



Figures and figure supplements

Unique-region phosphorylation targets LynA for rapid degradation, tuning its expression and signaling in myeloid cells

Ben F Brian IV et al

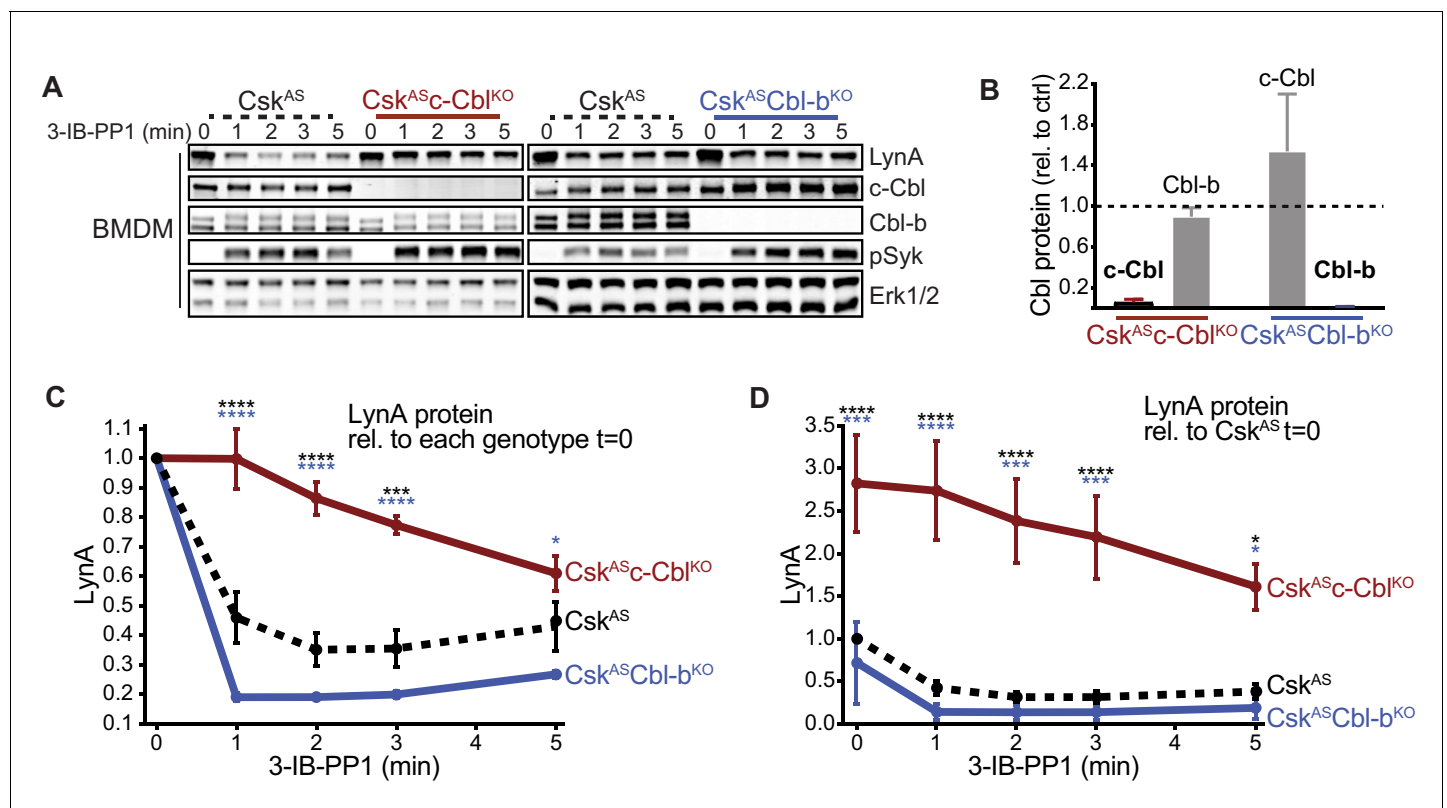


Figure 1. Loss of c-Cbl expression in macrophages leads to steady-state upregulation and delayed degradation of LynA protein. (A) Immunoblots showing LynA, c-Cbl, and Cbl-b protein in *Csk^{AS}*, *Csk^{AS}c-Cbl^{KO}*, and *Csk^{AS}Cbl-b^{KO}* BMDMs treated with 3-IB-PP1 for the indicated times. Interdomain-B-phosphorylated Syk^{pY352} (pSyk, Yan et al., 2013; Wang et al., 2010; Law et al., 1996), an SFK target and prerequisite for Syk activation, is shown as a control for 3-IB-PP1-initiated signaling; total Erk1/2 is shown as a loading control. (B–D) Densitometry quantification of relative levels of Cbl and LynA protein, corrected for total protein content using REVERT Total Protein Stain (TPS). (B) Quantification of c-Cbl (red) and Cbl-b (blue) in *Csk^{AS}c-Cbl^{KO}* and *Csk^{AS}Cbl-b^{KO}* BMDMs relative (rel.) to their steady-state levels in *Csk^{AS}* BMDMs (dotted line). Expression of the other Cbl family member is shown in gray. Error bars reflect the standard error of the mean (SEM), n = 4 from three *Csk^{AS}Cbl^{+/−}* mice and n = 3 from two *Csk^{AS}Cbl^{b−/−}* mice. (C–D) Quantification of LynA relative to the steady-state level in each genotype (C) or relative to the steady-state level in *Csk^{AS}* BMDMs (D). SEM, n = 8 for *Csk^{AS}*, n = 5 from three *Csk^{AS}Cbl^{+/−}* mice, n = 3 from two *Csk^{AS}Cbl^{b−/−}* mice. The significance (Sig.) from two-way ANOVA (ANOVA₂) with Tukey's multiple comparison test (-Tukey) are as follows: [*Csk^{AS}c-Cbl^{KO}* vs. *Csk^{AS}* black asterisks], [*Csk^{AS}c-Cbl^{KO}* vs. *Csk^{AS}Cbl-b^{KO}*, blue asterisks]; ****p<0.0001, ***p=0.0001–0.0005, * P=0.0171–0.0459. No significant difference was detected between other pairs (ns). Note: some of the error bars are smaller than the line width. Refer to **Figure 1—source data 1**. Paradoxical changes in Erk1/2 phosphorylation in Cbl-deficient cells are shown in **Figure 1—figure supplement 1**. Supporting siRNA studies are shown **Figure 1—figure supplement 2**.

DOI: <https://doi.org/10.7554/eLife.46043.002>

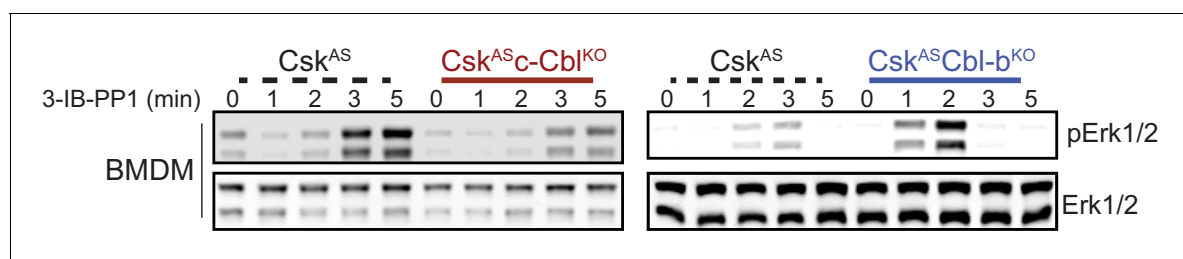


Figure 1—figure supplement 1. Paradoxical changes in Erk1/2 phosphorylation in Cbl-deficient cells obscure the contribution of LynA to signaling. Immunoblot showing impaired activating phosphorylation of Erk1/2^{pT202/pY204} (pErk1/2) in Csk^{AS}-Cbl^{KO} BMDMs and enhanced Erk phosphorylation in Csk^{AS}-Cbl-b^{KO} BMDMs compared to Csk^{AS} BMDMs treated with 3-IB-PP1.

