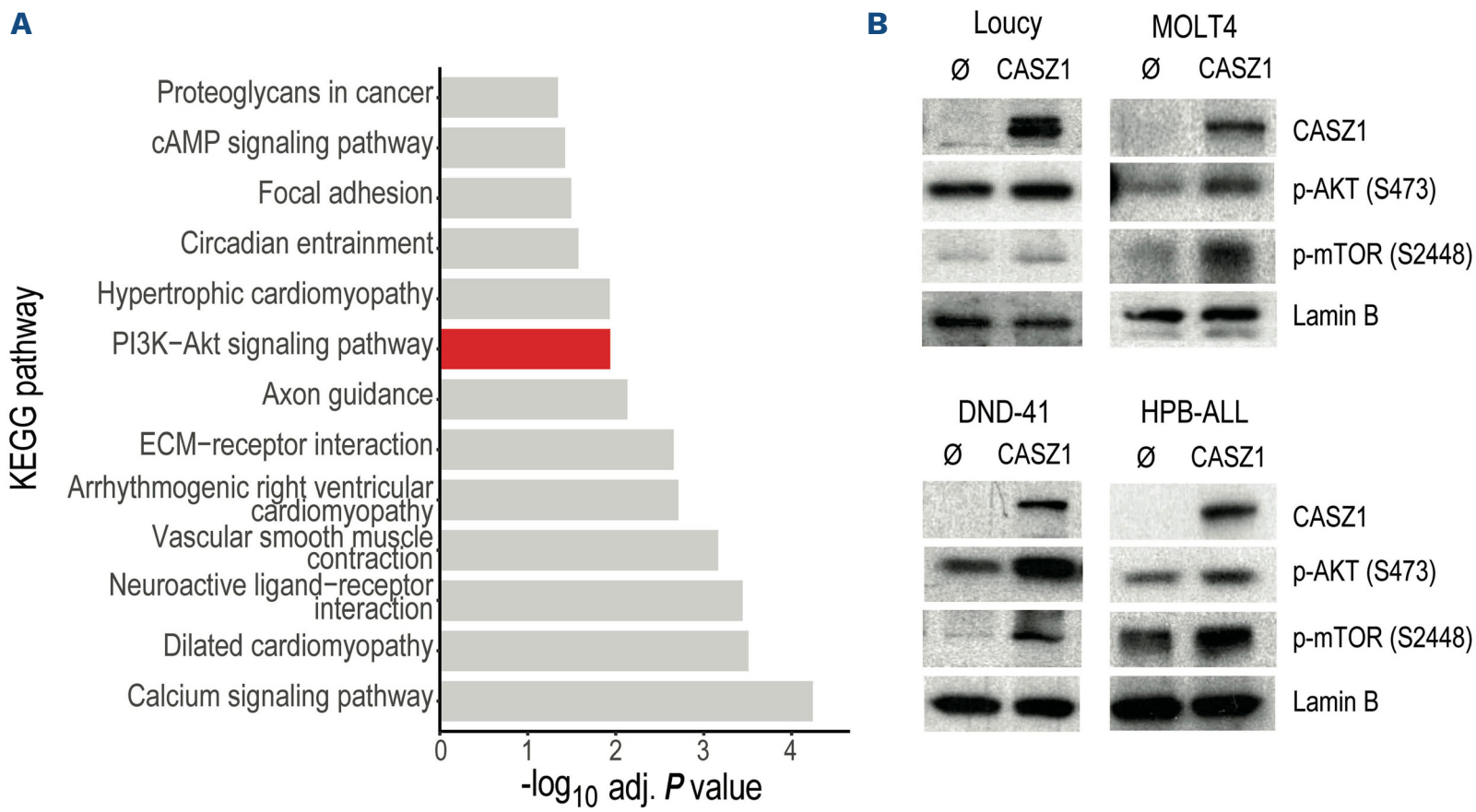

Figure 4. CASZ1 overexpression activates PI3K-AKT-mTOR signaling in T-cell acute lymphoblastic leukemia. (A) KEGG pathway analysis of the genes whose expression levels display a correlation coefficient of at least 0.7 with *CASZ1* expression in a cohort of 14 T-cell acute lymphoblastic leukemia (T-ALL) patient samples (GEO database, accession number GSE18497). (B) T-ALL cell lines without (Ø) or with CASZ1 overexpression were assessed for PI3K signaling activation by immunoblot detection of the phosphorylation levels of AKT and mTOR. Total CASZ1 levels were also examined. Lamin B was used as loading control. Data are representative of 2 independent experiments.

of the PI3K-AKT-mTOR pathway inhibitor was specific, as shown by the downregulation of S6 phosphorylation levels (Figure 5E, F). Taken together, these results clearly indicate that CASZ1 promotes *in vivo* tumorigenesis by activating PI3K-AKT-mTOR signaling.

