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ATP citrate lyase is an essential player in the metabolic rewiring induced by PTEN loss during T-ALL development.

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Abstract:

Alterations inactivating the tumor suppressor gene PTEN drive the development of solid and hematological cancers, such as T-cell acute lymphoblastic leukemia (T-ALL), whereby PTEN loss defines poor-prognosis patients. We investigated the metabolic rewiring induced by PTEN loss in T-ALL, aiming at identifying novel metabolic vulnerabilities. We showed that the enzyme ATP citrate lyase (ACLY) is strictly required for the transformation of thymic immature progenitors and for the growth of human T-ALL, which remain dependent on ACLY activity even upon transformation. Whereas PTEN mutant mice all died within 17 weeks, the concomitant ACLY deletion prevented disease initiation in 70% of the animals. In these animals, ACLY promoted BCL-2 epigenetic upregulation and prevented the apoptosis of pre-malignant DP thymocytes. Transcriptomic and metabolic analysis of primary T-ALL cells next translated our findings to the human pathology, showing that PTEN-altered T-ALL cells activate ACLY and are sensitive to its genetic targeting. ACLY activation thus represents a metabolic vulnerability with therapeutic potential for high-risk T-ALL patients.

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ATP citrate lyase is an essential player in the metabolic rewiring induced by PTEN loss during T-ALL development.

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Key points

1. The enzyme ATP citrate lyase (ACLY) is required for the transformation of thymic immature progenitors upon PTEN loss.
2. ACLY activation represents a metabolic vulnerability with therapeutic potential in high-risk PTEN-altered human T-ALL.

Abstract

Alterations inactivating the tumor suppressor gene *PTEN* drive the development of solid and hematological cancers, such as T-cell acute lymphoblastic leukemia (T-ALL), whereby *PTEN* loss defines poor-prognosis patients. We investigated the metabolic rewiring induced by *PTEN* loss in T-ALL, aiming at identifying novel metabolic vulnerabilities. We showed that the enzyme ATP citrate lyase (ACLY) is strictly required for the transformation of thymic immature progenitors and for the growth of human T-ALL, which remain dependent on ACLY activity even upon transformation. Whereas *PTEN* mutant mice all died within 17 weeks, the concomitant ACLY deletion prevented disease initiation in 70% of the animals. In these animals, ACLY promoted BCL-2 epigenetic upregulation and prevented the apoptosis of pre-malignant DP thymocytes. Transcriptomic and metabolic analysis of primary T-ALL cells next translated our findings to the human pathology, showing that *PTEN*-altered T-ALL cells activate ACLY and are sensitive to its genetic targeting. ACLY activation thus represents a metabolic vulnerability with therapeutic potential for high-risk T-ALL patients.

Introduction

The PI3K/AKT signaling pathway regulates essential cellular functions such as proliferation, survival, migration and metabolism. Directly opposing PI3K/AKT activation, the lipid phosphatase PTEN, (phosphatase and tensin homolog deleted on chromosome 10), fine-tunes these functions ensuring the maintenance of tissue homeostasis. *PI3K-AKT* activating mutations and/or alterations inactivating *PTEN* lead to the development of distinct human pathologies including cancer, where they are amongst the most frequent oncogenic events. This holds true not only for solid tumors but also for numerous hematological malignancies such as those arising from the transformation of thymic progenitor cells. During physiological conditions, PTEN regulates major developmental checkpoints in these cells, namely the beta selection¹ and the positive and the negative selections². During pathological conditions, instead, these cells undergo multiple genomic and non-genomic mechanisms which inactivate PTEN^{3,4}, thus driving transformation. As such, PTEN function is lost in both adult and pediatric T-cell acute lymphoblastic leukemia (T-ALL)^{3,5}. Similarly, *PTEN* alterations have been found also in pediatric T-cell lymphoblastic lymphoma (T-LBL)⁶, a closely related immature T-cell neoplasm⁷. In both these malignancies, genomic PTEN alterations have a negative prognostic impact and they identify a subgroup of patients at high-risk relapse^{5,6,8,9,10}. Genomic PTEN inactivation moreover correlates with therapy resistance in T-ALL cohorts^{5,10,11}. An unmet clinical challenge is therefore to provide novel therapies for these PTEN-altered patients with dismal outcomes.

PTEN supra-physiological expression induces a switch in the cellular metabolism, which protects the cells from the oncogenic transformation¹². Conversely, in distinct cell types PTEN loss increases lipid biogenesis, glycolysis, protein synthesis and affects mitochondrial metabolism¹³. As in solid tumors, leukemic cells need to rewire their metabolism to face cell growth and survival. In this context, PTEN loss is likely to play an important role. We addressed this aspect in T-ALL cells, to identify novel metabolic vulnerabilities and related therapeutic targets. Combining metabolic and transcriptomic profiling, a murine T-ALL/lymphoma model and T-ALL patient-derived xenografts, we identified ATP citrate lyase (ACLY) as a crucial player in the metabolic rewiring induced upon PTEN loss during T-ALL development. This central metabolic enzyme uses coenzyme A (CoA) and mitochondrial citrate to synthesize acetyl-coenzyme A (acetyl-CoA), which is indispensable for multiple anabolic reactions such as the synthesis of fatty acids, cholesterol, and specific amino acids¹⁴. Activated by AKT1 in response to oncogenic

signaling and cytokines stimulation¹⁵, ACLY promotes histone acetylation in both cancer and immune cells as acetyl-CoA is also the essential cofactor of histone acetyltransferases^{16,17,18,19}. In this study, we demonstrated that ACLY is a metabolic player required for the oncogenic transformation of thymic T-cell progenitors as well as for the growth of established human T-ALL cells, where it opens a therapeutic vulnerability.

Methods

Mice

PTEN^{flox/flox} (B6.129S4-Ptenth1Hwu/J), CD4^{Cre} (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ), ROSAYFP^{flox/flox} (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J) and ACLY^{flox/flox} mice (Aclyt1.1Welk/Mmjax) were purchased from Jackson laboratory and NOD Scid Gamma Mice (NSG) (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) from Charles River Laboratories. Mice were maintained as described in supplemental Methods. All experiments were ethically approved (APAFIS#25489-2019091313386161v3)

RNA sequencing of patient samples

Diagnostic peripheral blood or bone marrow samples from adult T-ALL patients were analyzed after informed consent was obtained according to the Declaration of Helsinki. Patients were included in the GRAALL03/05 trials registered at [ClinicalTrials.gov](https://clinicaltrials.gov) (GRAALL-2003, NCT00222027; GRAALL-2005, NCT00327678). The screening of oncogenetic abnormalities and immuno-phenotype was performed as previously described²¹.

Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen) and converted into cDNA using SuperScript III Reverse transcription (Thermo Fisher Scientific). Libraries were prepared using the SureSelect XT HS2 RNA System (Agilent) and sequenced on a NovaSeq (Illumina). Differential expression and pathway enrichment analyses were performed as previously described²¹. For metabolic-specific analyses, the entire dataset was reduced to metabolic genes as described³⁷. Analyses were carried out on a subset of 4,871 metabolic genes obtained from the Reactome Pathway database (ID R-HSA-1430728). PROGENy scores were computed on the entire transcriptome as previously reported^{21,22}.

In vitro stable isotope tracing

Cells were seeded in DMEM deprived of glucose and supplemented with 10% FBS and chased with 1mM labeled ¹³C glucose for 1,4 or 24 hours. 10⁶ live cells per replicate were collected, washed twice in pre-chilled PBS (400g, 5 min, 15°C centrifugation), snap frozen and stored at -80°C for further LC-MS-based metabolomic analyses.

Metabolic analysis

Targeted LC-MS metabolomics analyses were conducted on a QExactive Plus Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe coupled to a Dionex UltiMate 3000 uHPLC system (Thermo) as detailed in supplemental Methods. Intracellular cholesterol levels were quantified in 2.10^6 viable PDX blasts using the Amplex Red Cholesterol Assay Kit (Invitrogen), according to the manufacturer's instructions.

Statistical analysis

Whereas otherwise indicated, significance levels of data were determined by Student's t test, ANOVA or non-parametric equivalent tests depending on the normality of the data for the differences in mean values. Whereas otherwise indicated data are shown as average \pm SE in at least 3 independent experiments, each including at least $n=2$ per group. Asterisks refer to the following p-values: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$

Results

PTEN deletion activates ATP citrate lyase in pre-leukemic thymic cells.

Since PTEN regulates the cellular metabolism in distinct tissues, we hypothesized that PTEN loss could transform thymocytes by altering their cellular metabolism. To address this point, we employed a CD4Cre;PTEN^{flox2};ROSA^{YFP}^{flox2} model whereby PTEN deletion occurs in late double negative thymic progenitors and it can be monitored based on the expression of the YFP reporter. Using this model, which recapitulates PTEN-loss-induced T-ALL/lymphoma development^{1,24,25}, we performed targeted metabolomic profiling of pre-malignant PTEN mutant thymocytes (hereby referred as PTEN^{Δ/Δ})^{(1,2,25} and data not shown) and flox controls. Consistent with a PTEN-mediated regulation of glycolysis²⁵, pre-leukemic mutant thymocytes showed decreased glucose and glycolytic intermediates (Fructose 1,6 biphosphate, glyceraldehyde 3 phosphate, and pyruvate) and a parallel increased in lactate production (**Fig. 1A-D**). Mutant cells also depicted a decrease in glutamine, which paralleled an increase in α -ketoglutarate. Interestingly, moreover, fatty acids such as palmitic and oleic acids were among the most up-regulated metabolites in the mutant thymocytes cluster, whereas acetyl-CoA, the two-carbon donor for fatty acid synthesis, was among the most down-regulated metabolites. This was associated with a decrease in citrate and coenzyme A (**Fig 1B-D**). These findings suggest that, at a pre-leukemic stage, PTEN mutant cells might activate glycolysis, glutaminolysis and *de novo* lipogenesis²⁷. At the crossroads of these metabolic pathways, there is the enzyme ATP citrate lyase (ACLY), which converts citrate into acetyl-CoA. Notably, ACLY is a direct target of AKT, which triggers its phosphorylation on serine 455, a post-translational modification that increases the enzyme activity¹⁵. Based on these data, we investigated whether ACLY is phosphorylated in PTEN mutant thymocytes. As shown in **Fig. 1E**, ACLY phosphorylation on ser455 was increased in pre-malignant PTEN mutant thymocytes as compared to their control counterpart. Taken together, these results indicate that *Pten* loss increases ACLY phosphorylation, consistent with increased activity, in pre-malignant thymocytes.

Genetic ACLY ablation prevents PTEN-loss-induced T-ALL/lymphoma development.

To investigate whether ACLY could promote PTEN-loss-induced leukemogenesis, we genetically ablated ACLY in PTEN mutant mice using an ACLY^{flox2} model²⁸ (**Suppl. fig 1**). ACLY deletion in PTEN mutant mice prevented PTEN-loss induced changes in all glycolytic intermediates (**Fig 2A-E**) and it restored wild-type levels of α -ketoglutarate,

citrate and palmitate **Fig 2C-D**). Mice deleting both PTEN and ACLY (hereafter PTEN^{Δ/Δ};ACLY^{Δ/Δ}) also showed increased levels of acetyl-coA when compared to PTEN mutants (**Fig 2C-D**), albeit this did not reach statistical significance when a correction for multiple test hypothesis was applied. In line with this, the increased **extracellular acidification rate** and oxygen consumption rate occurring in double positive thymic cells upon PTEN deletion was reversed in the absence of ACLY (**Fig. 2E-F**). Taken together, these data indicate that, at a pre-malignant stage, PTEN loss induces a metabolic rewiring which is reversed by ACLY deletion. In addition, they suggest that the increased acetyl-coA production driven by PTEN loss is unlikely to originate from an increased glycolysis but rather from a general metabolic rewiring, which occurs upon PTEN loss and renders the cells more dependent on ACLY activity.

In turn, the metabolic changes induced by ACLY deletion were accompanied to a significant impact on disease development. As expected, within 140 days, all PTEN^{Δ/Δ} mutant mice died of T-ALL/lymphoma (**Fig. 3A**) and showed an accumulation of thymic cells bearing oligoclonal/monoclonal rearrangements of the TCRβ receptor locus and a DP TCRαβ⁺ phenotype (**Fig. 3B-C**). In line with previous findings, leukemic blasts predominantly showing a CD4 SP phenotype accumulated in blood, lymph nodes, spleen and bone marrow of PTEN mutant mice (**Fig. 3D-F** and data not shown).

Interestingly, however, only 30% of mice deleting both PTEN and ACLY (hereafter PTEN^{Δ/Δ};ACLY^{Δ/Δ}) succumbed from an oligoclonal and infiltrating disease (**Fig. 3A and data not shown**), developing with a significantly slower latency when compared to PTEN mutants (**Fig. 3A and suppl. fig 2A**).

The metabolic profile of double mutant mice developing the disease closely resembled the metabolic profile of PTEN mutants (**Suppl. fig.2B**). As such, in these animals, we did not observe a rescue of PTEN-loss induced decrease in acetyl-coA levels, which occurred instead in non-leukemic double mutant mice. (**Suppl. fig.2C**)

Strikingly, moreover, 70% of double mutant mice survived over one year without showing any signs of disease development. In these mice, leukemic cells were not observed in the peripheral organs or in the thymus (**Fig. 3D-F**), whereby normal polyclonal rearrangements of the TCRβ receptor locus were detected (**Fig. 3B-C**). When compared to their age-matched control counterparts, moreover, 1-year old PTEN^{Δ/Δ};ACLY^{Δ/Δ} mice presented a similar cellularity of lymphoid organs (**Suppl. fig 3F-I**) as well as a similar distribution of double positive (DP) and single positive (SP4 and SP8) thymic progenitors (**Suppl. fig 3E**). ACLY deletion *per se* did not alter mice survival (**Fig. 3A**), nor the cellularity of

lymphoid organs (**Suppl. fig 4A-D**), nor the frequencies of CD4+CD8+TCR $\alpha\beta$ + cells (**Suppl. Fig 5A-D**).