DOI: <https://doi.org/10.7554/eLife.46043.003>

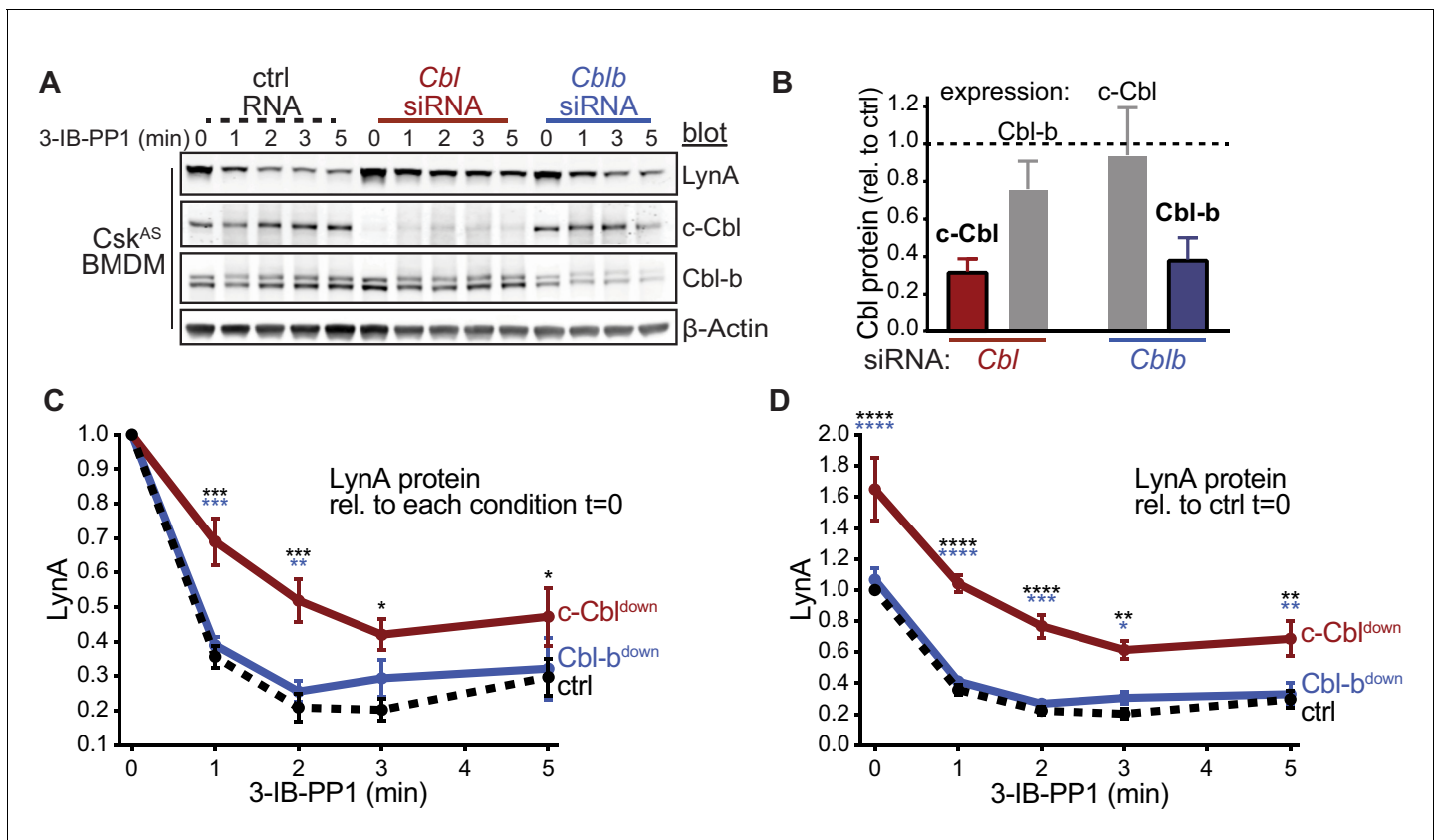


Figure 1—figure supplement 2. siRNA knockdown of c-Cbl expression in macrophages leads to steady-state upregulation and delayed degradation of LynA protein. (A) Immunoblots showing LynA, c-Cbl, and Cbl-b protein in Csk^{AS} BMDMs transfected with non-targeting RNA (ctrl) or with siRNA constructs targeting *Cbl* or *Cblb* mRNA. Rested cells were treated with 3-IB-PP1 for the indicated times; β-Actin is shown as a loading control. (B–D) Quantification of relative c-Cbl, Cbl-b, and LynA protein, corrected for total protein content (TPS). SEM, n = 3. (B) Quantification of c-Cbl protein (red) and Cbl-b protein (blue), reported relative to their expression in ctrl BMDMs (dotted line). Remaining expression of the other Cbl family member is shown in gray. (C–D) Quantification of LynA, reported relative to the steady-state level in each siRNA condition (C) or relative to the level in the ctrl sample at steady state (D) in ctrl (black dotted), c-Cbl^{down} (red), and Cbl-b^{down} (blue) samples. Sig. from ANOVA₂-Tukey: [c-Cbl^{down} vs. ctrl, black asterisks], [c-Cbl^{down} vs. Cbl-b^{down}, blue asterisks]; ****p<0.0001, ***p=0.0001–0.0005, **p=0.0015–0.0058, *P=0.0105–0.0451. Other pairs ns. Refer to **Figure 1—figure supplement 2—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.004>

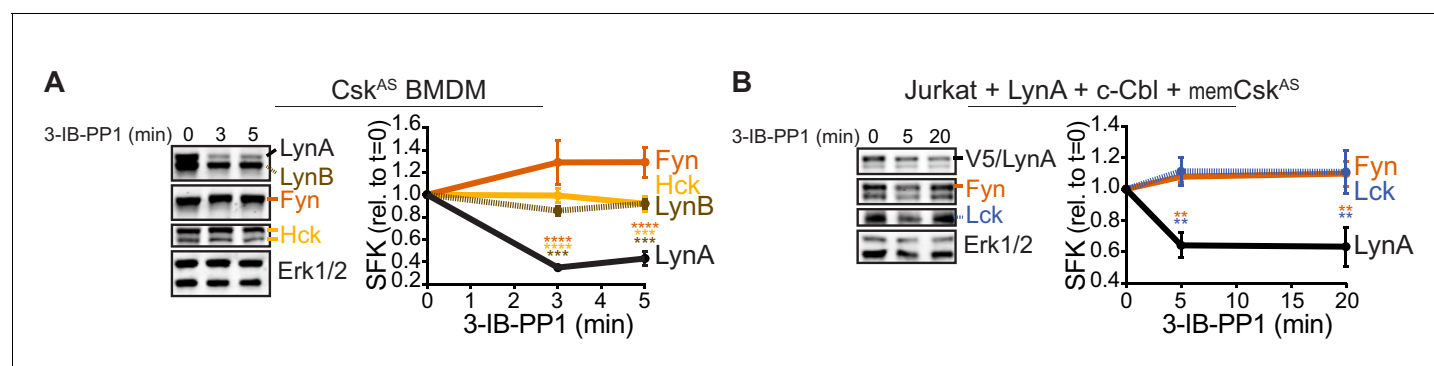


Figure 2. LynA is degraded more rapidly than Fyn and Lck during 3-IB-PP1 treatment. Immunoblots and quantification of SFK levels over the course of 3-IB-PP1 treatment. In both panels total Erk1/2 protein is shown as a loading control. Quantified values are corrected for total protein content (TPS) and reported relative to the steady-state level of each SFK. **(A)** Levels of Fyn (dark orange), Hck (light orange), LynA (black), and LynB (brown dashed) in 3-IB-PP1-treated Csk^{AS} BMDMs. SEM, $n = 3$. Sig. from ANOVA₂-Tukey: [Fyn vs. LynA, dark orange asterisks], [Hck vs. LynA, light orange asterisks], [LynB vs. LynA, brown asterisks]; **** $p < 0.0001$, *** $p = 0.0006$ – 0.0009 , ** $p = 0.0034$. **(B)** Levels of Fyn (dark orange) and Lck (blue dashed) and HisV5-tagged LynA (black) in 3-IB-PP1-treated Jurkat T cells cotransfected with HisV5-tagged LynA, memCsk^{AS}, and c-Cbl. SEM, $n = 4$. Sig. from ANOVA₂-Tukey: [Lck vs. LynA, blue asterisks], [Fyn vs. LynA, dark orange asterisks]; ** $p = 0.0018$ – 0.0048 . Other pairs ns. Refer to **Figure 2—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.007>