To determine whether CASZ1 promotes tumorigenesis in the context of T-ALL, we next tested whether CASZ1 accelerates NOTCH-induced T-cell leukemia in zebrafish.[39,40] We generated mosaic zebrafish lines expressing intracellular *NOTCH1* (ICN1) and/or *CASZ1*. Although with long latency, we found that CASZ1 accelerated the development of thymic hyperplasia with subsequent invasion of adjacent tissues (Figure 6A) leading to leukemia/lymphoma development in six of 20 animals expressing CASZ1 and ICN1 (starting at day 77, median survival: 259 days), as opposed to only one of 41 animals expressing ICN1 alone that developed leukemia by day 190 (median survival not reached during the duration of the experiment; Figure 6B). The lymphoid neoplastic cells from CASZ1+ICN1 zebrafish displayed a typical blast morphology, which contrasted to the cells collected from the vast majority of the ICN1 animals (Figure 6C). As expected, leukemia/lymphoma cells were of T-cell origin, as determined by the expression of *CD4*, *CD8* and *LCK*, and the absence of B-cell markers such as *PAX5*, *IGM* or *CD79α* (Figure 6D). Finally, malignant cells in CASZ1+ICN1 zebrafish displayed decreased apoptosis (Figure 6E, F) compared to control cells in ICN1 zebrafish. Altogether, our results indicate that CASZ1 can have a tumorigenic effect in lymphoid cells and cooperate with ICN1 to promote T-cell leukemia *in vivo*.

CASZ1 protects human T-cell acute lymphoblastic leukemia cells from death triggered by serum starvation and chemotherapy

To investigate the biological impact of CASZ1 in human T-ALL, we tried to silence *CASZ1* in T-ALL cell lines. However, we were able to do so only mildly in Jurkat cells, after transfection with small interfering RNA (siRNA) for CASZ1. Although CASZ1 knock down was only around 25-30% (*Online Supplementary Figure S10A*), there was a tendency for decreased viability in cells with lower CASZ1 levels (*Online Supplementary Figure S10B*) and a statistically significant negative impact on proliferation (*Online Supplementary Figure S10C*). We then ectopically expressed CASZ1 in several T-ALL cell lines using lentiviral transduction (*Online Supplementary Figure S11A*). Overexpression of CASZ1 had no impact on T-ALL cell viability (*Online Supplementary Figure S11B*) or proliferation (*Online Supplementary Figure S11C*). Basal viability and growth rate of T-ALL cell lines in regular culture conditions are high. This may mask positive effects of some oncogenes. For instance, *in vitro* prosurvival effects of TAL1 in T-ALL