Taken collectively, these results demonstrate that ACLY plays a crucial role in PTEN-loss-induced T-ALL development by promoting disease initiation.

ACLY promotes apoptosis resistance in pre-leukemic DP progenitors and it epigenetically regulates Bcl-2 expression.

As previously shown, in PTEN mutant mice the disease originates from pre-leukemic DP cells which are transformed into leukemia-initiating cells following the acquisition of additional oncogenic events^{29,30}. Since ACLY was required to initiate the disease in PTEN mutant mice, we next addressed whether, on a mechanistic level, this could result from ACLY ability to promote apoptosis resistance in the cells of origin of the disease.

To investigate this hypothesis, we first analyzed whether ACLY activation selectively occurs in pre-leukemic PTEN null DP cells. Despite PTEN ablation activated AKT in double negative (DN), double positive (DP), CD4 single positive (SP4) and CD8 single positive (SP8) thymic progenitors, a significant increase in phospho ser455 ACLY levels was only observed in mutant DP cells (**Fig. 4A-B**), thus demonstrating that PTEN loss activates ACLY exclusively in the cells of origin of the disease. We next analyzed whether ACLY activation in these cells modulates the expression of anti-apoptotic proteins. As shown in **Fig 4C-D**, we did not observe significant differences in the expression of Bcl-XL, the main anti-apoptotic protein in DP thymic cells^{31,32}. Yet, in these cells, which developmentally express very low Bcl-2 during physiological conditions^{33,34}, PTEN loss induced a dramatic increase in Bcl-2 expression. Interestingly, this was prevented by ACLY deletion (**Fig. 4EC-E**). Since ACLY not only controls metabolic pathways but also epigenetically activates gene transcription, we next wondered whether ACLY-mediated Bcl-2 regulation occurred on a transcriptional level. PTEN loss up-regulated Bcl-2 transcripts and this was instead abrogated in the absence of ACLY (**Fig. 4F**). This ACLY-mediated transcriptional regulation of Bcl-2 moreover specifically occurred in DP cells but not other thymic populations such as DN cells which physiologically express high Bcl-2 levels (**Fig. 4G**). In line with this, in PTEN mutants we observed an increased H3K27 acetylation at the Bcl-2 main P1 promoter³⁵, which was instead reduced in double mutant mice (**Fig. 4H**).

These data, showing that ACLY is instrumental to epigenetically up-regulate BCL-2 in pre-leukemic PTEN null DP cells, next prompted us to functionally examine whether its

deletion prevents PTEN-loss induced resistance to apoptosis in these cells. Whereas DP cells sorted from PTEN mutants did not undergo apoptosis when treated *ex-vivo* with dexamethasone, DP cells sorted from PTEN^{Δ/Δ};ACLY^{Δ/Δ} mice were highly sensitive to dexamethasone-induced apoptosis (**Fig. 4I**). In line with this, ACLY deletion in PTEN null mice reduced the *in vivo* dexamethasone-resistance of pre-leukemic DP cells (**Fig. 4J**). Taken together, these data indicate that ACLY activation upon PTEN loss promotes the resistance of pre-leukemic DP progenitors to apoptotic stimuli.

Human PTEN null T-ALL cells modulate ACLY activity

We next investigated whether our findings could be recapitulated in the human setting. To do so, we first investigated whether PTEN silencing in a panel of T-ALL PTEN germ-line cell lines was able to activate ACLY. We thus transduced five T-ALL cell lines, expressing distinct PTEN levels (**Fig 5A**), with a lentiviral vector carrying an shRNA PTEN or an shRNA control (hereby shRNA Ctrl A). In these cells, PTEN silencing significantly increased ACLY phosphorylation on ser455 (**Fig. 5A-B**) and acetyl-CoA levels (**Fig. 5C**), consistent with an AKT1-mediated activation of the enzyme in these cells (**Fig 5A-B**). To further confirm that an increase ACLY activity occurs following PTEN loss in dynamic settings, we performed stable isotope tracing assays. Given that glucose is a major carbon source contributing to synthesis of ACLY's substrate citrate, we analyzed [13C]-glucose flux into acetyl-coA. **In cell lines, the flux of [13C]-glucose through the glycolysis and the TCA cycle was comparable in all experimental conditions (Fig. 5D-E). As compared to control cells, however, the acetyl-coA isotopologue labeled from glucose (acetyl-coA m+2) showed a more pronounced increase over time in cells deprived of PTEN. Moreover, its levels were 2-fold decreased when both PTEN and ACY were silenced, thus indicating that PTEN silencing increases glucose flux into acetyl-coA in an ACLY-dependent manner. Upon 4 hours [13C]-glucose incubation, acetyl-coA m+2 levels increased in control cells, whereas they remained low in ACLY silenced cells, in line with ACLY genetic silencing. Upon longer [13C]-glucose incubation times (24 hours), albeit remaining lower than in control cells, acetyl-coA m+2 levels showed an increase in ACLY silenced cells, which may result from compensatory mechanisms intervening in the absence of ACLY during longer labeling time.** Taken together, these evidences indicate that the increased levels of acetyl-coA in PTEN null cells are likely to be a direct consequence of an increased enzymatic conversion of citrate by ACLY rather than indirectly arising from a PTEN-loss induced increased glycolytic flux.

In line with this, ACLY targeting by a shRNA (**Suppl. Fig 6A**) prevented the growth of three cell lines tested but only when PTEN was also silenced (**Fig. 5F**). This suggests that only cells deprived of PTEN are specifically vulnerable to ACLY inhibition.

To further exclude off-target effects, we next silenced ACLY in a PTEN null Jurkat line, engineered to re-express PTEN following doxycycline exposure (**Suppl. Fig 6B**)²⁰. ACLY silencing reduced cell proliferation of these Jurkat cells but it did not once PTEN was re-expressed (**Fig. 5F**). Furthermore, reintroducing ACLY (**Suppl. Fig 7A**) in cell lines deprived of PTEN and ACLY, rescued cell growth defects in all cell lines tested (**Suppl. Fig 7B-E**), thus excluding off-target effects due to an shRNA-mediated ACLY silencing.

Since during disease initiation ACLY regulates Bcl-2 levels and apoptotic resistance, we next functionally explored whether ACLY regulates a Bcl-2 dependency in human T-ALL cell lines. To this end, we examined the sensitivity of T-ALL cell lines lacking PTEN, ACLY or both to the Bcl-2 inhibitor venetoclax. In addition, since mature T-ALL cells, such as those lacking PTEN⁵, are more dependent on Bcl-xL than Bcl-2³⁶, we also examined the sensitivity to the Bcl-xL inhibitor navitoclax. ACLY targeting did not affect steroid resistance nor the sensitivity of PTEN-null T-ALL cells to venetoclax and navitoclax (**Suppl. Fig 8A-G**), thus indicating that ACLY-mediated Bcl-2 regulation is dispensable once the leukemia is already developed while it is instead required during transformation.

To further translate our results to the patients setting, we performed a metabolic analysis of 8 patients derived xenografts (PDX). This included 3 patients with PTEN abnormalities, 1 patient with PI3KCA mutations and 4 patients with a germ line status for PTEN, PI3KCA, PI3KR1 and AKT1 (**Table 1**). Consistent with the reported genomic alterations, with the exception of one PTEN altered case, *PTEN/PI3KCA* altered patients displayed significantly higher phosphorylated AKT (ser347) levels (**Table 1**). This mirrored the clustering identified by the principal component analysis (**Fig. 6A**). Patients bearing genomic abnormalities in the PI3K/PTEN pathway displayed a lower levels of coenzyme A and acetyl-coA (**Fig. 6B-C and E**) and a parallel increase in palmitic acid and stearic acid (**Fig. 6E**). Albeit after correction for multiple testing hypothesis the changes in these metabolites lost statistical significance (Wilcoxon sum rank test $p=0.029$, $p_{adj}=0.065$), the trend observed for these metabolites in PTEN altered PDX parallels the data observed in the murine model. Acetyl-coA, coA and palmitic acids were for instance amongst the 21 metabolites mostly changed both in Pten mutant mice and PTEN altered PDX (**Fig. 6F**). Enrichment analyses revealed the prevalence of tRNA synthesis, amino acids and unsaturated fatty acids, consistent with acetyl-coA consumption (**Fig. 6D and Suppl. Fig. 9A-C**). Hence, our results suggest that PTEN loss of function shifts the blasts metabolism

towards fatty acid synthesis by modulating ACLY. To further investigate this aspect, we carried out a transcriptomic analysis of 155 primary T-ALL by RNA sequencing. Differential expression analysis of 4,871 metabolic genes revealed that most of the PTEN-altered cases compose a cluster (**Fig. 6G**). In line with our metabolomic analysis, the over-representation analysis of deregulated metabolic genes in PTEN-altered cases revealed an up-regulation of fatty acids and long-chain fatty acyl-CoA esters synthesis, and a down-regulation of CoA synthesis (**Fig. 6H**). We next stratified primary samples based on inferred PI3K activity as determined by PROGENy. This analysis confirmed that an elevated PI3K signaling is associated with the up-regulation of fatty acid synthesis and the TCA cycle (**Fig. 6I-K**). A strong PI3K activity also correlated with the enrichment in a cholesterol signature (**Fig. 6L**) and an increased cholesterol synthesis was observed in PTEN-altered PDX-derived blasts (**Fig. 6M**). Taken together, these analyses demonstrated that ACLY activation is at the center of a crucial metabolic rewiring induced by PTEN loss in T-ALL cells, which is likely to provide fatty acids and cholesterol necessary to their growth.

PTEN-altered T-ALL cells are sensitive to ACLY targeting

To further corroborate our findings, we next analyzed phospho ser455ACLY levels in additional 14 PDX-derived blasts T-ALL cells bearing *PTEN/PI3KCA* alterations and showing active AKT signaling. These patients displayed higher ACLY phosphorylation, consistent with an AKT1-mediated activation of ACLY (**Fig. 7A-B**). Based on this, we next silenced ACLY in five different PDX-derived blasts by transducing these cells with a baboon-pseudotyped lentiviral vector carrying an ACLY shRNA ACLY or a control shRNA³⁰. Remarkably, in 4/5 PTEN-altered PDX which we analyzed, ACLY silencing reduced their *in vitro* viability already 48 hours after the transduction. As shown in **Fig. 7C**, at this time point already a few viable GFP-positive cells were left among those transduced with the shRNA ACLY-carrying vector. In the fifth PDX derived sample, ACLY targeting did not affect the cellular viability ex-vivo, prompting us to examine its effect in vivo. We performed therefore a competitive transplantation assay, whereby transduced cells were allowed to compete with non-transduced cells in a 20:80 ratio following their injection into NSG mice. PDX-derived blasts lacking ACLY were out-competed by non-transduced cells, whereas GFP control cells were not (**Fig. 7D**). We next investigated whether ACLY represents a metabolic vulnerability specific to PTEN-altered T-ALL cases. We thus genetically silenced either PTEN or ACLY or both of them in cells derived from three distinct PDX displaying a germ line PTEN status. Although we could technically only

evaluate three PDX, we observed a similar trend in the three cases analyzed, which displayed a reduction in the number of viable cells when ACLY was silenced in combination with PTEN (**Fig. 7E**). Taken together, these results indicate that ACLY promotes the survival of PTEN altered T-ALL cells, where it represents a metabolic vulnerability.

Discussion

We demonstrated that ACLY is a metabolic player not only required for the oncogenic transformation of thymic T-cell progenitors but also for maintaining an established human T-ALL, where it represents an important metabolic vulnerability.

Combining metabolomics and a murine T-ALL/lymphoma model, we showed that PTEN loss selectively activates ACLY in pre-malignant DP thymocytes and that this is instrumental to drive disease initiation. From a mechanistic point of view, we show that this arises, at least in part, from an ACLY-mediated regulation of apoptosis resistance in these cells, which are the cells-of-origin of the disease in Pten conditional murine models^{29,30}. Whereas during physiological T-cell development, DP progenitors down-regulate BCL-2 in favor of BCL-XL, we show that in pre-leukemic conditions, PTEN loss does not affect BCL-XL expression but it rather induces supra-physiological levels of BCL-2 and that this occurs via ACLY activation. Mechanistically, we show that in these cells ACLY regulates Bcl-2 on a transcriptional level by acetylating the main Bcl-2 P1 promoter³⁵. Being PTEN-loss induced leukemogenesis in the mouse a multiple-hit process²⁹, our data support a model whereby ACLY-mediated apoptosis resistance via Bcl-2 up-regulation favors the selection of select pre-malignant DP, hence enabling them to undergo additional multiple oncogenic events, which fully transform these cells. In this context, it is worth noticing that despite ACLY is required for the growth of fully established leukemias, in already transformed cells ACLY does not functionally regulate Bcl-2 nor apoptotic resistance. These evidences, which indicate that ACLY-mediated Bcl-2 up-regulation occurs only during leukemia ontogeny, further strengthen its role during transformation. Along the same line, albeit during physiological conditions, ACLY regulates apoptosis in thymic DP cells, in this context it operates via a Bcl-2 independent mechanism (unpublished). This further argues in favor of a specific requirement for ACLY-mediated Bcl-2 up-regulation during the leukemic ontogeny in the absence of Pten. Taken together, these findings provide at least one possible mechanism underlying the survival of Pten ACLY double mutants. Yet, given the strikingly survival rate of these animals, it is likely that the multiple additional mechanisms intervene in this context. Further studies will be thus necessary to fully elucidate these aspects.

As our translational investigations point-out, ACLY role in T-ALL development relates not only the oncogenic transformation of immature thymic progenitors but also the maintenance of an established leukemia, which remains dependent on ACLY activity even upon transformation.