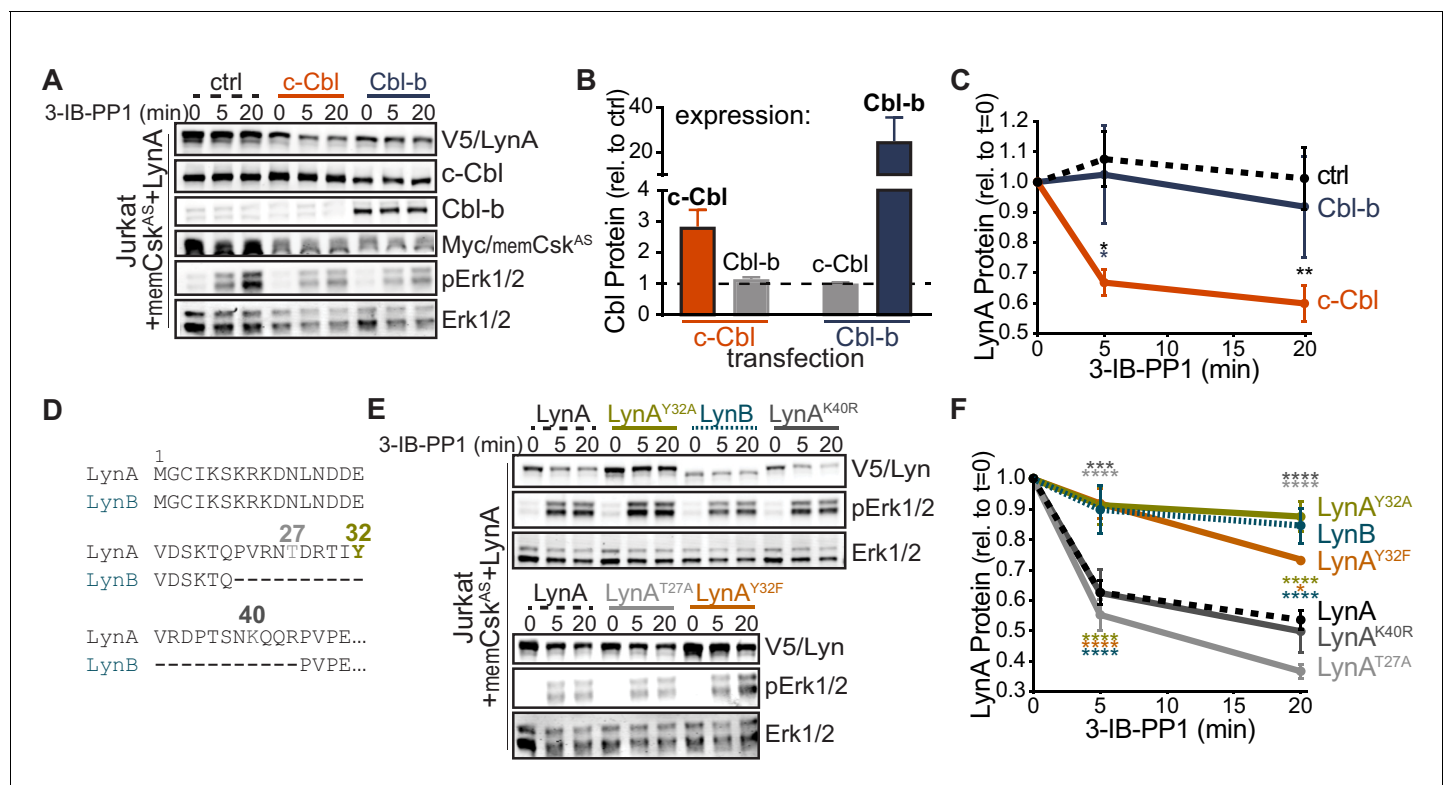


Figure 3. Unique-region tyrosine 32 is required for activation-induced degradation of LynA. (A) Immunoblots of Jurkat cells cotransfected with His₆V5-tagged LynA, Myc-tagged memCsk^{AS}, and either empty vector (ctrl), c-Cbl, or Cbl-b and treated with 3-IB-PP1. Phosphorylated Erk1/2^{pT202/pY204} (pErk1/2) is shown as a qualitative control for 3-IB-PP1-initiated signaling from the combined effects of transfected LynA and endogenous Lck; total Erk1/2 is shown as a loading control. (B–C) Quantification of relative c-Cbl, Cbl-b, and His₆V5-tagged LynA protein, corrected for total protein content (TPS). SEM, n = 4. (B) Quantification of overexpressed c-Cbl (orange) and Cbl-b (dark blue) relative to endogenous (ctrl) levels (dotted line). Endogenous expression of the other Cbl family member in each condition is shown in gray. (C) Quantification of LynA protein during 3-IB-PP1 treatment relative to the steady-state level for transfections of empty-vector ctrl (black dotted), c-Cbl (orange), or Cbl-b (dark blue). Sig. from ANOVA₂-Tukey: [c-Cbl vs. ctrl, black asterisks], [c-Cbl vs. Cbl-b, blue asterisk]; **p=0.0092, *p=0.0270–0.0102. Other pairs ns. (D) N-terminal amino-acid sequences of mouse LynA and LynB (Yi et al., 1991; Stanley et al., 1991), including the 21 residue insert in the unique region of LynA, highlighting residues Y32 (olive), T27 (light gray), and K40 (dark gray). (E) Immunoblots of 3-IB-PP1-treated Jurkat cells cotransfected with c-Cbl, Myc-tagged memCsk^{AS}, and His₆V5-tagged Lyn constructs, including wild-type LynA, LynA^{Y32A}, LynB, LynA^{K40R}, LynA^{T27A}, and LynA^{Y32F}. (F) Quantification of protein levels of V5-tagged LynA (black dotted), LynA^{Y32A} (olive), LynB (teal dotted), LynA^{K40R} (dark gray), LynA^{T27A} (light gray), and LynA^{Y32F} (orange) over the course of 3-IB-PP1 treatment, corrected for total protein content (TPS) and reported relative to the steady-state level for each Lyn variant. SEM, n = 7 for LynA, n = 4 for LynA^{Y32A} and LynB; n = 3 for LynA^{K40R}, LynA^{T27A}, and LynA^{Y32F}. Sig. from ANOVA₂-Tukey: [LynA^{Y32A} vs. LynA, olive asterisks], [LynB vs. LynA, teal asterisks], [LynA^{Y32F} vs. LynA, orange asterisks], [LynA^{K40R} vs. LynA^{Y32A}, dark gray asterisks], [LynA^{T27A} vs. LynA^{Y32A}, light gray asterisks], [LynB vs. LynA^{K40R}, p=0.013, p=0.01], [LynB vs. LynA^{T27A}, p<0.0001], [LynA^{Y32F} vs. LynA^{K40R}, p=0.012, 0.0157], [LynA^{T27A} vs. LynA^{Y32F}, p<0.0001], [LynA^{Y32A} vs. LynA^{K40R}, p<0.0001]; ****p<0.0001, ***p=0.0006, *p=0.0166. Other pairs ns. Note: the error bar for LynA^{Y32F} is smaller than the line width. Refer to Figure 3—source data 1. Refer to Figure 3—figure supplement 1 for microscopy showing localization of unique-region-only constructs of Lyn. Refer to Figure 3—figure supplement 2 for quantification of relative expression levels of LynA variants and LynB.