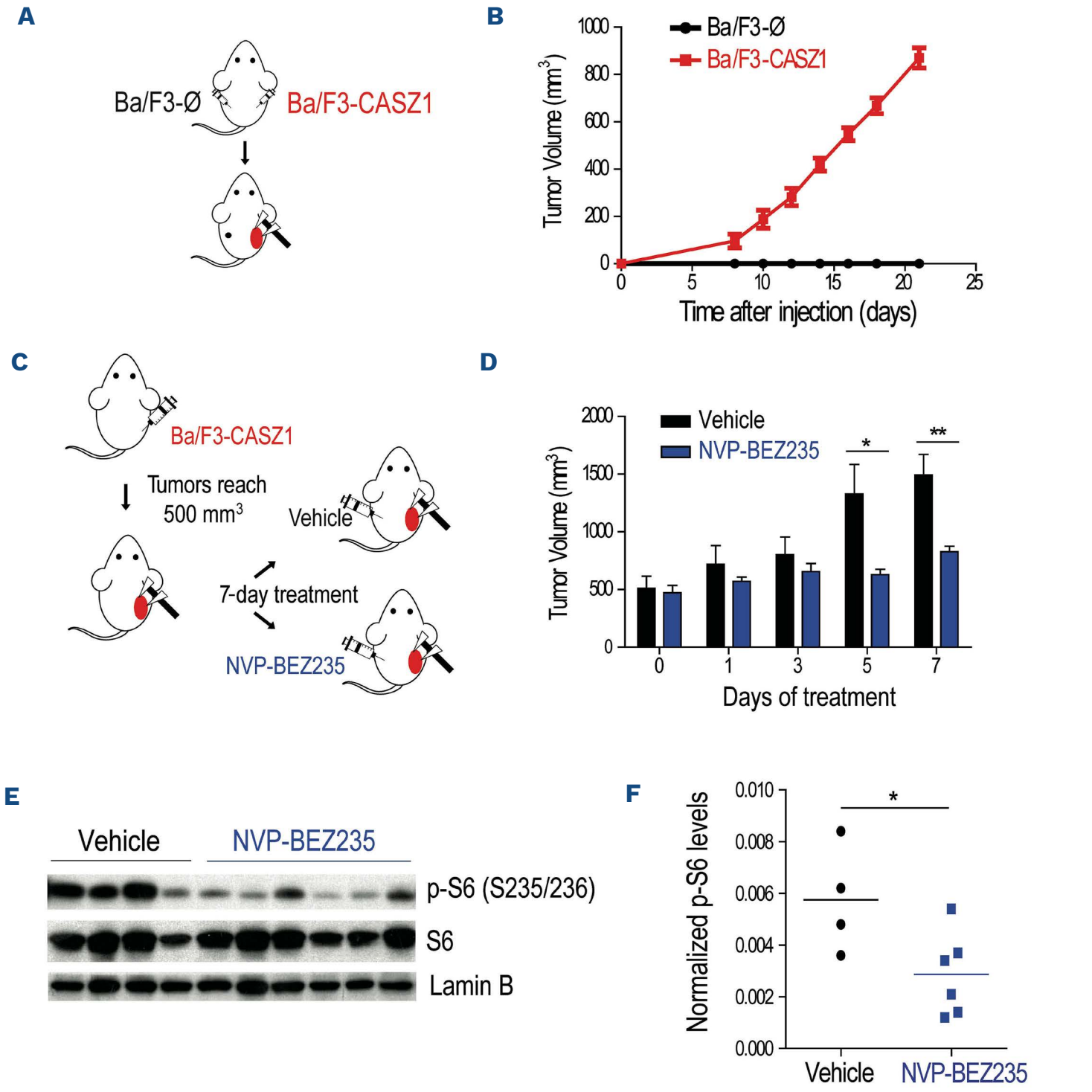

Figure 5. CASZ1 overexpression in Ba/F3 cells drives *in vivo* tumorigenesis by activating the PI3K-AKT-mTOR signaling pathway. (A, B) Eight-week old NOD/SCID mice (N=10) were injected subcutaneously with 10^7 Ba/F3-Ø or Ba/F3-CASZ1 cells and tumor growth was measured as described in the methods. (A) Schematic representation of the experimental layout. (B) Longitudinal analysis of tumor growth. (C-F) Eight-week-old NSG mice were injected subcutaneously with 10^7 Ba/F3-CASZ1 cells. Once the tumors reached 500 mm^3, mice were randomized to receive either NVP-BEZ235 (N=6) or vehicle (N=5) for 7 consecutive days. (C) Schematic representation of the experimental layout. (D) Longitudinal analysis of tumor growth upon treatment initiation. **P*<0.05; ***P*<0.01, one-way analysis of variance. (E) Vehicle- or NVP-BEZ235-treated tumors were collected and phosphorylation and total levels of S6 assessed by immunoblot analysis. Lamin B was used as loading control. (F) S6 phosphorylation levels in (E) were measured by densitometry analysis, and then normalized to total S6 and Lamin B levels. **P*<0.05; ***P*<0.01, student's *t* test.

cells were exposed only upon demonstration that TAL1 protected leukemia cells from stress-induced apoptosis.[41] Similar to TAL1, CASZ1 overexpression protected T-ALL cells from serum starvation-induced apoptosis (Figure 7A). Moreover, CASZ1 conferred resistance to treatment with daunorubicin, dexamethasone and L-asparaginase (Figure 7B), chemotherapeutic drugs currently used to treat patients with T-ALL.