In this context, ACLY anabolic functions are likely to play an important role since in *PTEN/PI3K* altered cells metabolic, genetic and transcriptomic profiling of human T-ALL cells indicate an increased need for ACLY to supply acetyl-coA for fatty acids and cholesterol biosynthesis. These findings, which recapitulate the results obtained with the murine model, are in line with previous data highlighting a role for cholesterol biosynthesis in the growth of immature ETP-ALL cells³⁷. Consistent with its activation in PTEN-altered patients, ACLY sustains the growth of T-ALL cells that underwent PTEN loss. As evidenced in both cell lines and primary T-ALL, T-ALL cells deprived of PTEN were sensitive to ACLY silencing.

Highlighting a metabolic vulnerability linked to PTEN/PI3K genetic alterations, our data suggest that high-risk T-ALL patients bearing these abnormalities may benefit from pharmacological ACLY targeting. Albeit ACLY inhibitors have been under development, the only FDA-approved ACLY inhibitor (Bempezoic acid) also stimulates AMPK activity and thus lacks selectivity. Conversely, the ACLY inhibitors which showed good specificity did not reach clinical studies, as in the case of SB-204490 a poor tissue-specific distribution in humans has limited them³⁸. In light of this, our results hold the promises of boosting the development of novel specific ACLY inhibitors suitable for clinical intervention.

In conclusion, our study revealed that PTEN loss defines a metabolic rewiring in T-ALL cells, whereby ACLY is a major player favoring not only the transformation of thymic progenitor cells but also the growth of established human leukemias. Primary T-ALL cells dependency on ACLY activity thus represents a novel metabolic vulnerability with therapeutic potential.

Data sharing

For original data, please contact melania.tesio@inserm.fr or vahid.asnafi@aphp.fr

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Authorship contributions

G.P.A. provided methodology, investigation, analysis, validation, data curation, writing of the original draft preparation; G.H. provided investigation and analysis; E.B., C.C, E.V M.S. provided provided resources; O.A. provided biostatistical analysis, I.N. provided metabolic investigations, G.P. provided resources and writing (reviewing and editing); K.W. and E.M. provided resources, V.A. undertook methodology, validation, resources, data curation, writing supervision, funding acquisition and M.T. undertook conceptualization, methodology, validation, resources, data curation, writing supervision, project administration, and funding acquisition

Disclosure of Conflicts of Interest

The authors declare no competing financial interests

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Table 1

PDX	PTEN	PIK3CA	PIK3R1	AKT1	pAKT levels (geo mean from FACS analysis)
UPN 663	p.R233WA biallelic	GL	GL	GL	15,1
UPN 534	Type I monoallelic deletion	GL	GL	GL	14,7
UPN 374	GL	c.1624G>A biallelic	GL	GL	19,4
UPN 519	p.R233SfsX14 biallelic	GL	GL	GL	24,5
UPN 584	GL	GL	GL	GL	0
UPN 567	GL	GL	GL	GL	0
UPN 613	GL	GL	GL	GL	934
UPN 610	GL	GL	GL	GL	1,7

Table 1. PTEN, PI3K3A, PI3R1, AKT1 status and phospho AKT levels in 8 PDX which underwent metabolomic analysis

Figure legends

Figure 1. Metabolic analysis of pre-leukemic *pten* mutant thymocytes.

(A) Principal component analysis of 4 *pten* mutant and 5 *ptenflox2* thymi issued from pre-leukemic 8 weeks old mice subjected to unsupervised metabolic analysis. (B) Heatmaps of the 50 most deregulated metabolites, (C) glycolytic intermediates, Krebs cycle intermediates, glutamine, glutamate, citrate, coenzyme A, acetyl-coA and fatty acids and (D) pathway analysis in 4 pre-leukemic *PTEN* Δ/Δ thymocytes or 5 flox control counterparts. (E) Phosphorylated ser455-ACLY in the thymi of pre-leukemic *PTEN* Δ/Δ mutant or control animals in 3 independent experiments, with at least n= 2 per group.

Figure 2. Metabolic analysis of thymi from *PTEN* Δ/Δ , *ACLY* Δ/Δ , *PTEN* Δ/Δ ;*ACLY* Δ/Δ or *PTENflox2*;*ACLYflox2* mice.

(A) Principal component analysis of 4 *PTEN* Δ/Δ mutant, 3 *ACLY* Δ/Δ mutant, 3 *PTEN* Δ/Δ ;*ACLY* Δ/Δ and 5 *PTENflox2*;*ACLYflox2* thymi from pre-leukemic 8-weeks old mice undergoing unsupervised metabolic analysis. (B) Heatmaps of the 50 most deregulated metabolites, (C) analysis of glycolytic intermediates, Krebs cycle intermediates, glutamine, glutamate, citrate, coenzyme A, acetyl-coA and fatty acids and (D) pathway analysis in 4 *PTEN* Δ/Δ mutant, 3 *ACLY* Δ/Δ mutant, 3 *PTEN* Δ/Δ ;*ACLY* Δ/Δ and 5 *PTENflox2*;*ACLYflox2* thymi. Extracellular acidification rate (ECAR) (E) and oxygen consumption rate (OCR) (F) of sorted DP thymocytes in 2 independent experiments, with at least n=2 per group.

Figure 3. Genetic *ACLY* ablation prevents *PTEN*-loss induced T-ALL/lymphoma development.

(A) Kaplan–Meier survival curve for *PTEN* Δ/Δ mutant mice (n=43), *ACLY* Δ/Δ mutant (n=16), *PTEN* Δ/Δ ;*ACLY* Δ/Δ double mutant mice (n=22) and floxed controls (n=40). TCR β rearrangements (B) and phenotypic analysis of the thymus (C) in *PTEN* Δ/Δ mutant, *ACLY* Δ/Δ mutant, *PTEN* Δ/Δ ;*ACLY* Δ/Δ animals or flox controls. CD4⁺ absolute numbers cells in the spleen (D), bone marrow (E) and lymph nodes (F) in 14-weeks old *PTEN* Δ/Δ mutant, *ACLY* Δ/Δ mutant, *PTEN* Δ/Δ ;*ACLY* Δ/Δ animals or flox controls (including *PTENflox2*, *ACLYflox2* and *PTENflox2*;*ACLYflox2* mice).

Figure 4. *ACLY* ablation abrogates apoptosis resistance in mutant DP progenitors

(A) Phospho ser473-AKT and **(B)** and phospho ser455-ACLY on DN, DP, SP4 and SP8 from pre-leukemic PTEN Δ/Δ mutant or PTENflox2 controls. In mutant mice, carrying the ROSAYFPflox2 reporter, DN, DP, SP4 and SP8 cells are gated on YFP⁺ cells. Levels of Bcl-xL **(C and D)** and Bcl-2 **(C and E)** in DP cells from controls or mice lacking PTEN, ACLY or both. Bcl-2 relative expression to actin in DP cells **(F)** and DN **(G)** sorted from 4 PTEN Δ/Δ mutant, 3 ACLY Δ/Δ mutant, 4 PTEN Δ/Δ ;ACLY Δ/Δ double mutant mice or 3 flox controls. **(H)** H3K27Ac enrichment at the Bcl-2 P1 promoter in 3 PTEN Δ/Δ mutant, 3 ACLY Δ/Δ mutant, 3 PTEN Δ/Δ ;ACLY Δ/Δ double mutants and flox controls. Data represent percentages of input normalized to the actin promoter. Sensitivity of DP cells to dexamethasone ex vivo **(I)** or in vivo (2 mg/kg/day for 3 consecutive days) **(J)** in PTEN Δ/Δ mutant, ACLY Δ/Δ mutant, PTEN Δ/Δ ;ACLY Δ/Δ animals or flox controls (including PTENflox2, ACLYflox2 and PTENflox2;ACLYflox2 mice).

Figure 5. PTEN loss activates ACLY in human T-ALL cells to sustain cell growth

PTEN, pAKTser473 and pACLYser455 **(A-B)** and acetyl-coA levels **(C)** in human T-ALL cell lines transduced with a lentiviral vector carrying an shRNA PTEN or a control shRNA (shRNA Ctrl A). Relative abundance of unlabeled and labelled metabolites mapped to glycolysis **(D)** and TCA **(E)** in DND41-cells exposed to [13C]-glucose for 1 hour, 4 hours or 24 hours. **(F)** Growth of DND41, RPMI-8402 and HBP-ALL transduced with a shRNA PTEN, a shRNA ACLY or the combination of the two and relative control shRNA (sh RNA Ctrl A as control for the shRNA targeting PTEN and shRNA Ctrl B as control for the shRNA targeting ACLY). **(G)** Growth of Jurkat cells transduced with an shRNA ACLY or control shRNA in the presence or in the absence of doxycycline-mediated PTEN re-expression.

Figure 6. Targeted metabolic and transcriptomic analysis of patients-derived T-ALL cells

(A) Principal component analysis of 8 PDX undergoing targeted metabolomic analysis. **(B)** Most up-regulated and down-regulated metabolites in PTEN-altered cases. **(C)** Heatmaps of the most 50 deregulated metabolites and **(D)** pathways enriched in PTEN-altered patients. **(E)** Metabolic analysis of citrate, coenzyme A, acetyl-coA, glutamine, glutamate, alpha-ketoglutarate, palmitic acid, stearic acid and oleic acid in the 8 PDX shown in A. **(F)** Venn diagramme for metabolites mostly changed in both PTEN mutant mice and PTEN altered PDX ($p < 0.05$). **(G)** Metabolic transcriptome-based clustering of 155 patients **(H)** Pathway enrichment analyses based on the differential metabolic gene expression of PTEN-altered ($n=33$) vs wild-type ($n=122$) patients determined using EnrichR. **(I)**

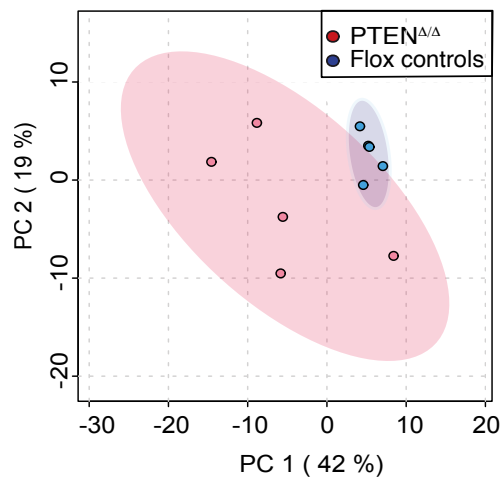
PROGENy score computation from whole transcriptomics data from 155 patients. The median score of the series was used to stratify patients into high and low-score groups. **(J)** Volcano plot depicting differentially expressed metabolic genes in high vs low PI3K score. **(K-L)** Enriched metabolic gene sets enrichment in high vs low PI3K score as determined by GSEA. **(M)** Intracellular levels of cholesterol in WT or altered PI3K signaling T-ALL PDX blasts.

Figure 7. ACLY silencing in primary PTEN null T-ALL cells affects their viability

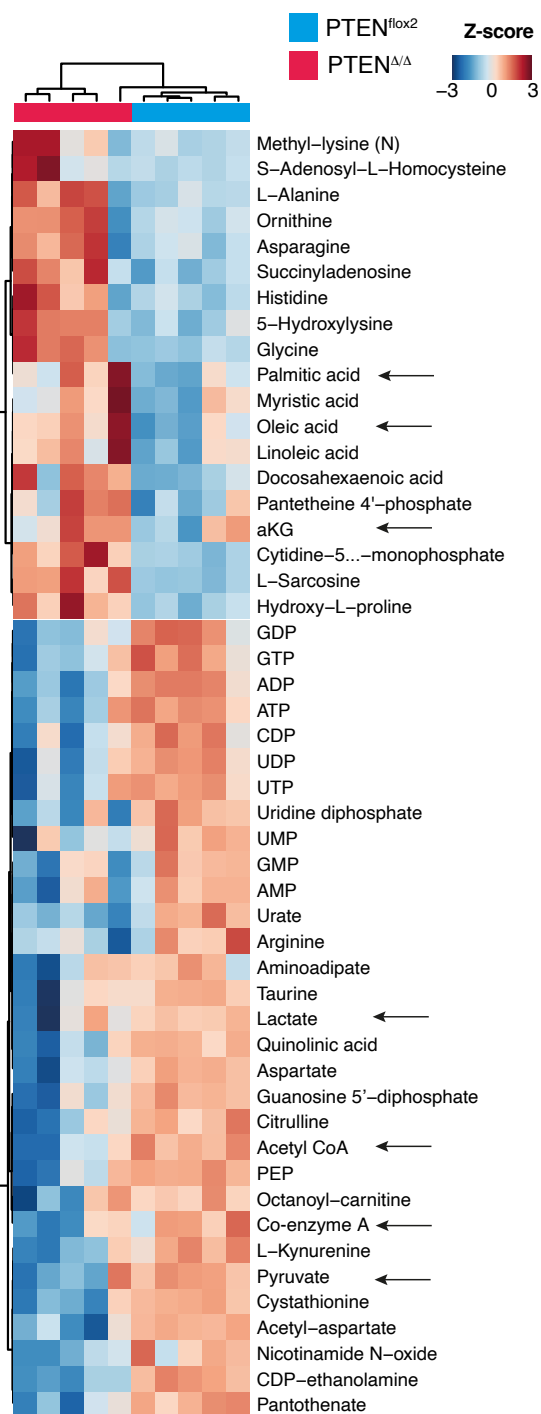
(A) pACLYser455 levels in 8 PTEN/PI3KCA altered and 6 WT PDX-derived T-ALL blasts. **(B)** Representative FACS plot depicting pAktser473 and pACLYser455 in a PTEN/PI3KCA WT patient or a PTEN-deleted patient. **(C)** Percentages of GFP+PTEN altered PDX-derived blasts remaining viable 48 hours post-transduction with lentivirus vectors carrying shRNA ACLY or a control construct. **(D)** Competitive transplantation assay, whereby PDX-derived cells transduced with a shRNA ACLY or a shRNA control competed with non-transduced cells in a 20:80 ratio following injection into NSG mice. The relative ratio of transduced and not transduced cells found in the bone marrow of NSG mice is shown. **(E)** Number of GFP+ PTEN germ-line PDX-derived blasts remaining viable 3, 4 or 5 days post-transduction.