DOI: <https://doi.org/10.7554/eLife.46043.009>

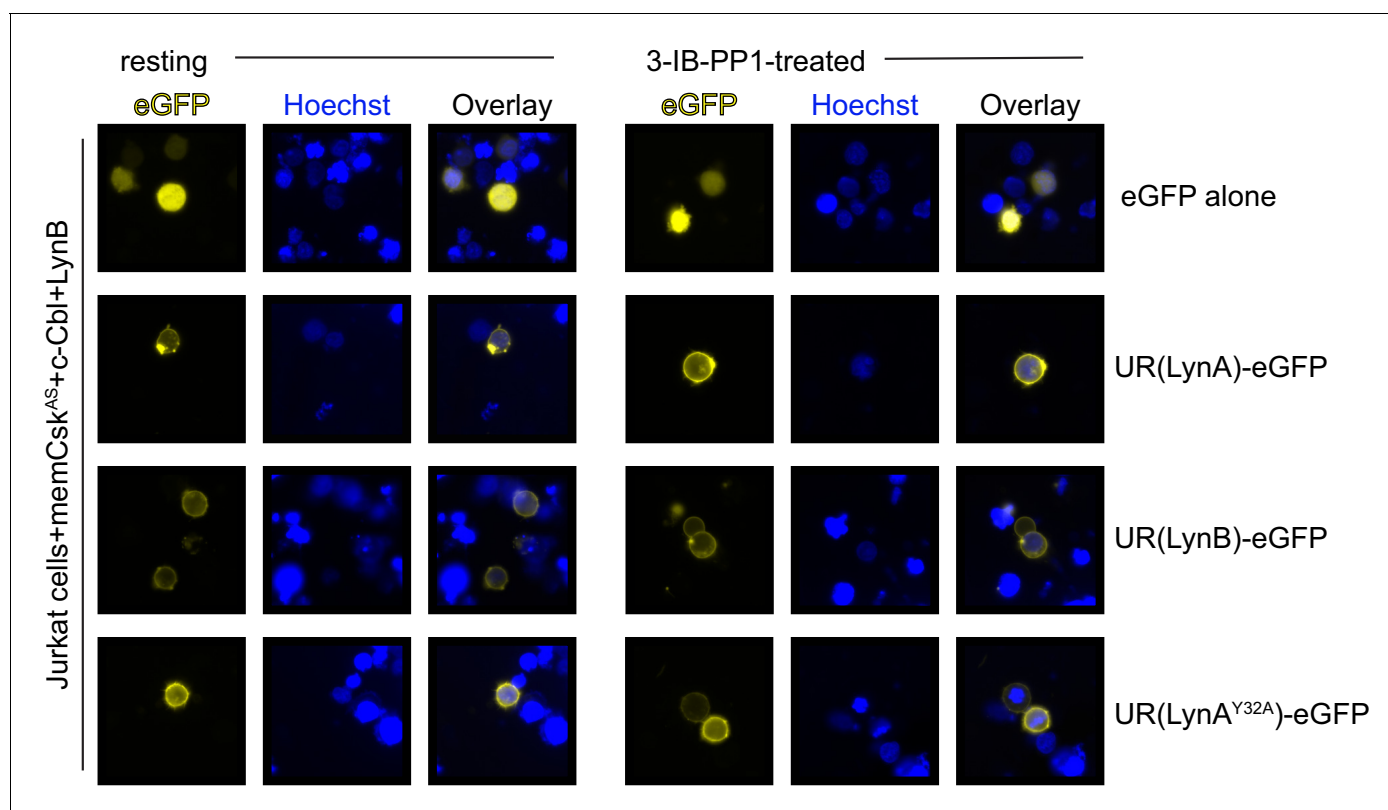


Figure 3—figure supplement 1. The unique regions of LynA, LynB, and LynA^{Y32A} are membrane-localized in resting and 3-IB-PP1-treated cells. Representative epifluorescence images of GFP-tagged unique-region (UR) constructs of LynA, LynB, and LynA^{Y32A}, showing localization in Jurkat cells before and after treatment for 5 min with 3-IB-PP1.

DOI: <https://doi.org/10.7554/eLife.46043.010>

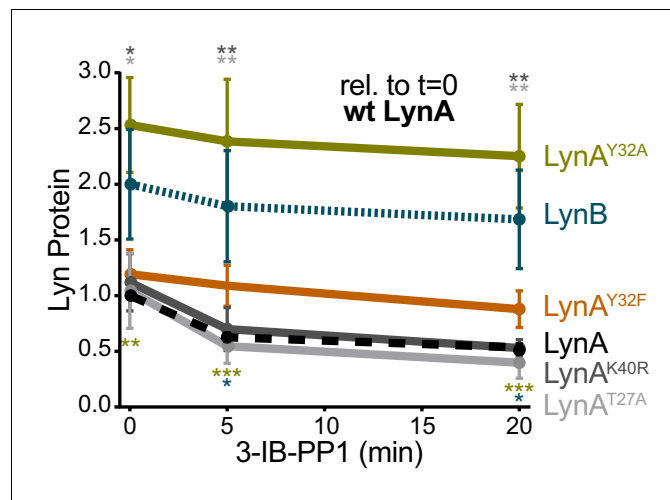


Figure 3—figure supplement 2. LynA^{Y32A} and LynB are more highly expressed than wild-type LynA when expressed ectopically in Jurkat cells. Quantification of His₆V5-tagged LynA protein in Jurkat cells, reported relative to the steady-state expression of transfected wild-type His₆V5-tagged LynA protein. Sig. from ANOVA₂-Tukey: [LynA^{Y32A} vs. LynA, olive asterisks], [LynB vs. LynA, teal asterisks], [LynA^{K40R} vs. LynA^{Y32A}, dark gray asterisks], [LynA^{T27A} vs. LynA^{Y32A}, light gray asterisks]; ***p=0.0002–0.0004, **p=0.0018–0.0062, *p=0.0216–0.0361. Other pairs ns. Refer to **Figure 3—figure supplement 2—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.011>