Based on our findings that CASZ1 overexpression could rescue T-ALL cell viability under stress conditions and contribute to *in vitro* resistance to chemotherapeutic drugs commonly used in the treatment of T-ALL patients, we hypothesized that high expression of *CASZ1* might correlate with unfavorable outcome. However, we did not observe any significant association between *CASZ1* expression levels and relapse-free or overall survival in children with newly diagnosed T-ALL enrolled in the St. Jude Children's Research Hospital Total Therapy 15 and 16 studies (*Online Supplementary Figure S12A, B*), even when specifically examining relapse cases (*Online Supplementary Figure S12C*).

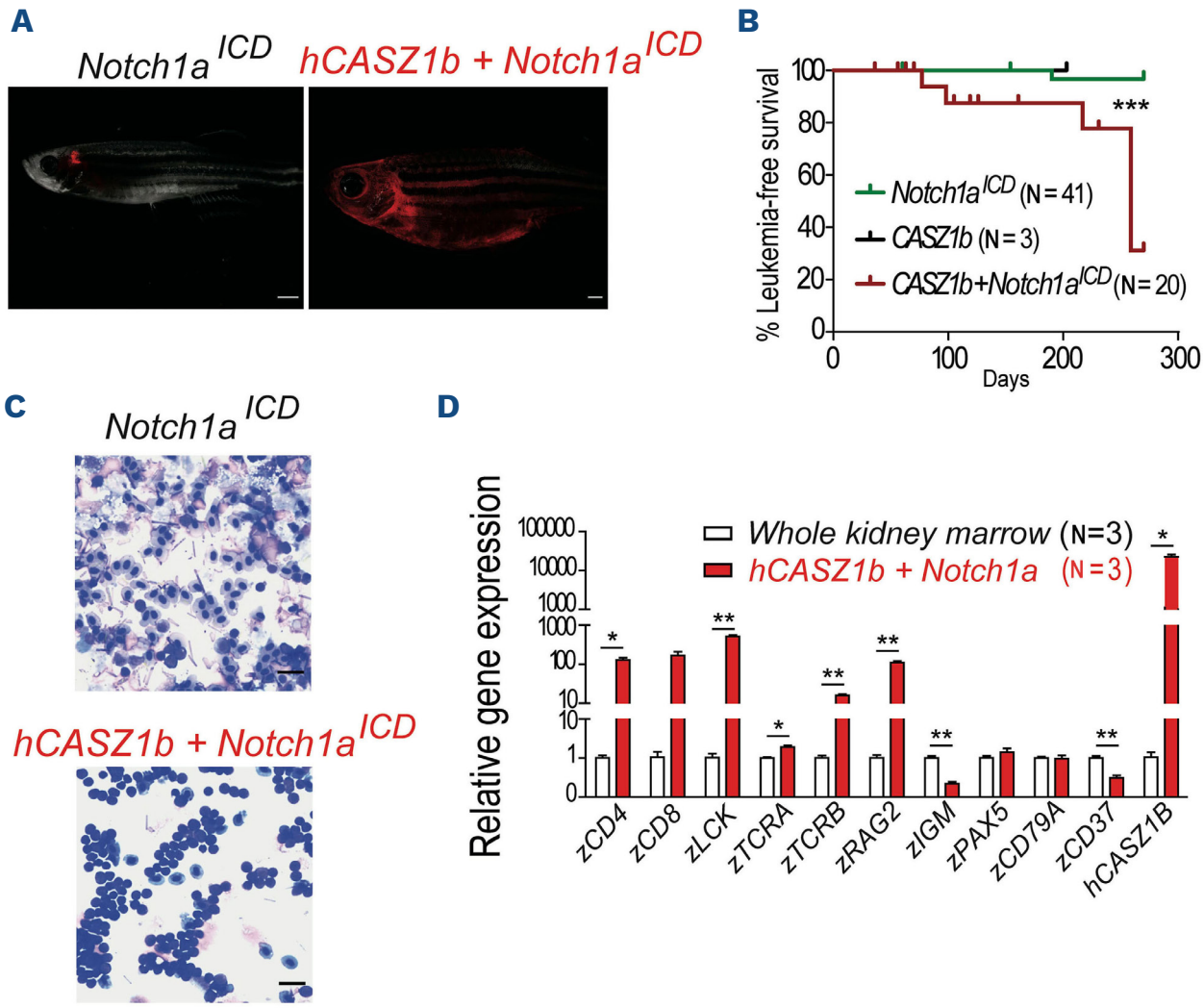

Figure 6. CASZ1 cooperates with NOTCH1 to drive T-cell leukemogenesis in transgenic zebrafish. Tu/AB strain embryos were injected at one cell stage with *rag2:notch1a*^{ICD}, *rag2:hCASZ1b* or a mixture of both. (A) Images representative of mosaic transgenic zebrafish at 77 days post injection. (B) Kaplan-Meier analysis of leukemic fish comparing *Notch1a*^{ICD} (N=15), *hCASZ1b* (N=3, sacrificed at 203 days without evidence of disease) and *hCASZ1b* + *Notch1a*^{ICD} (N=20). ***P<0.001, log-rank (Mantel-Cox) test. (C) May-Grünwald and Wright-Giemsa stained cytospins showing lymphoblast morphology; 400x magnification. Images are representative of at least 3 independent fish analyzed of each genotype up to 100 days post injection. Scale bar equals 