Figure 1

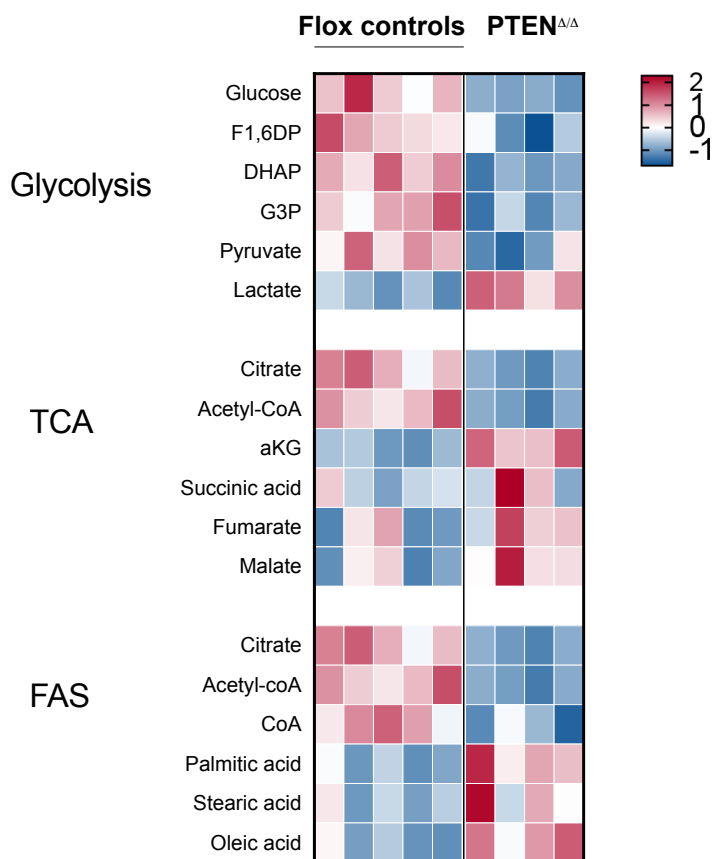
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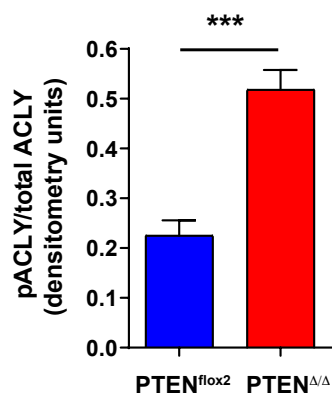
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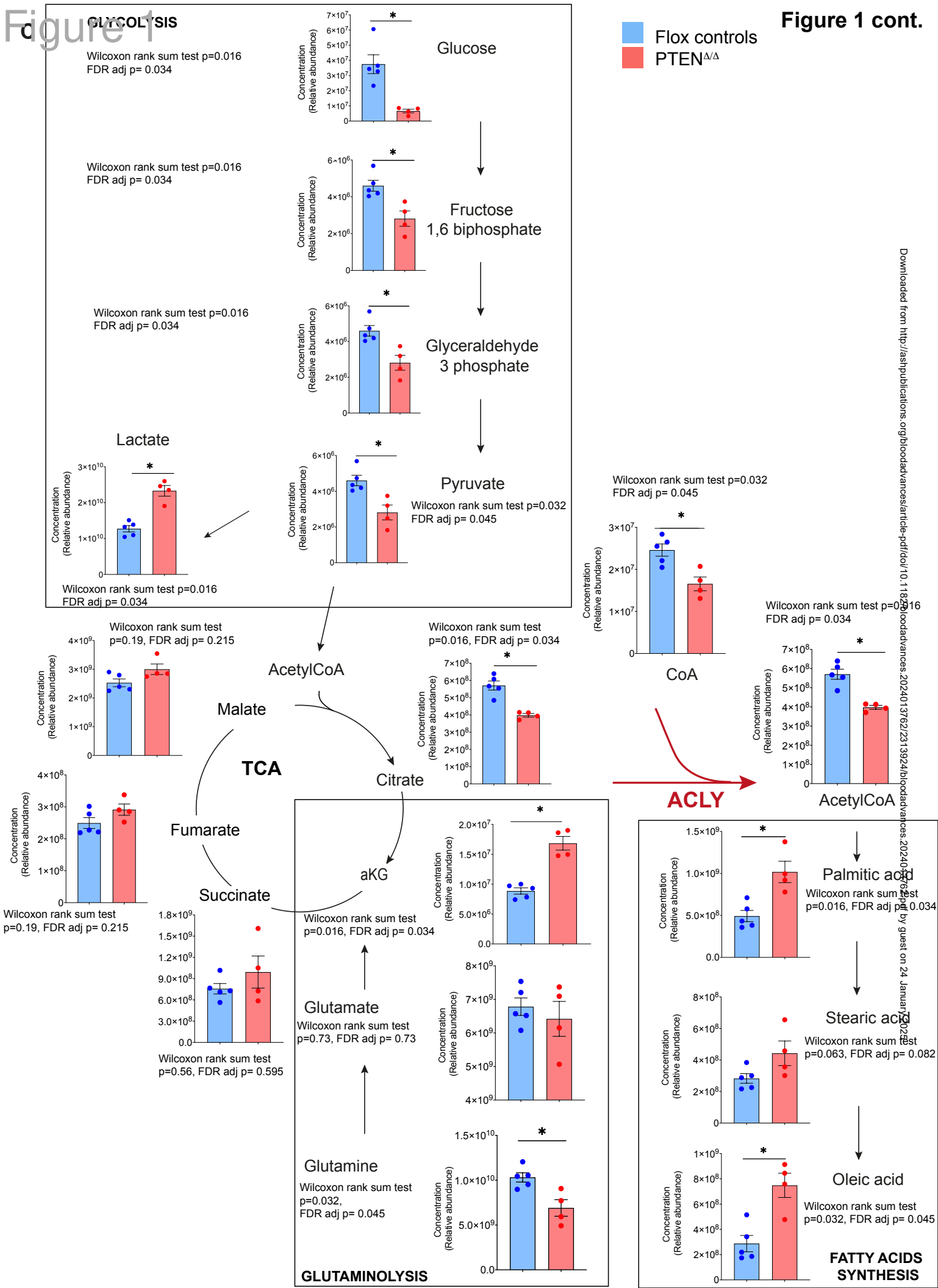
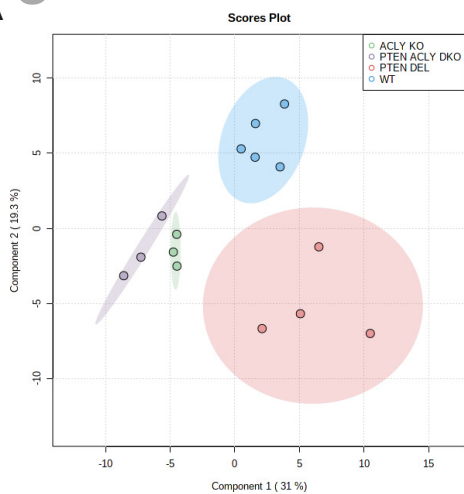
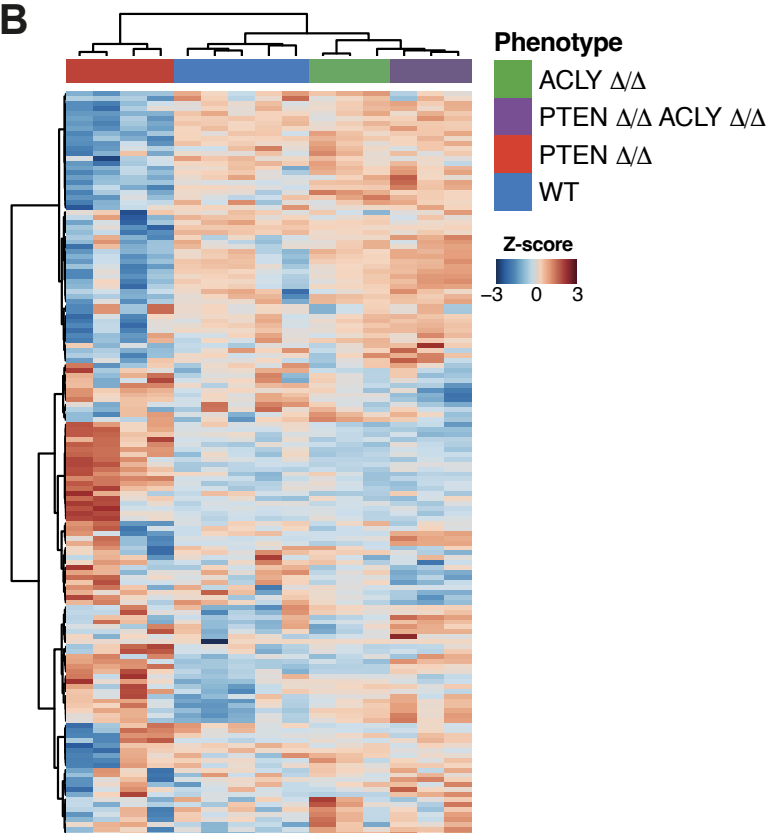