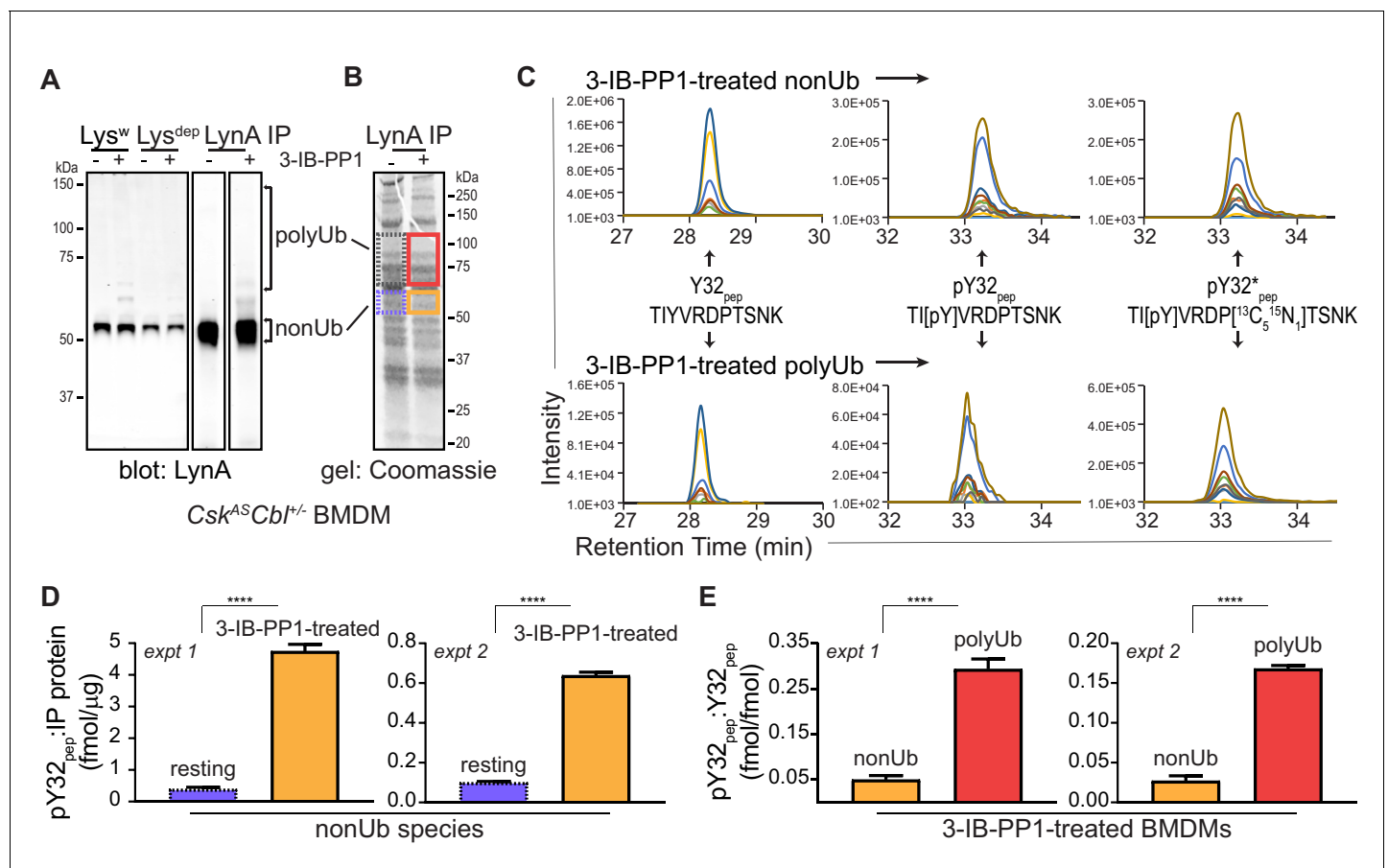


Figure 4. Tyrosine 32 is a site of activation-induced phosphorylation in macrophages. **(A)** Blots showing immunoprecipitation of LynA from BMDMs derived from *Csk^{AS}Cbl^{+/+}* mice with (+) or without (-) a 15 s treatment with 3-IB-PP1. Nonubiquitinated (nonUb) and polyubiquitinated (polyUb) LynA species in whole-cell and immunodepleted lysate (*Lys^w* and *Lys^{dep}*, respectively) and immunoprecipitate (IP) samples are shown. Uncropped immunoblots are shown in **Figure 4—figure supplement 1A**. **(B)** Coomassie-stained gel showing regions excised for LC-MS/MS analysis of polyUb LynA (higher boxes) and nonUb LynA (lower boxes); box colors correspond to the bar graphs below. Total IP protein content was quantified by applying densitometry to the whole lane and deriving a mass value via a BSA standard curve, shown in **Figure 4—figure supplement 1B**. Equivalent amounts of IP material were spiked with 125 fmol isotope-labeled control peptide (pY32_{pep}, a tryptic peptide containing phosphoY32) and subjected to in-gel trypsin digestion and LC-MS/MS. **(C)** Tryptic peptides Y32_{pep} (IP-derived unphosphorylated peptide), pY32_{pep} (IP-derived phosphopeptide), and added pY32_{pep} were quantified by using parallel reaction monitoring (PRM) to collect MS/MS spectra for peptide parent ions 687.3267 and 690.3336 *m/z*, respectively. Extracted ion chromatograms (XICs) were derived from Skyline for MS/MS fragment ions corresponding to y and b fragments from Y32, pY32, and pY32* peptides and used to quantify the ratios of pY32_{pep}:pY32*_{pep} or pY32_{pep}:Y32_{pep}. Representative ion-annotated XICs are shown in **Figure 4—figure supplement 2**. Assignment of XIC peaks using synthetic peptides is shown in **Figure 4—figure supplement 3**. Detection of phosphopeptides in IP samples is shown in **Figure 4—figure supplement 4**. Peptide calibration curves are shown in **Figure 4—figure supplement 5**. **(D–E)** Quantitative analysis of Y32 phosphorylation in resting and 3-IB-PP1-treated BMDMs, including nonUb species from resting BMDMs (blue dotted), nonUb species from 3-IB-PP1-treated BMDMs (orange), and polyUb species from 3-IB-PP1-treated BMDMs (red). Although we analyzed a gel fragment that could contain polyUb species in resting BMDMs (gray dotted), this species was so rare as to be unquantifiable. Error bars represent the standard deviation (stdev) of *n* = 3 technical replicates. Data from two biological replicates are shown side by side. **(D)** Quantification of pY32_{pep} in nonUb species from resting vs. 3-IB-PP1-treated BMDMs normalized to the total protein content of each IP. This analysis was used only for nonUb samples, which were roughly equimolar in resting and 3-IB-PP1-treated BMDMs. Sig. from two-tailed t tests for each experiment: [nonUb resting vs. nonUb 3-IB-PP1-treated, asterisks]; *****p* < 0.0001. **(E)** Quantification of pY32_{pep} in nonUb vs. polyUb species in 3-IB-PP1-treated BMDMs normalized to the amount of unphosphorylated Y32_{pep}. Sig. from two-tailed t tests for each experiment: [nonUb resting vs. nonUb 3-IB-PP1-treated, asterisks]; *****p* < 0.0001. Refer to **Figure 4—source data 1**. Raw mass spectrometry files are available at the Panorama repository <http://panoramaweb.org>, http://panoramaweb.org/Freedman_LynA.url, ProteomeXchange ID: PXD014621.

DOI: <https://doi.org/10.7554/eLife.46043.014>

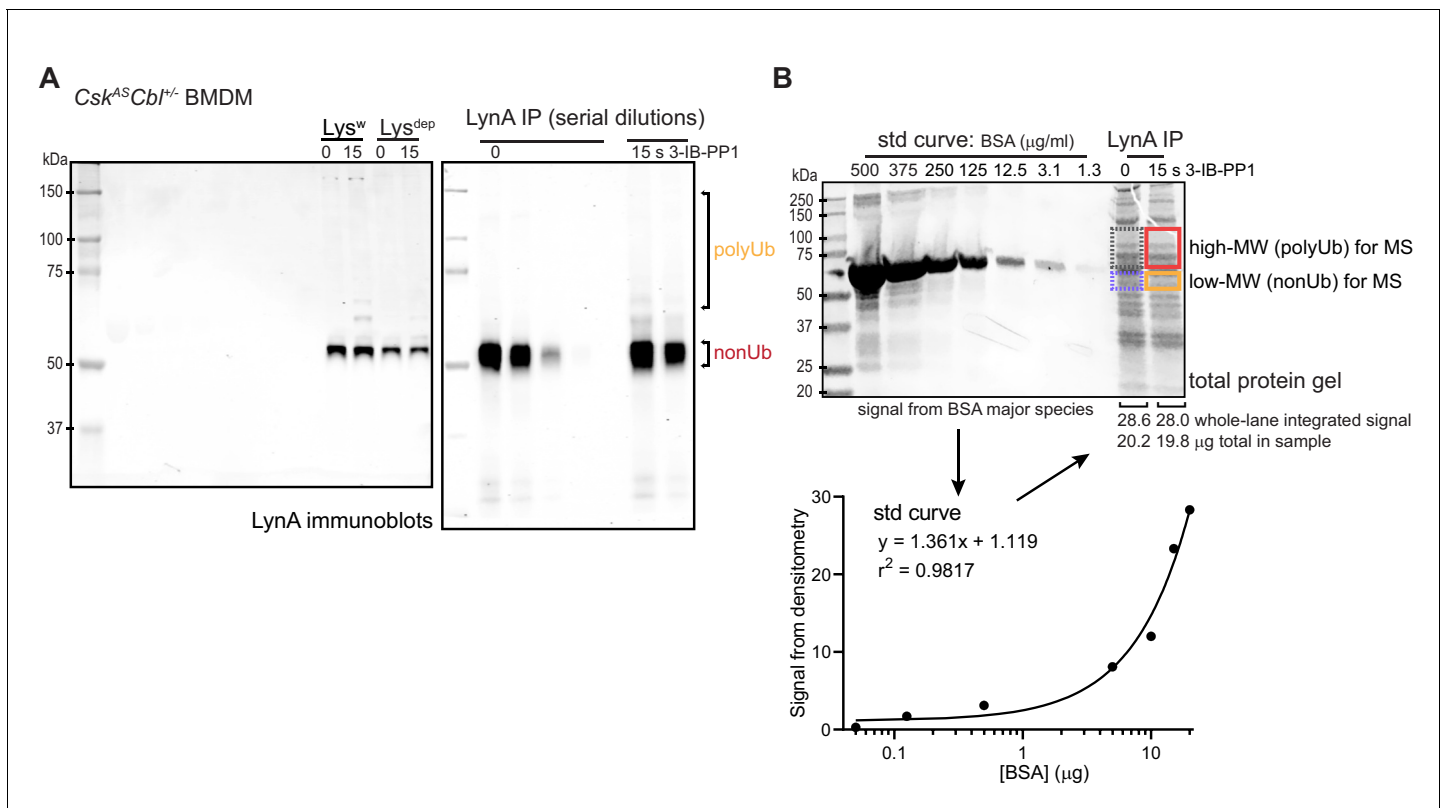


Figure 4—figure supplement 1. Uncropped blots and BSA standard curve for mass spectrometry samples. (A) Uncropped images of LynA immunoblots show positions of molecular weight markers and serially diluted LynA immunoprecipitates. (B) Uncropped Coomassie-stained gel showing the BSA standard curve (left lanes) and the total protein inputs from the IPs in (A). The lower panel shows densitometry quantification of protein-stain signal intensity from each BSA standard (std) and fitting of the standard curve. The mass of total protein in each immunoprecipitate was then quantified to normalize the sample input for LC-MS/MS. Refer to **Figure 4—figure supplement 1—source data 1** for BSA calibration of IPs.