10 μm. (D) Transcript levels of the indicated genes were determined by quantitative polymerase chain reaction (qPCR) and normalized to β-*actin*. The values were further normalized to the control condition (whole kidney marrow) and represent the mean ± standard deviation of at least 2 independent replicates. (E) Representative hematoxylin- and eosin-stained histological sections juxtaposed to immunohistochemistry for TUNEL. Scale bar equals 10 μm. (F) Values are the quantification of the immunohistochemistry data represented in (E). * P<0.05; **P<0.01, student's t test.

Nevertheless, in another cohort comprising exclusively of relapse T-ALL patients (GSE18497), for which gene expression and clinical parameters are publicly available,[36] high CASZ1 levels associated with particularly poor prognosis, as the CASZ1-high group displayed faster progression to disease relapse and decreased overall survival (*Online Supplementary Figure S12D-F*). Interestingly, in agreement with our findings that TAL1 regulates CASZ1 in T-ALL, *TAL1* was also associated with poor prognosis in the GSE18497 dataset (*Online Supplementary Figure S12G-I*). Overall, these analyses suggest that CASZ1 is not an independent prognostic factor in T-ALL, although it may associate with poor prognosis in relapse T-ALL depending on the specific treatment protocol.

Discussion

CASZ1 is a conserved zinc finger transcription factor that is essential for heart and neural development.[20,22-25,28,29] Not-