Figure 2

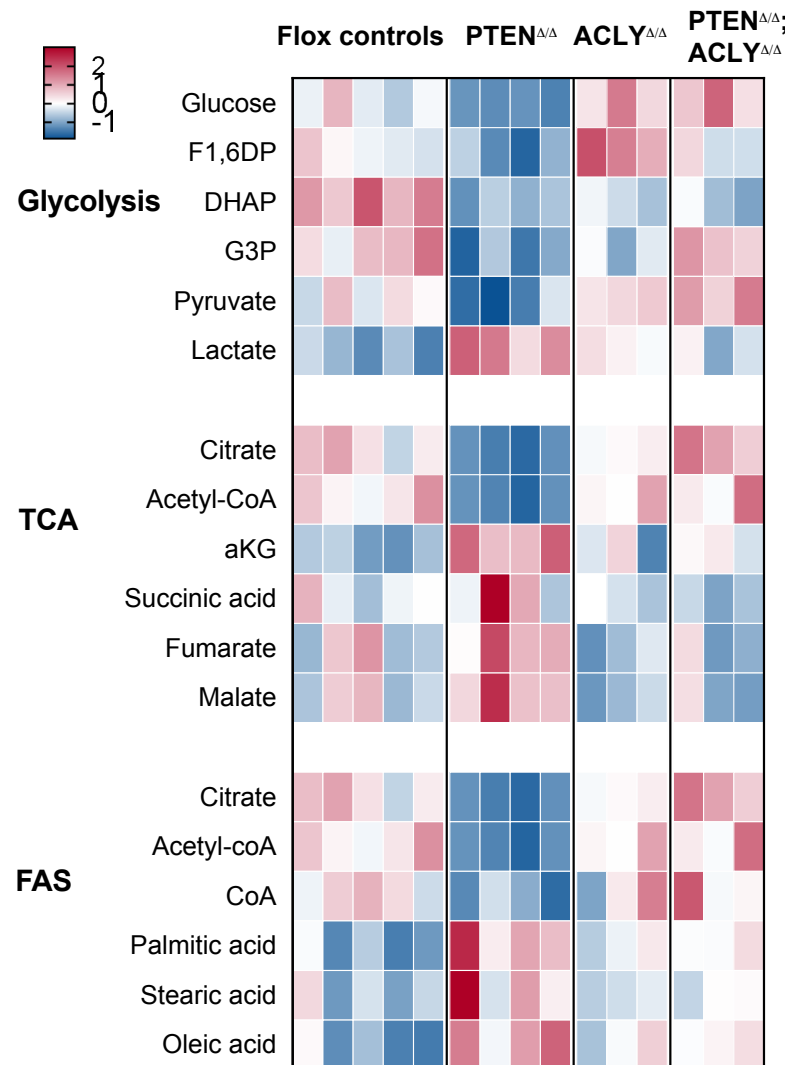
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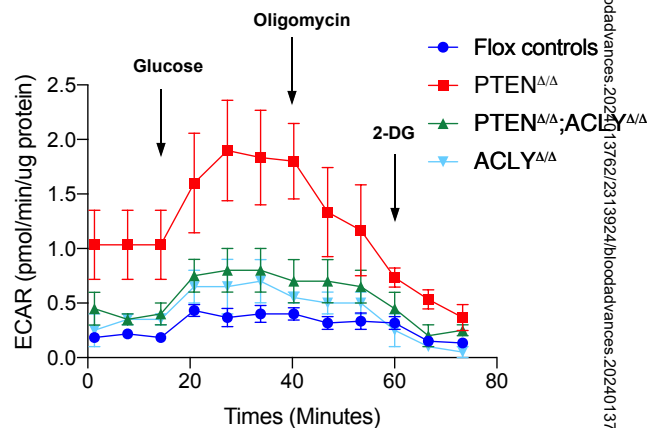
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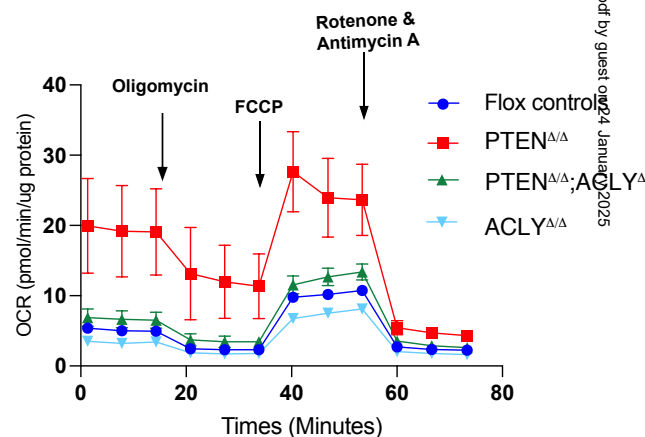
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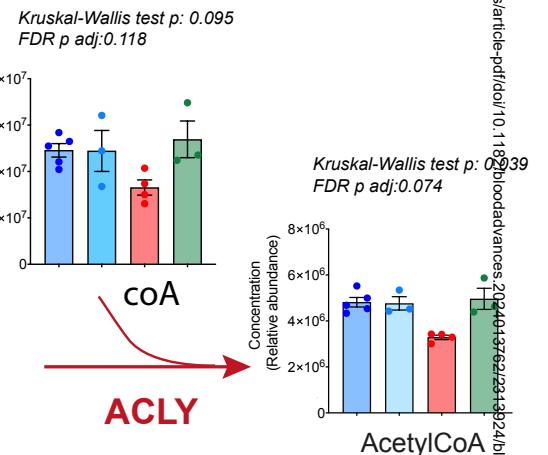
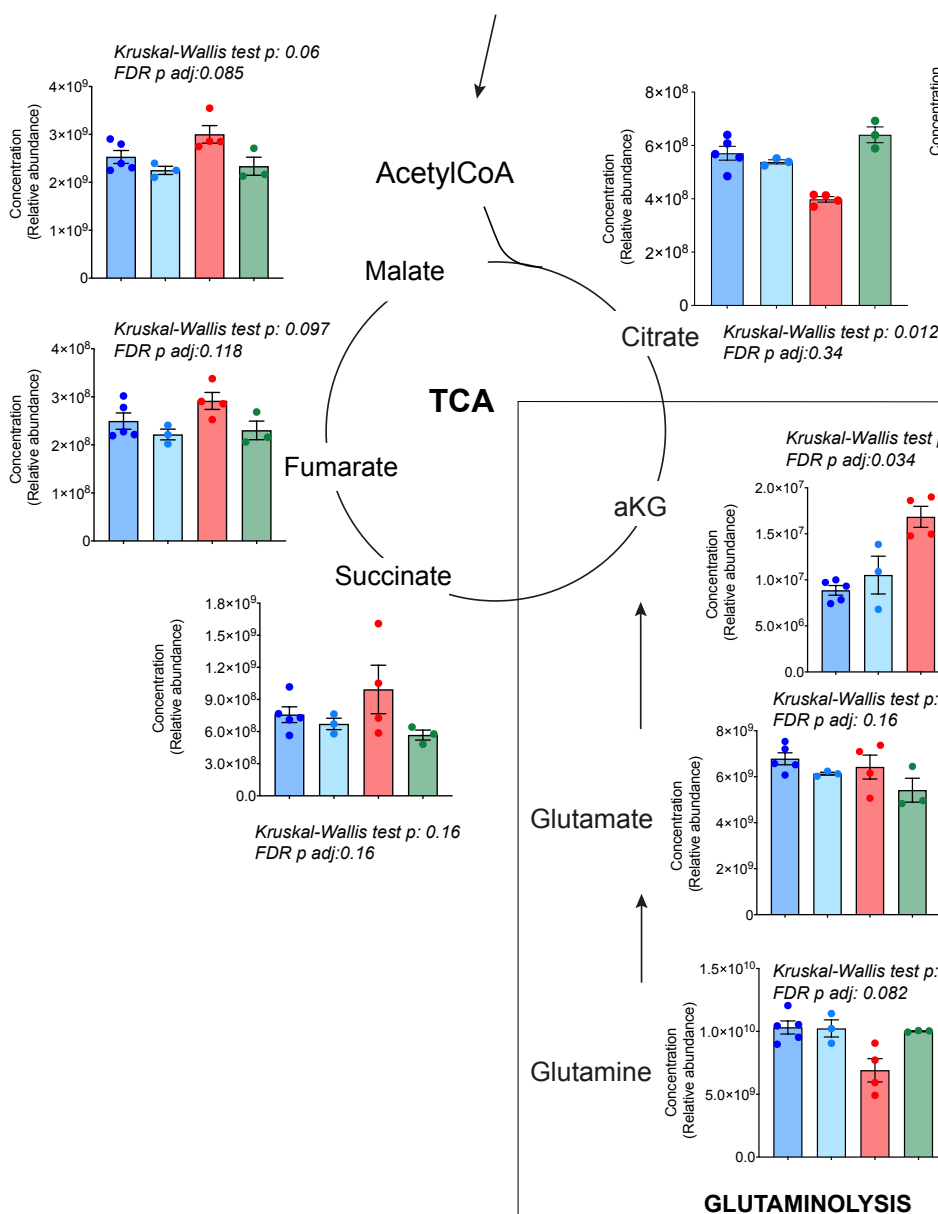
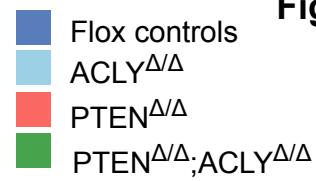
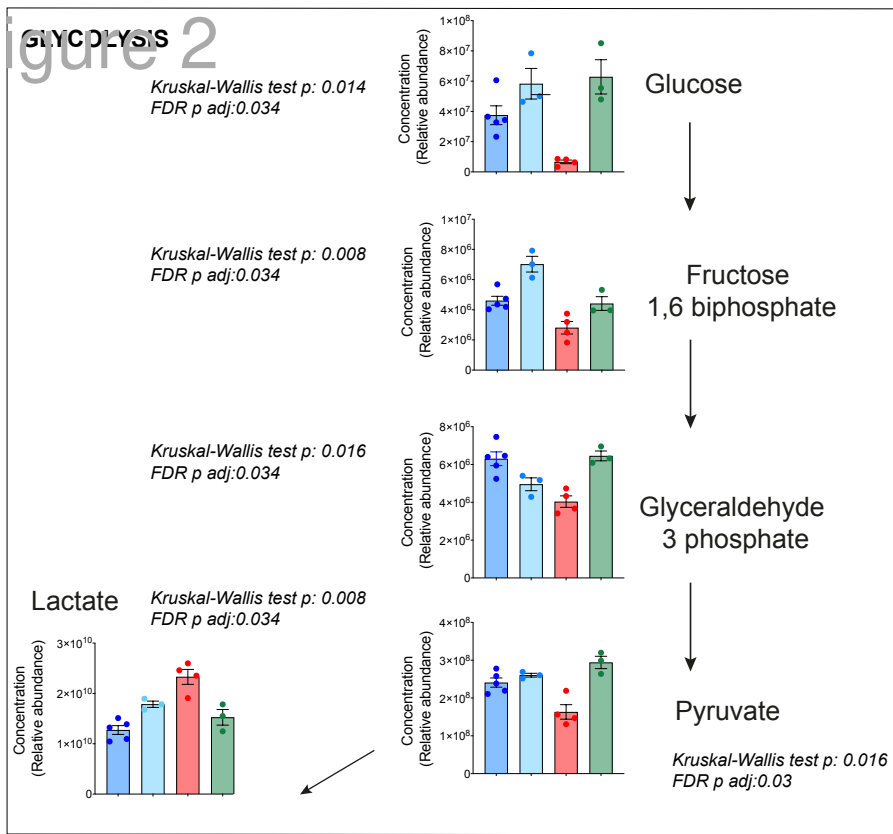


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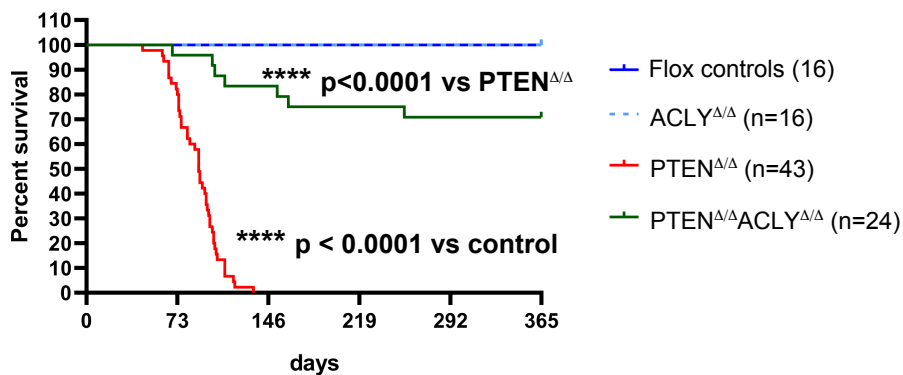
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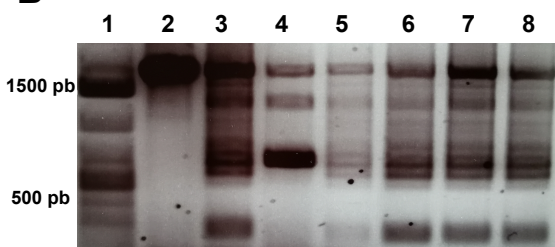
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Figure 3

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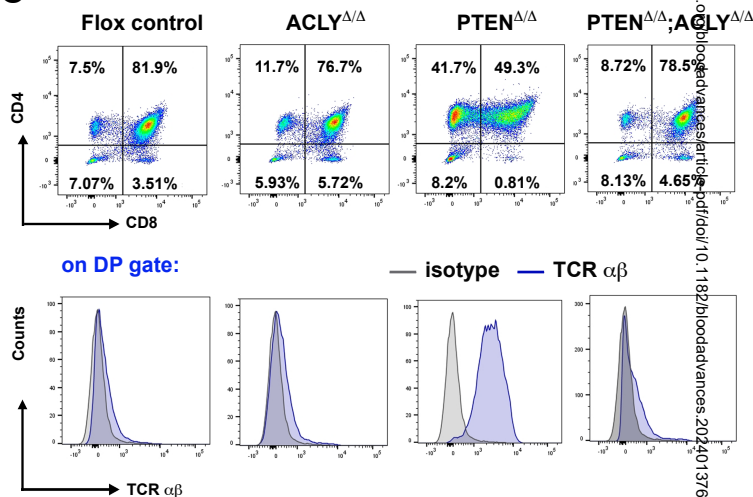


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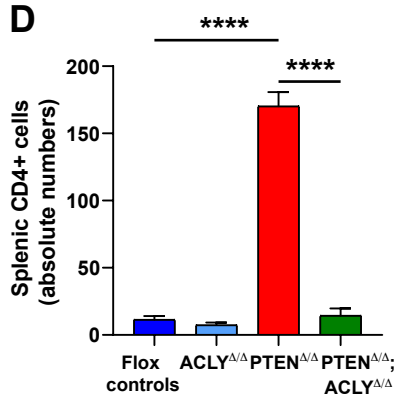


- 1: MW
- 2: Liver
- 3: PTEN^{flox2}
- 4: PTEN $\Delta\Delta$
- 5: PTEN^{flox2}, ACLY^{flox2}
- 6: PTEN $\Delta\Delta$, ACLY $\Delta\Delta$
- 7: ACLY^{flox2}
- 8: ACLY $\Delta\Delta$

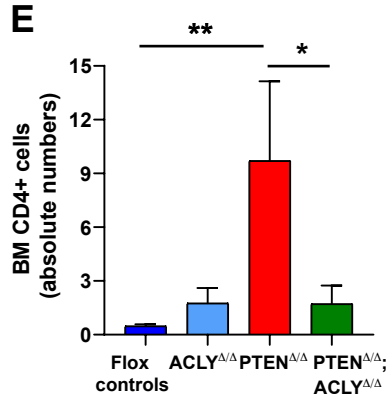
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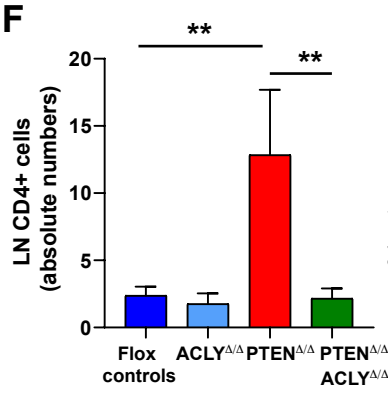