DOI: <https://doi.org/10.7554/eLife.46043.015>

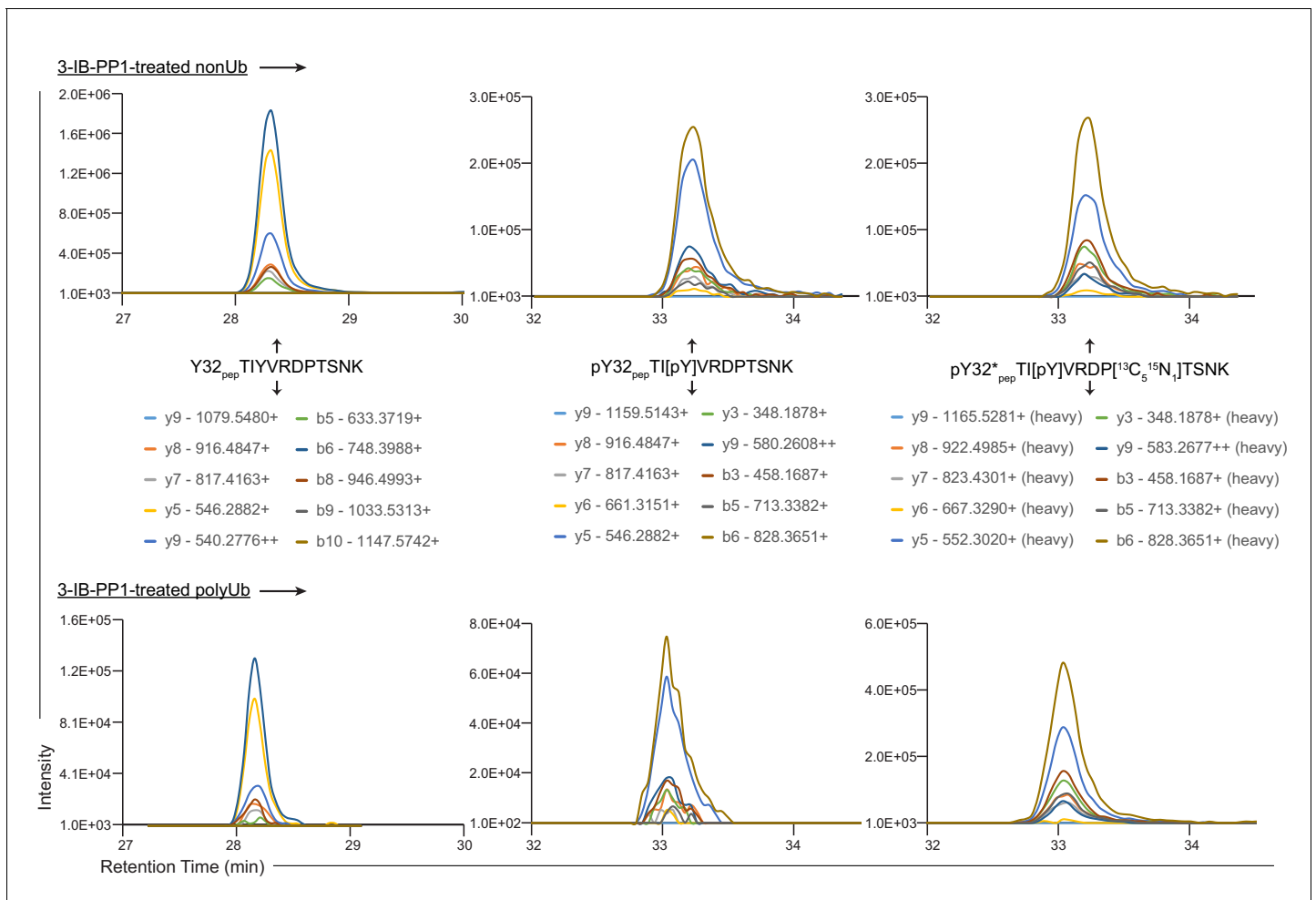


Figure 4—figure supplement 2. Annotated XICs. XICs from **Figure 4C** showing charge states and m/z values for y and b fragments from Y32_{peg}, pY32_{peg}, and pY32*_{peg}.