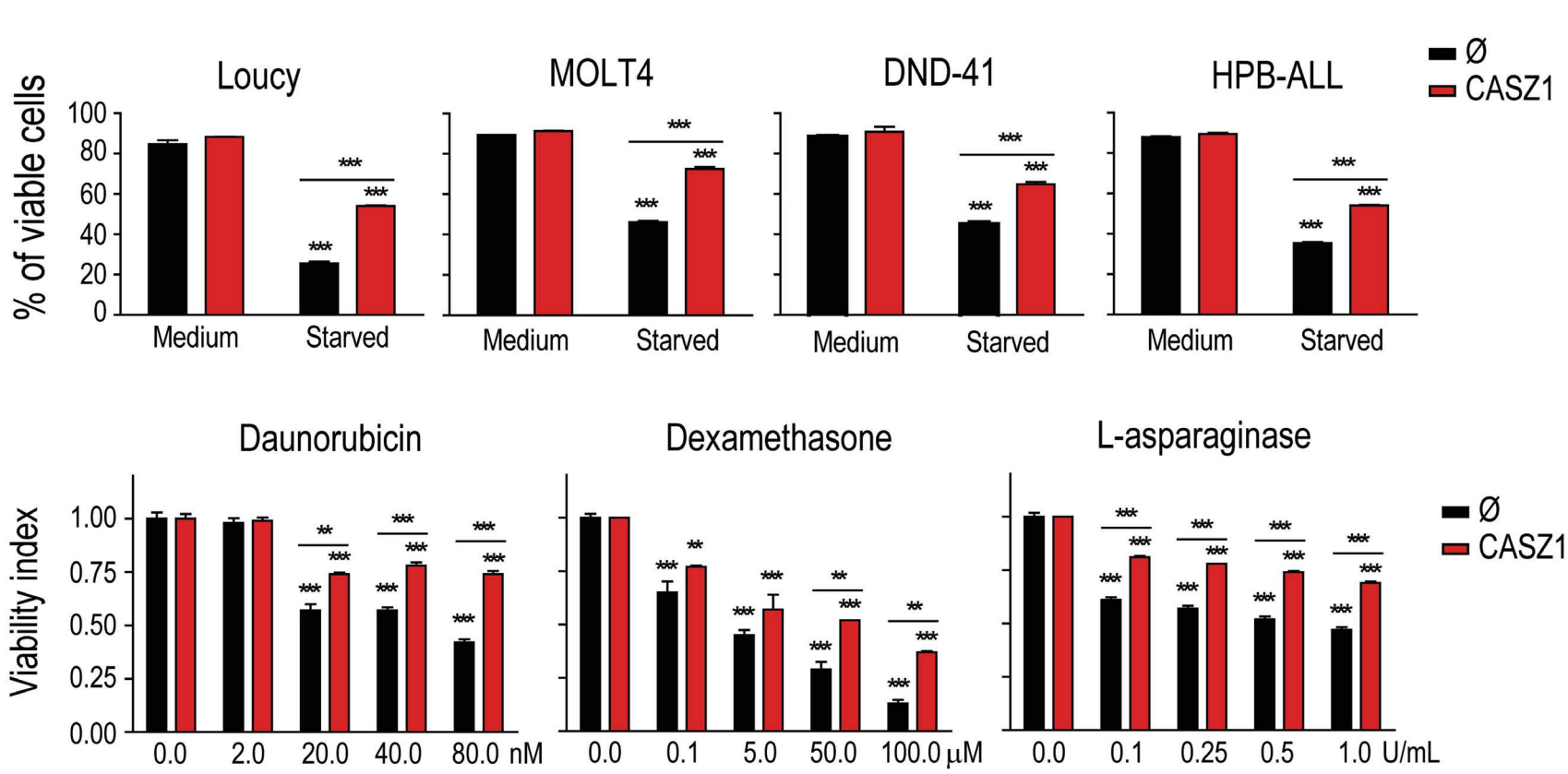

Figure 7. CASZ1 overexpression promotes T-cell acute lymphoblastic leukemia resistance to chemotherapy. (A) T-cell acute lymphoblastic leukemia (T-ALL) cells without (Ø) or with CASZ1 overexpression were cultured in regular culture conditions (medium) or under serum starvation (starved) for 48 hours [h] (Loucy), 72 h (MOLT4) or 144 h (DND-41 and HPB-ALL). (B) Loucy cells were cultured with the indicated concentrations of daunorubicin (72h), dexamethasone (72 h) and L-asparaginase (24 h). (A, B) Viability was determined, and the indicated values represent the mean ± standard deviation of at least 3 independent experiments **P*<0.05; ***P*<0.01; ****P*<0.001, student's *t* test.

withstanding its pivotal role in embryogenesis, CASZ1 has been also implicated in cancer, either by acting as tumor suppressor in neuroblastoma,[28,29] embryonal rhabdomyosarcoma[31] and hepatocellular carcinoma[30] or by promoting tumor metastasis in an ovarian cancer model.[32] Here, we demonstrate that CASZ1 is overexpressed and acts in an oncogenic manner in T-ALL.

The transcription factor TAL1 is a major oncogene in the context of T-ALL,[42] being overexpressed in a large portion of the patients and defining one of the subgroups of the disease.[43] TAL1 transgenic mice develop aggressive T-cell leukemia albeit with long latency.[44,45] Previous studies unveiled the transcriptional program associated with TAL1 in T-ALL.[46-48] Here, we demonstrated that TAL1 can regulate *CASZ1*. However, analysis of publicly-available ChIP-seq data revealed that TAL1 binding to the *CASZ1* locus in T-ALL cells is limited, with small and non-conserved peaks (*Online Supplementary Figure S13*), suggesting that TAL1 may have, at best, a mild direct regulatory effect on *CASZ1*. In addition, the negative impact of TAL1 genetic inactivation on *CASZ1* expression varied in degree amongst the T-ALL cell lines and primary leukemia cells we analyzed. This, together with the fact that T-ALL samples in general (including TAL1-negative cases) displayed upregulation of *CASZ1* as compared to normal thymocytes, suggests that additional factors should contribute to *CASZ1* upregulation in T-ALL. Preliminary analyses of publicly-available data and treatment of T-ALL cell lines with small molecule inhibitors did not find evidence for NOTCH1, MYC or EZH2 being consistent upstream regulators of CASZ1 (*data not shown*). Thus, the identification of CASZ1 upstream regulators in T-ALL, including transcription factors directly activating CASZ1, warrants further investigation.