Figure 4

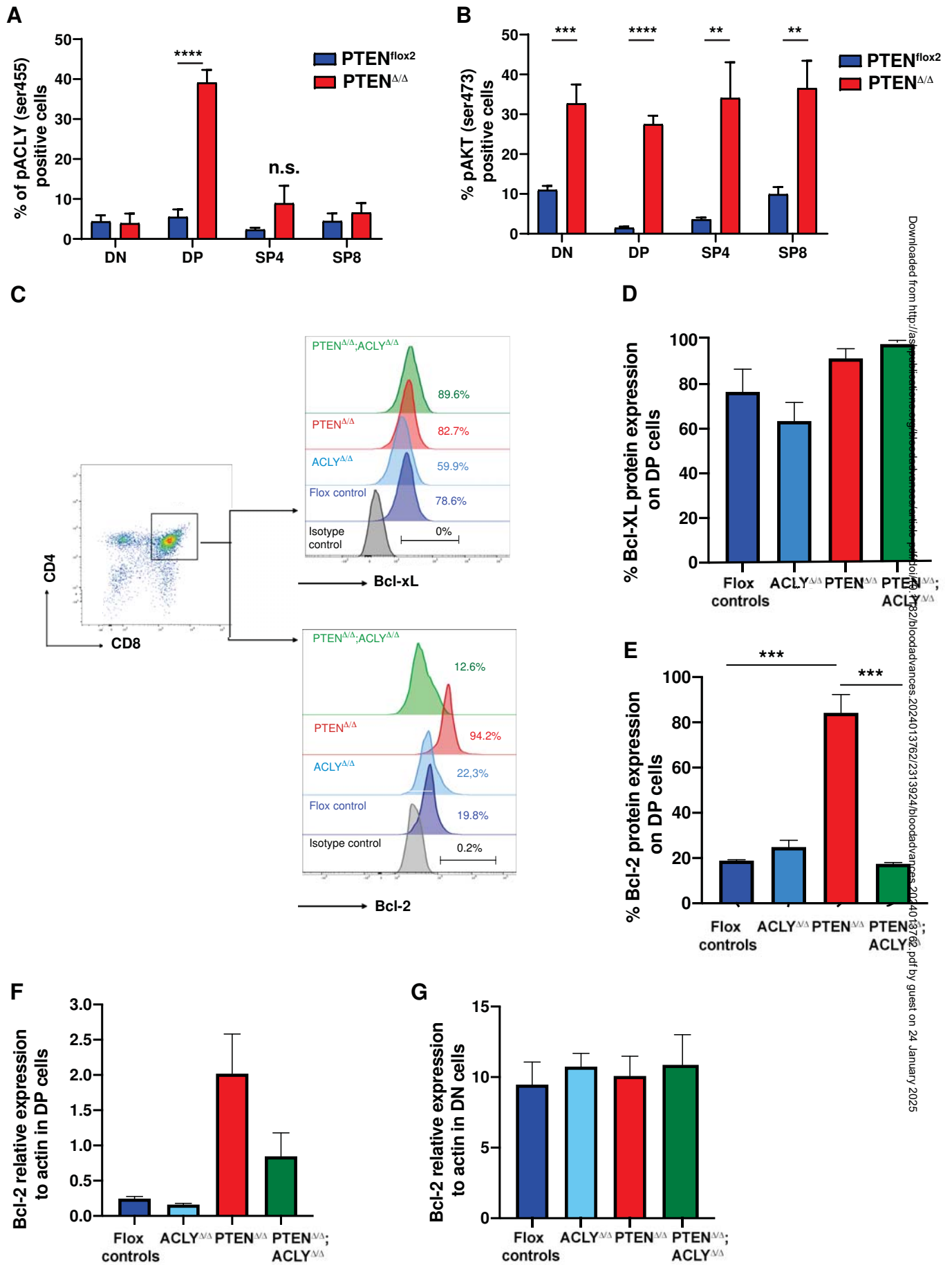
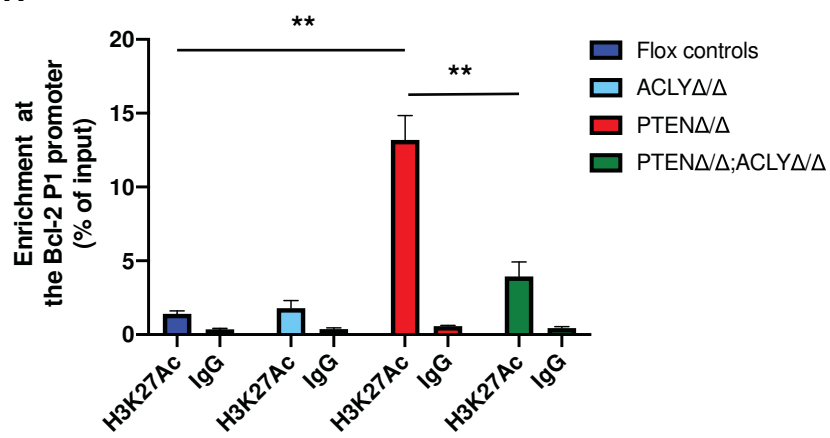
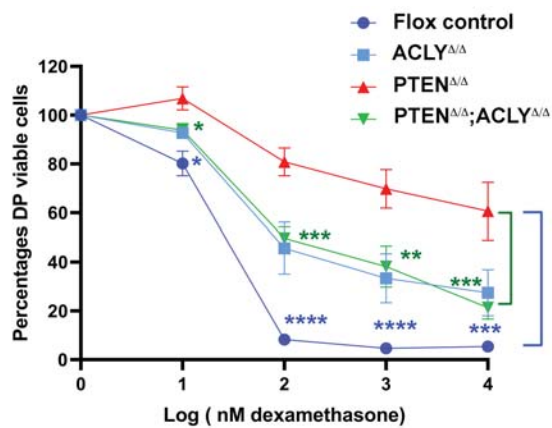


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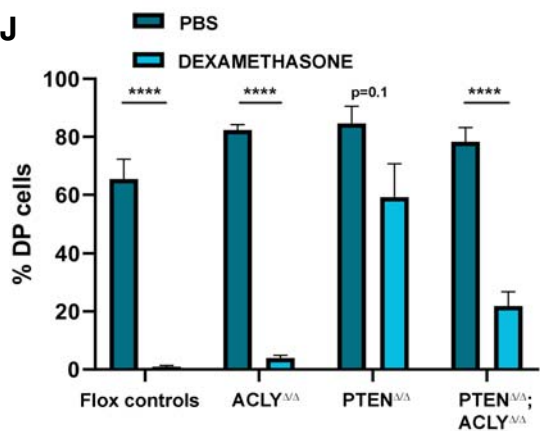
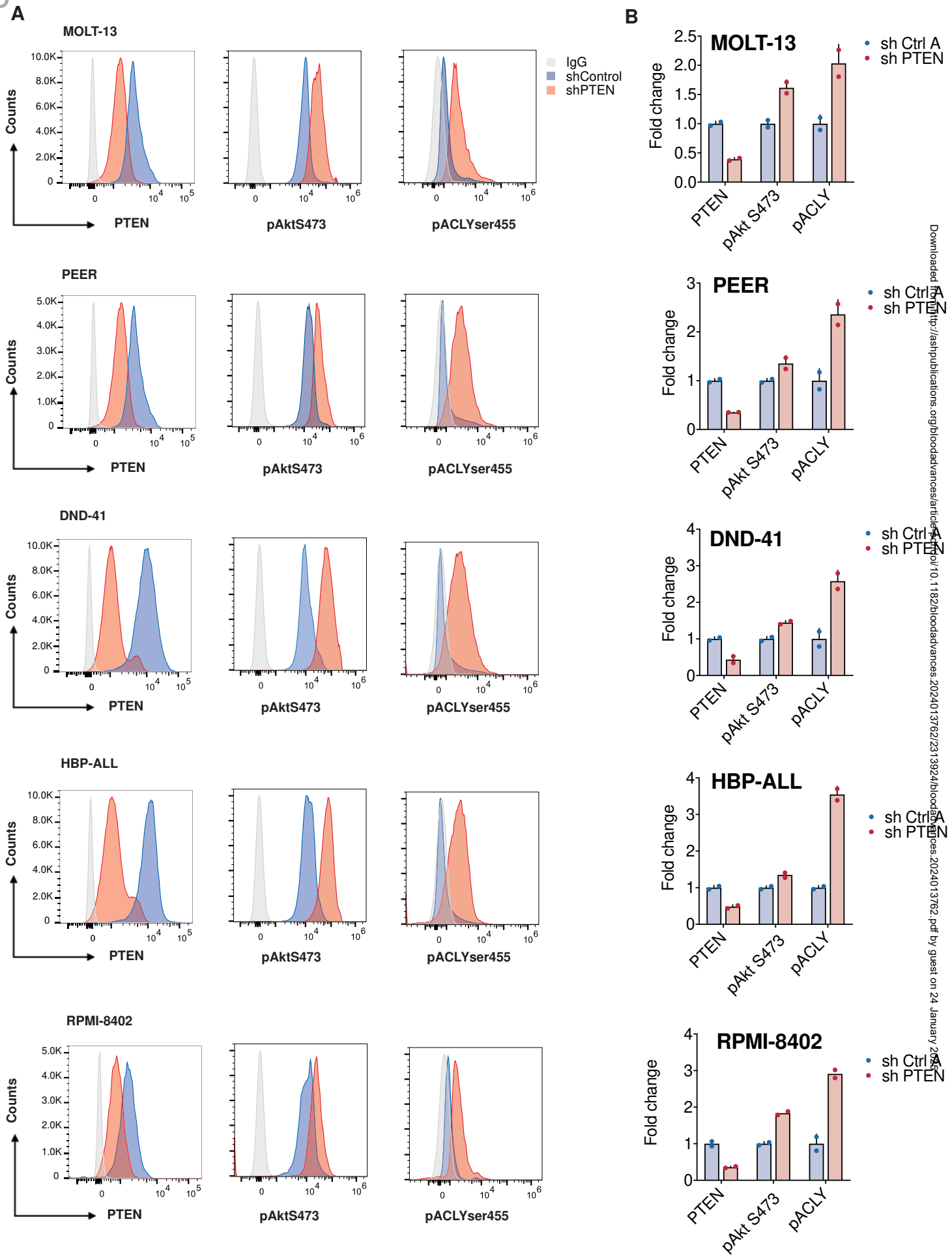
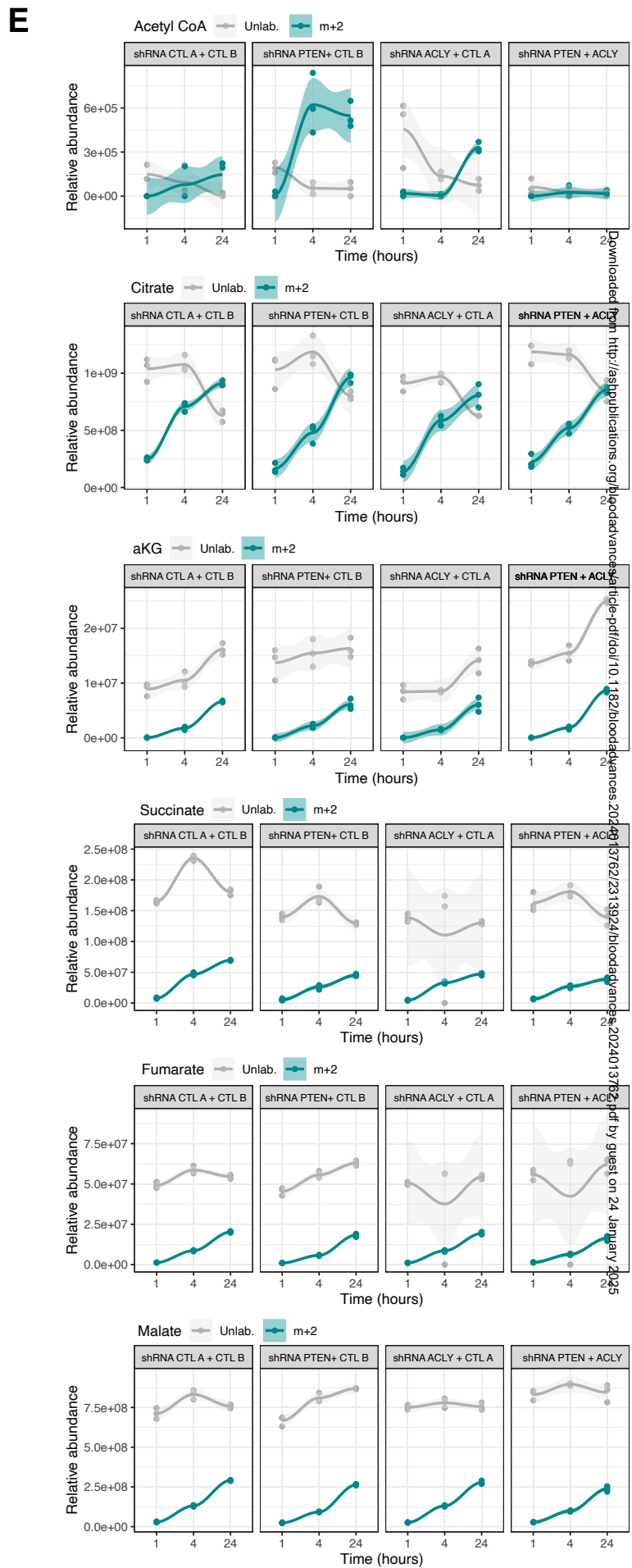
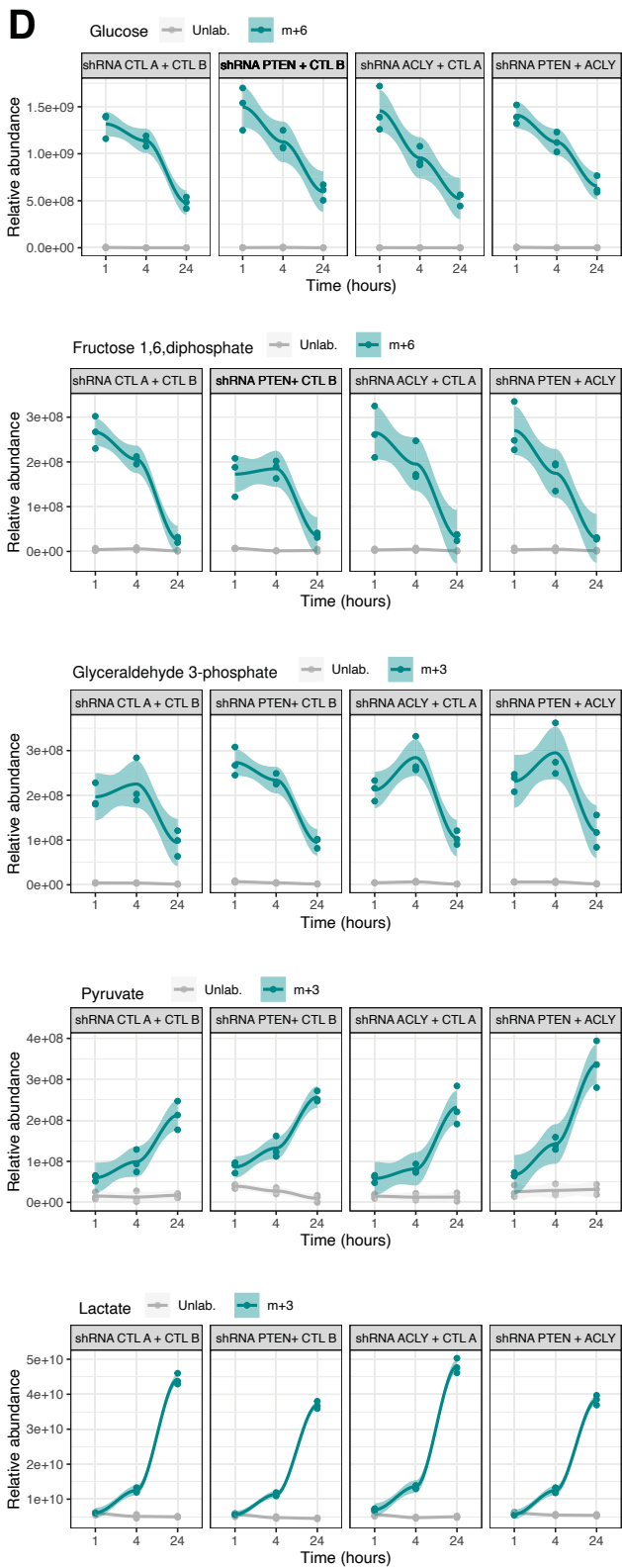