DOI: <https://doi.org/10.7554/eLife.46043.017>

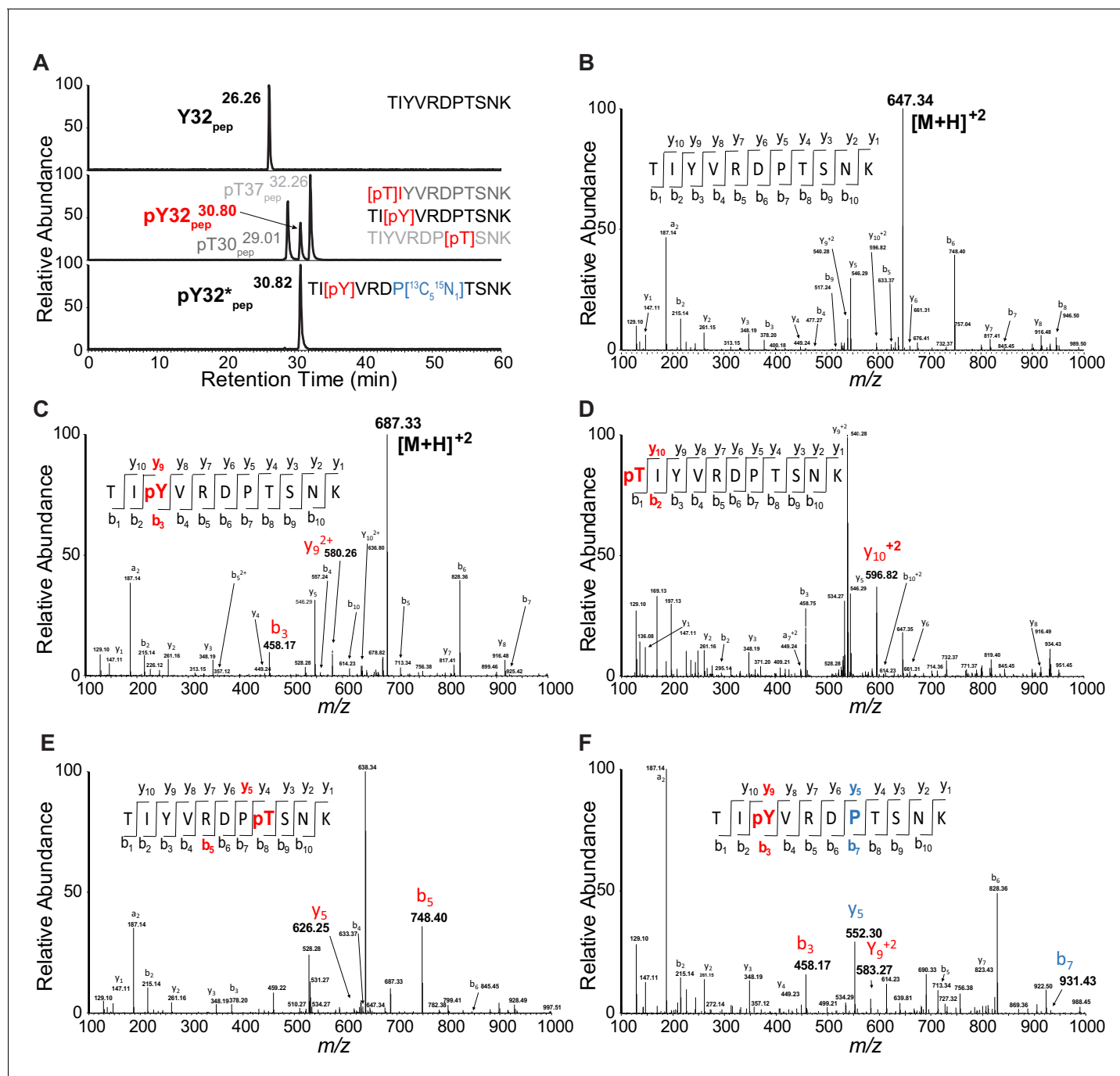


Figure 4—figure supplement 3. PRM chromatograms and spectra for unphosphorylated and phosphorylated LynA Y32 tryptic peptides. (A) PRM chromatograms illustrating resolution of synthetic phosphorylated and unphosphorylated peptides (top and middle panels), resolution of pY32 and synthetic threonine-phosphorylated (pT) species (middle panel), and coelution of unlabeled pY32_{pep} with isotope-labeled pY32*_{pep} (middle and bottom panels). Species detected in LynA IPs (Y32_{pep}, pY32_{pep}, and pY32*_{pep}) are indicated in bold type, phosphorylation sites are shown in red, and the isotope-labeled proline residue is shown in blue. (B–F) MS/MS spectra for synthetic peptides corresponding to tryptic peptides containing LynA Y32. The expected fragmentation pattern for each is indicated with phosphoions labeled in red/bold and isotope-labeled ions labeled in blue/bold. (B) MS/MS for Y32_{pep} run at 647.3435 m/z. (C) MS/MS for pY32_{pep} run at 687.3267 m/z, with highlighted fragment ions from phosphoY32. (D) MS/MS for pT30_{pep} run at 687.3267 m/z, with highlighted fragment ions from phosphoT30. (E) MS/MS for pT37_{pep} run at 687.3267 m/z, with highlighted fragment ions from phosphoT37. (F) MS/MS for pY32*_{pep} run at 687.3267 m/z, with highlighted fragment ions from phosphoY32 and the isotope-labeled proline residue.

DOI: <https://doi.org/10.7554/eLife.46043.018>

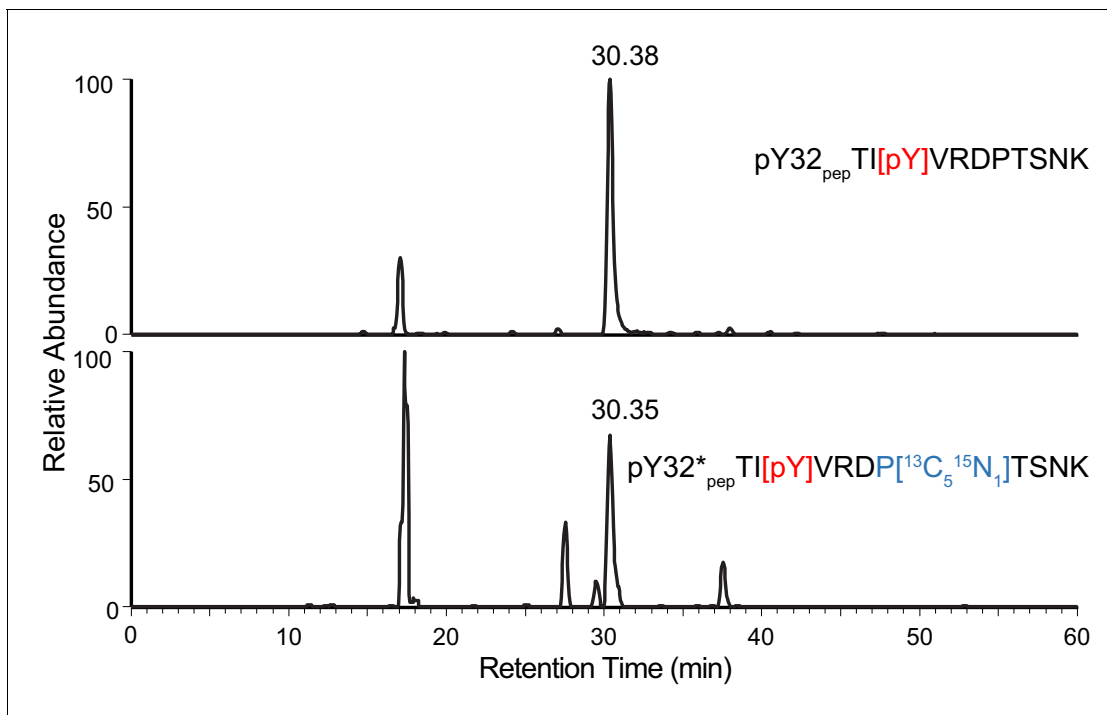


Figure 4—figure supplement 4. Y32 is the only site of phosphorylation detected in LynA Y32-containing tryptic peptide. XICs of 687.3267 *m/z* (top) and 690.3336 *m/z* (bottom) with a 10 ppm tolerance window for nonUb LynA in IPs from 3-IB-PP1-treated BMDMs. The top chromatogram demonstrates that pY32_{pep} TI[pY]VRDPTS NK but not pT30_{pep} [pT]IYVRDPTS NK or pT37_{pep} TIYVRDP[pT]SNK is detected under experimental conditions. The bottom chromatogram shows the co-isolation of isotope-labeled pY32*_{pep} TI[pY]VRDP[¹³C₅ ¹⁵N₁]TSNK from the same sample.

DOI: <https://doi.org/10.7554/eLife.46043.019>

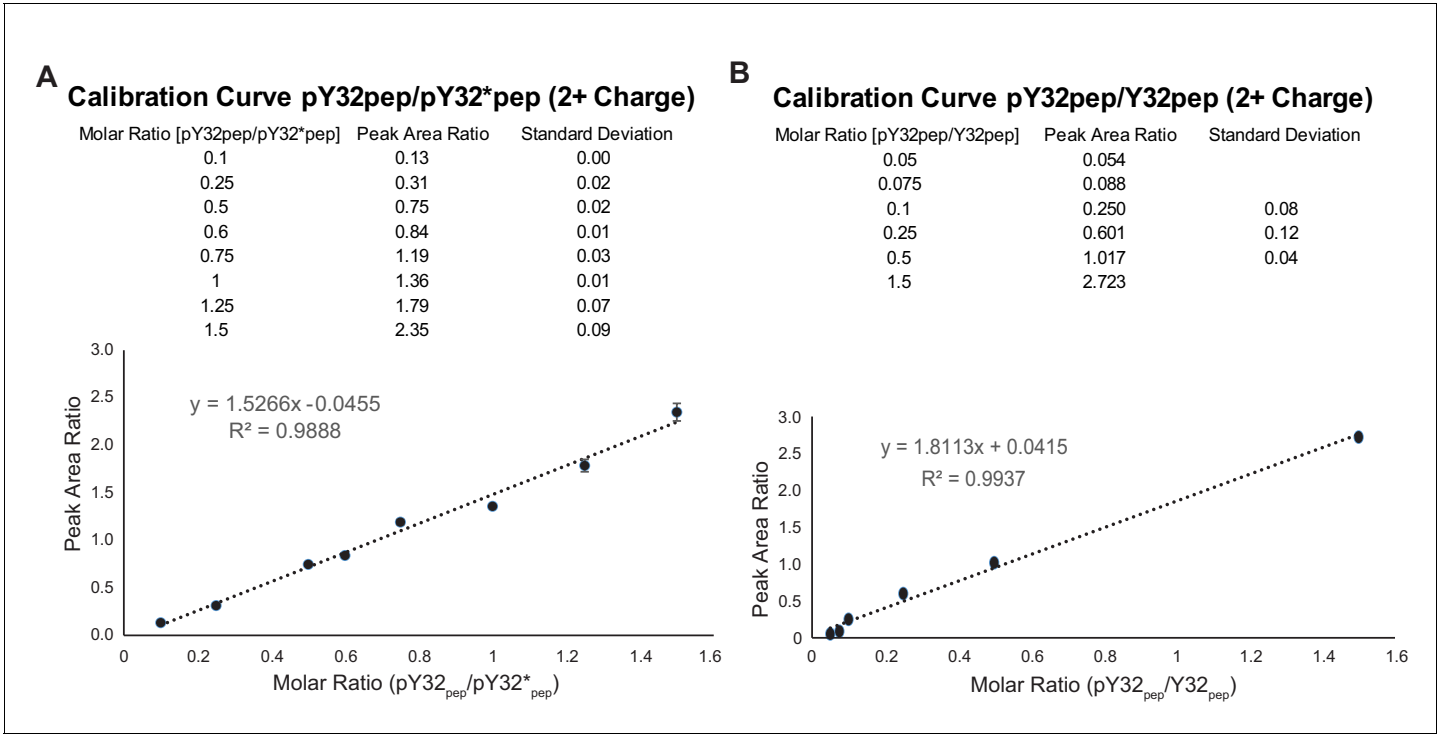


Figure 4—figure supplement 5. Peptide calibration curves. (A) Calibration curve for pY32 based on LC-MS/MS analysis. The results reflect the means and standard deviations of data acquired from three independent LC-MS/MS measurements. (B) Calibration curve for Y32 peptide. Refer to **Figure 4—figure supplement 5—source data 1, 2** for calibration curve data. Refer to **Figure 4—figure supplement 5—source data 3–6** for peptide quantification from Skyline analysis.