The role of CASZ1 in hematopoietic differentiation remains unaddressed. However, our analyses of a mouse dataset from the BloodSpot database[33] allowed us to trace *CASZ1* expression throughout T-cell ontogeny. *CASZ1* expression is low in developing and terminally differentiated T cells, paralleling that of TAL1 expression.[49] Compatible with an oncogenic role for CASZ1 overexpression, *CASZ1* levels are normally low in healthy T-cell progenitors, which are the targets for malignant transformation in T-ALL.[1] In agreement, we showed that CASZ1 is able to cooperate with ICN1 to drive T-cell leukemia in zebrafish, by promoting the survival of T-ALL blasts (effects that are recapitulated upon CASZ1-mediated transformation of Ba/F3 cells). Interestingly, in established human leukemia, the prosurvival role of CASZ1 was exposed (reminiscent of what happens with TAL1[41]) when T-ALL cells were cultured under stress conditions, either via serum starvation or treatment with chemotherapeutic drugs. This suggests that CASZ1 may protect, to some extent, against chemotherapy. In agreement with this assumption, we found that, among patients with relapsed T-ALL, high CASZ1 expression can associate with accelerated time to relapse and decreased overall survival. The PI3K-AKT-mTOR signaling pathway regulates multiple

cellular processes, including those that are associated with the tumorigenic process, such as viability, proliferation, migration and metabolism. The pivotal contribution of this signaling pathway to the malignant process in T-ALL has been established.[5,8,50] In the present study, we demonstrated that CASZ1 expression activates PI3K-AKT-mTOR signaling in both Ba/F3 cells and T-ALL cells, which is required for CASZ1-mediated cell transformation. These results demonstrate that one of the major molecular events elicited by CASZ1 expression in T-ALL is the activation of the PI3K-AKT-mTOR pathway. The mechanisms by which CASZ1 activates PI3K signaling in lymphoid cells warrant investigation. Our bioinformatics analyses of RNA-sequencing data also indicated that CASZ1 can activate JAK-STAT5 and MAPK pathways (Figure 5C). We confirmed these findings at the protein level (*Online Supplementary Figures S14* and *S15*). Whereas we did not test the functional impact of JAK-STAT signaling downstream from CASZ1, we found that MAPK is required for *in vitro* transformation of Ba/F3 cells but dispensable for CASZ1-mediated expansion of Ba/F3 cells *in vivo* (*Online Supplementary Figure S16*).

Overall, our studies demonstrate that CASZ1 is upregulated in T-ALL (at least in part via TAL1 activity), protecting T-ALL cells from stress induced apoptosis and inducing the activation of the PI3K-AKT-mTOR signaling pathway, which is required for transformation *in vitro* and tumor growth *in vivo*. Our findings identify CASZ1 as a putative novel oncogene in T-ALL.

Disclosures

No conflicts of interest to disclose.

Contributions

BAC designed and performed experiments, analyzed and interpreted data, and drafted the manuscript. MD, AG, RF, MLO, JRA, LRM, NCC, AV, LD, FM and ABS designed, performed and interpreted experimental data. ARG supervised and performed bioinformatics analysis together with JLN. SK, SJ, CC, C-HP and CGM were involved in the collection and analysis of data on gene expression versus clinical outcome (St. Jude's cohort). SFdA, PVV, JAY, DML and FP contributed to the design of the study with additional experimental suggestions. JTB designed the project structure, interpreted data, coordinated the studies and wrote the manuscript. All authors critically read and contributed to the final version of the manuscript.

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Data-sharing statement

For original data that are not publicly deposited, please contact the corresponding author.

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