Figure 5



Downloaded from <http://ashpublications.org/bloodadvances/article/doi/10.1182/bloodadvances.2024013762/313924/bloodadvances.2024013762.pdf> by guest on 24 January 2025

Figure 5



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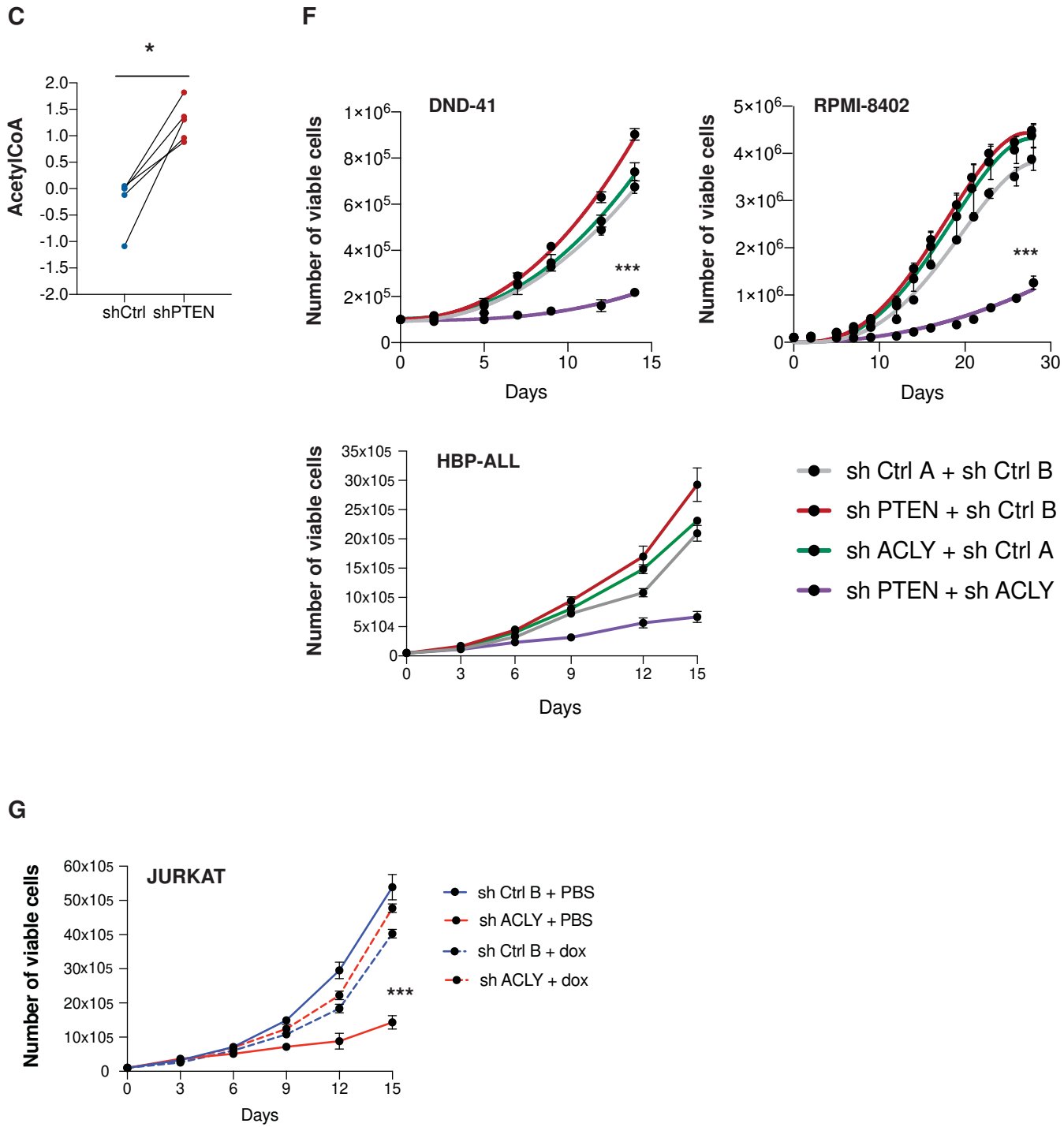
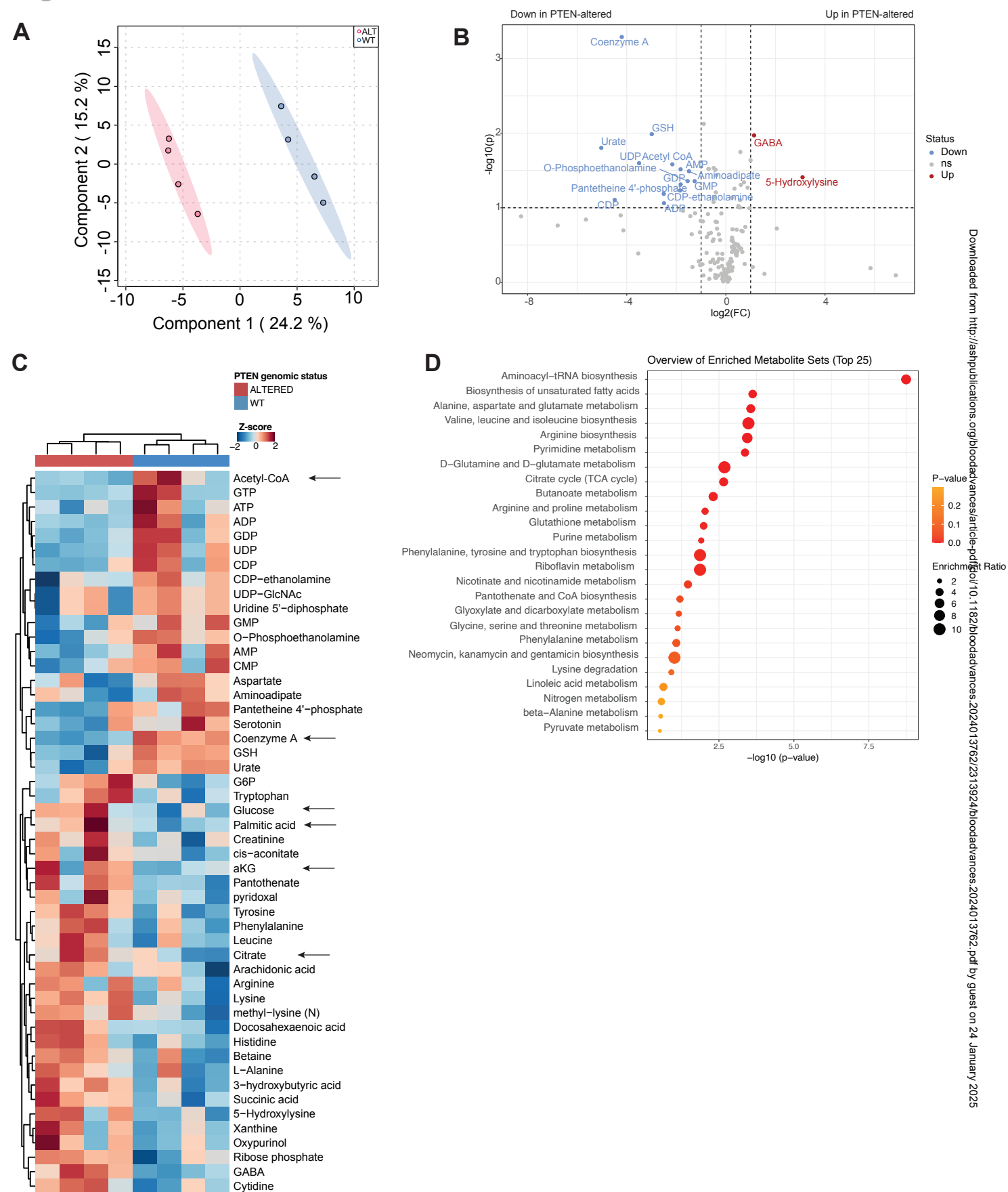
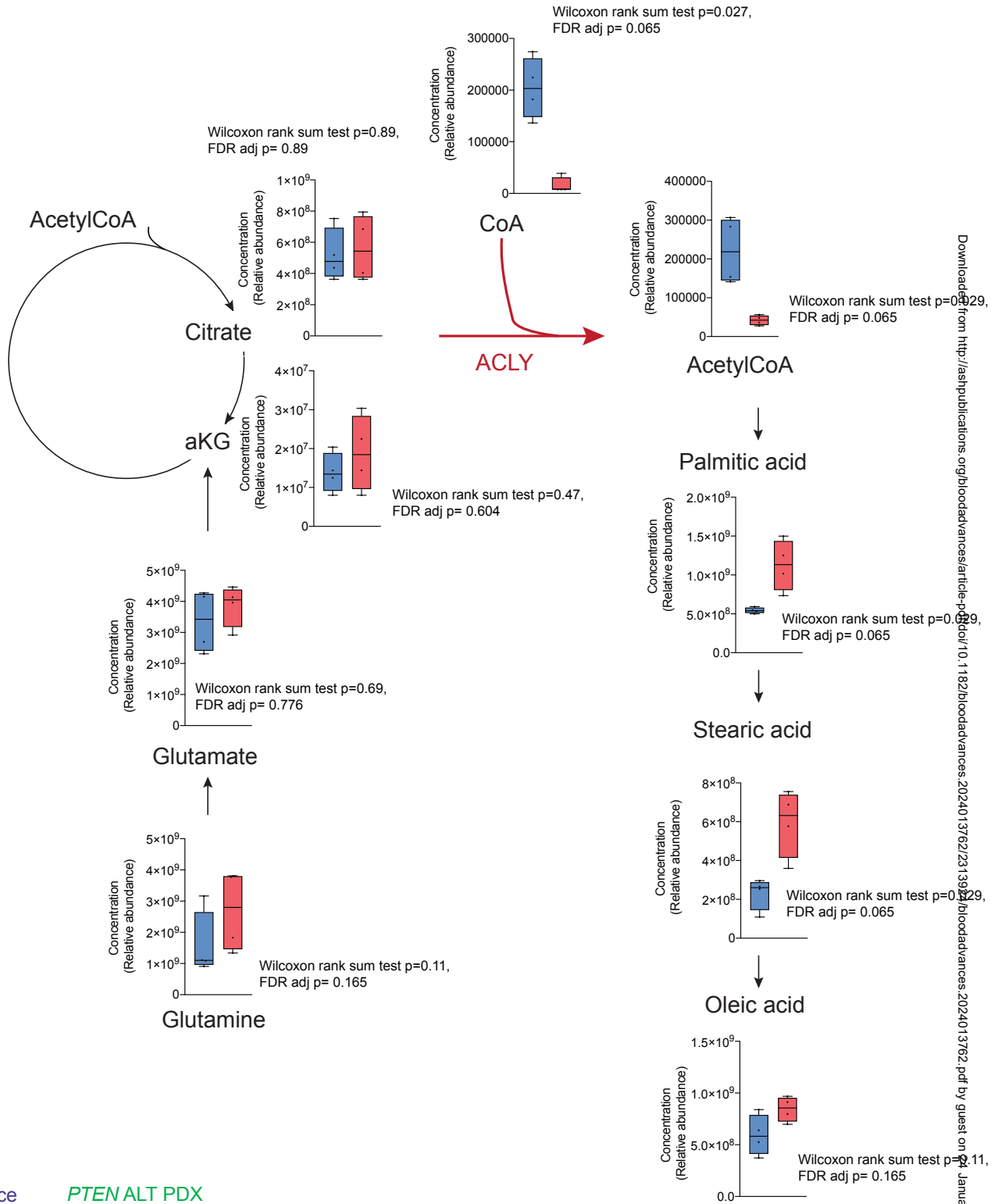


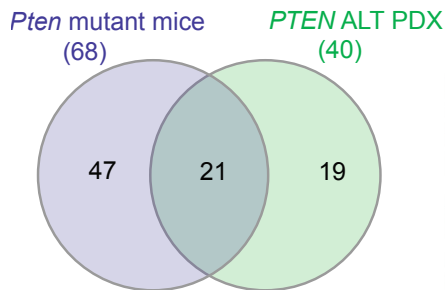
Figure 6



E



F



Mouse: Cystathionine, Quinolinic acid, Ornithine, dihydroxyacetone phosphate, Aspartate, Asparagine, Citrate, Lactate, Glyceraldehyde 3-phosphate, Taurine, Guanosine 5-diphospho-D-mannose, Xanthosine, Cytidine-5-monophospho-N-acetylneuraminic acid, L-Kynurenine, Tryptophan, L-Alanine, CDP-ethanolamine, PEP, fructose 1,6-diphosphate, Pyruvate, acetyl-aspartate (N), oxypurinol, Aminoacidipate, Proline, L-Sarcosine, Succinyladenosine, 5-Hydroxylysine, Lysine, Threonine, Argininosuccinate, NADP+, Glycine, nicotinamide N-oxide, Orotic acid, ATP, ADP, Hydroxy-L-proline, UTP, NAD+, Decanoyl-Carnitine, Palmitoleic acid, UDP, Malate, GTP, Pantetheine 4'-phosphate, Octanoyl-carnitine, GDP, GLN.

Human: Inosine,3-hydroxybutyric acid, Stearic acid, guanosine, Arachidonic acid, urate, GABA, Creatine, SuccinylGSH, Creatinine, Ribose phosphate, Butyric acid, Hexanoic acid, cis-aconitate, Dodecanoic acid/Lauric acid, pyridoxal, guanine, Succinic acid, Eicosapentaenoic acid.

Mouse and human: Acetyl CoA, aKG, Myristic acid, Serine, Glucose, Citrulline, Oleic acid, Coenzyme A, Pantothenate, Palmitic acid, FAD, Phenylalanine, Tyrosine, betaine, Linoleic acid, Cytidine, Docosahexaenoic acid, Leucine, GSH, Xanthine, Histidine.

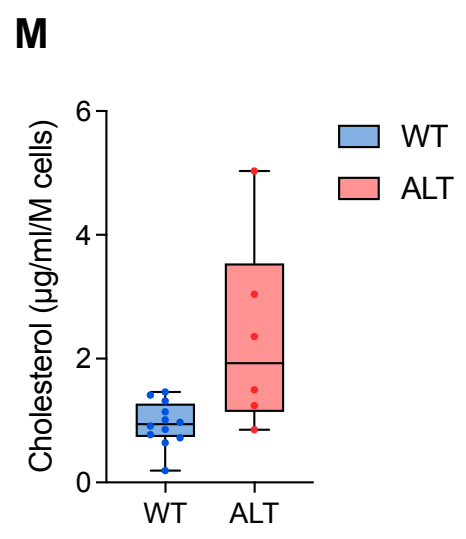
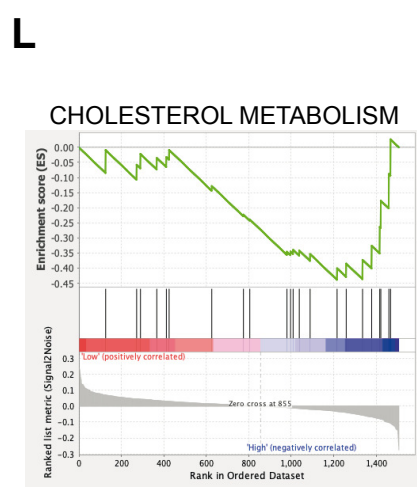
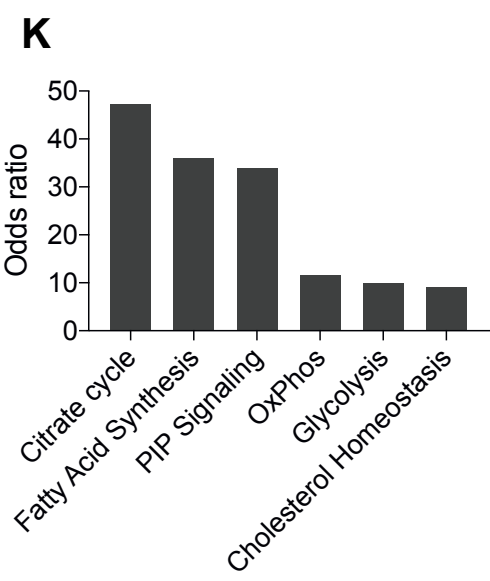
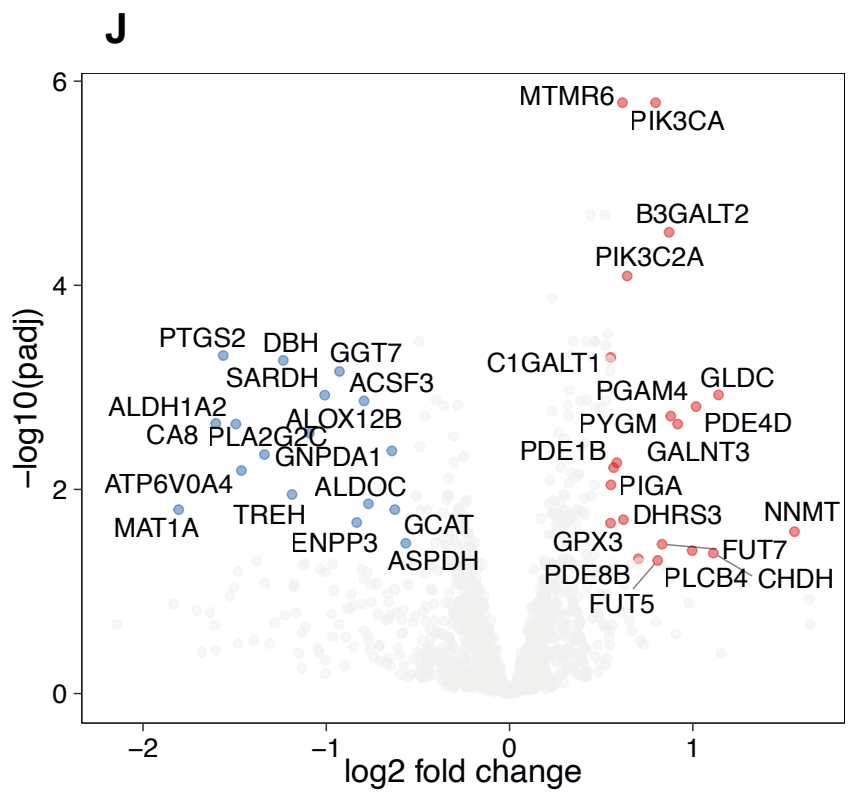
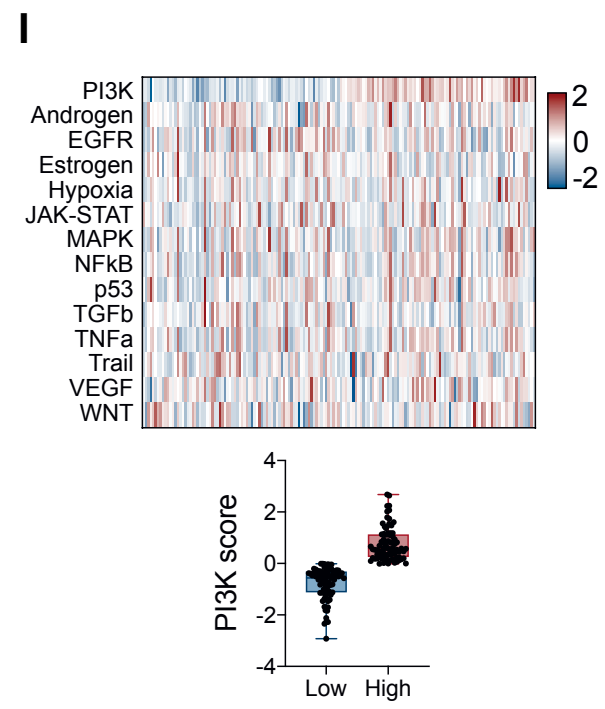
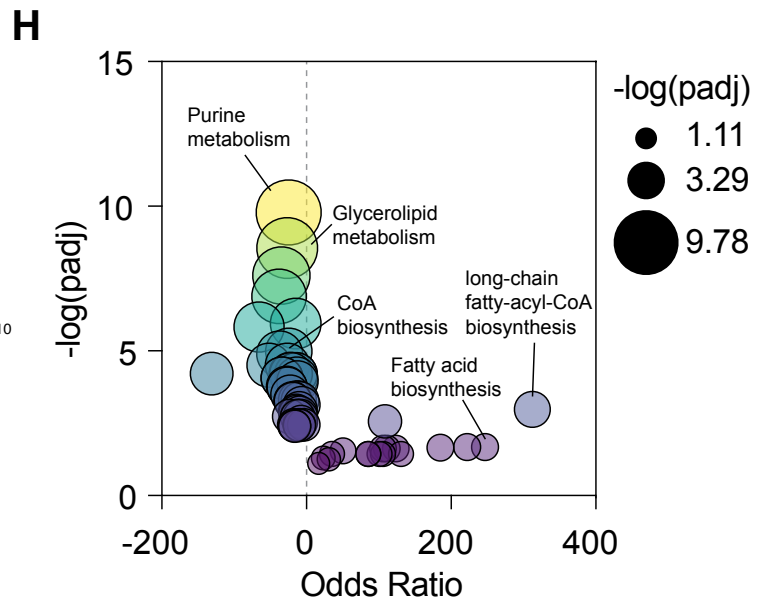
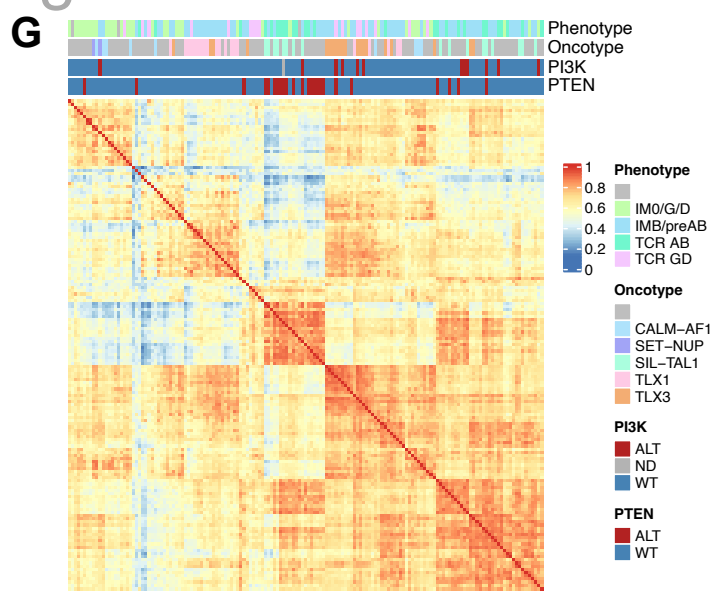


Figure 7

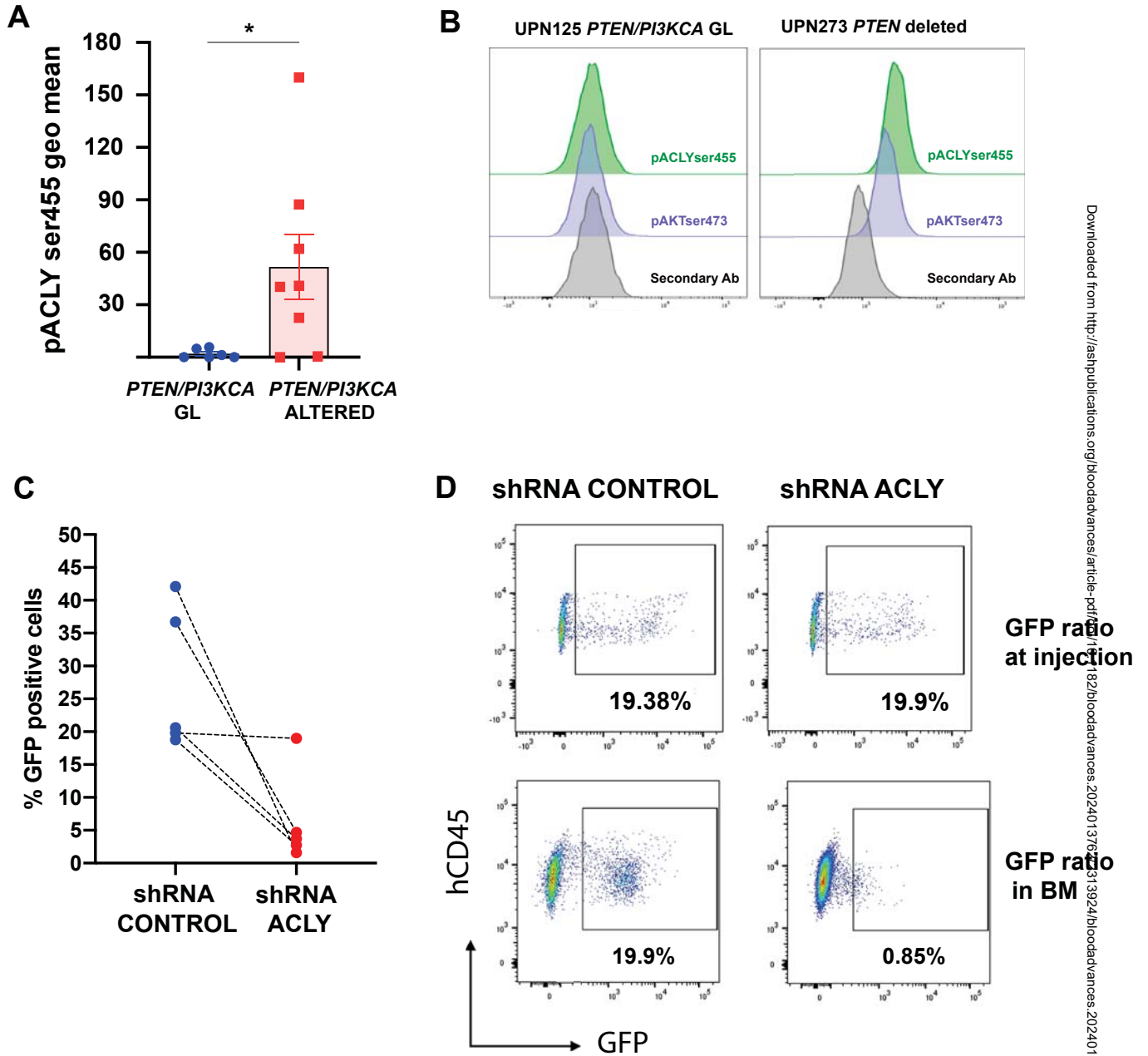


Figure 7 cont.

E