DOI: <https://doi.org/10.7554/eLife.46043.020>

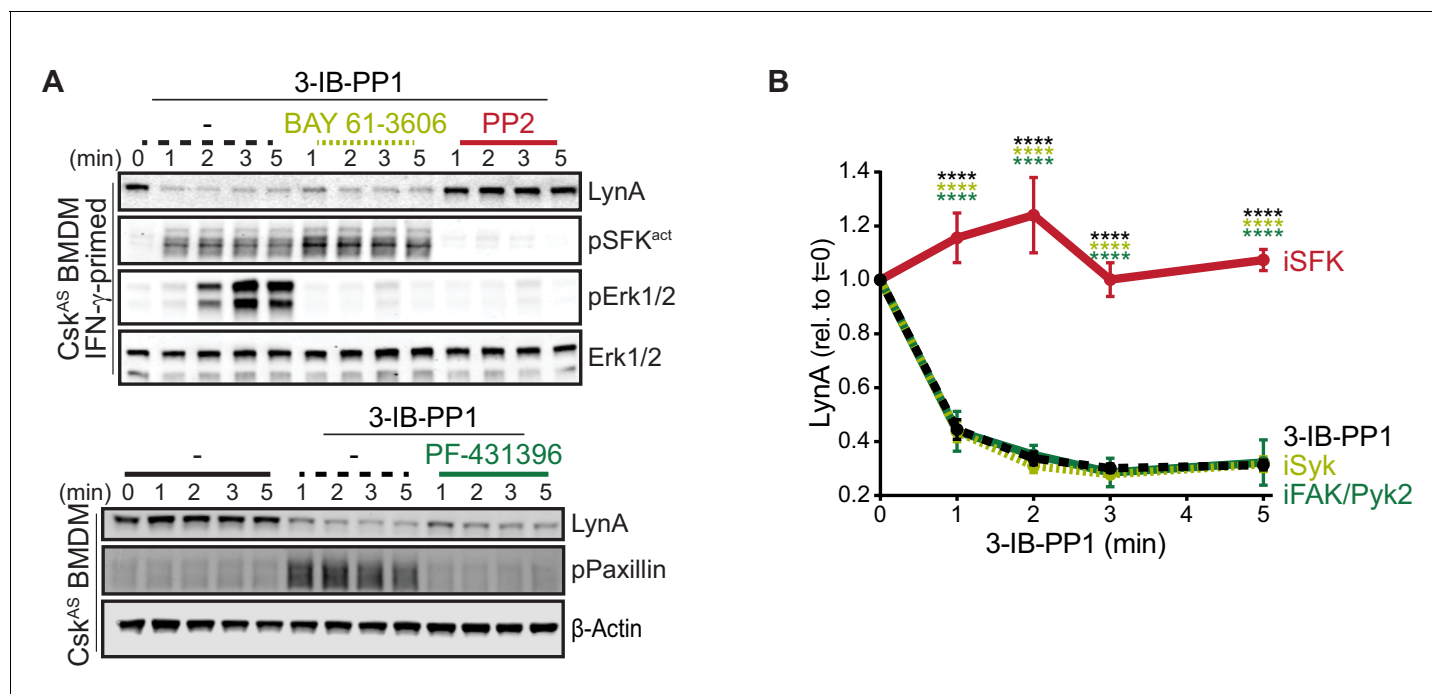


Figure 5. Syk, FAK, and Pyk2 kinase activities are not necessary for the rapid degradation of LynA. (A) Immunoblots showing expression of endogenous LynA protein in Csk^{AS} BMDMs treated with 3-IB-PP1 only or cotreated with 3-IB-PP1 and the Syk inhibitor BAY 61–3606, the SFK inhibitor PP2, or the FAK/Pyk2 inhibitor PF-431396. Activated signaling proteins including SFKs (pSFK^{act}, activation-loop-phosphorylation corresponding to Src^{pY416}), pErk1/2, and Paxillin^{pY118} (pPaxillin) are shown as controls for inhibitor function; β -Actin and total Erk1/2 are shown as loading controls. (B) Quantification of LynA protein during 3-IB-PP1 treatment only (black dotted) or with the inhibitors BAY 61–3606 (iSyk, light olive), PP2 (iSFK, red), or PF-431396 (iFAK/Pyk2, green), corrected for total protein content (TPS) and reported relative to the steady-state level of LynA. SEM, n = 3. Sig. from ANOVA₂-Tukey: [3-IB-PP1 only vs. 3-IB-PP1+PP2, black asterisks], [3-IB-PP1+BAY 61–3606 vs. 3-IB-PP1+PP2, light olive asterisks], [3-IB-PP1+PF-431396 vs. 3-IB-PP1+PP2, green asterisks]; ****p<0.0001. Other pairs ns. Refer to **Figure 5—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.028>

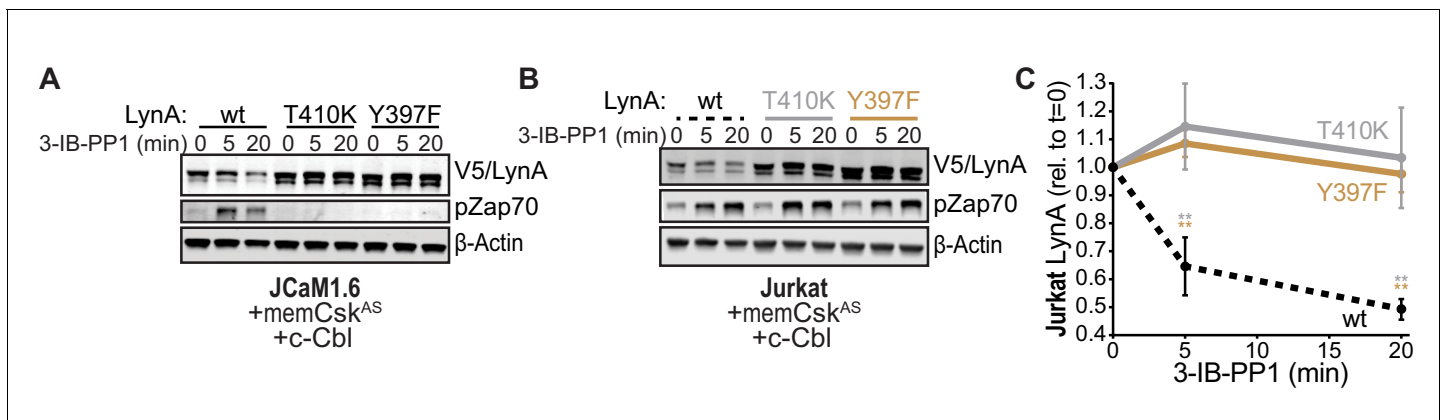


Figure 6. SFK activity is required for rapid degradation of LynA. **(A)** Immunoblots showing expression of transfected wild-type (wt), substrate-binding-impaired (T410K), or activation-loop-mutated (Y397F) His₆V5-tagged LynA protein coexpressed in JCaM1.6 cells with memCsk^{AS} and c-Cbl and treated with 3-IB-PP1. Interdomain-B-phosphorylated Zap70^{pY319} (pZap70), an SFK target and prerequisite for Zap70 activation, reflects the catalytic activity of LynA; β-Actin is shown as a loading control. **(B)** Overexpression of LynA variants in Jurkat cells. pZap70 reflects 3-IB-PP1-induced Lck activation. **(C)** Quantification of V5-tagged LynA variants wt (black dotted), T410K (gray), and Y397F (orange) in Jurkat cells during 3-IB-PP1 treatment, corrected for total protein content (TPS) and reported relative to the steady-state level for each variant of LynA. SEM, n = 3. Sig. from ANOVA₂-Tukey: [T410K vs. wt, gray asterisks], [Y397F vs. wt, orange asterisks]; **p=0.0015–0.0083. Other pairs ns. Refer to **Figure 6—source data 1**. Refer to **Figure 6—figure supplement 1** for additional experiments with the catalytic-site mutant LynA^{K275R}.

DOI: <https://doi.org/10.7554/eLife.46043.030>

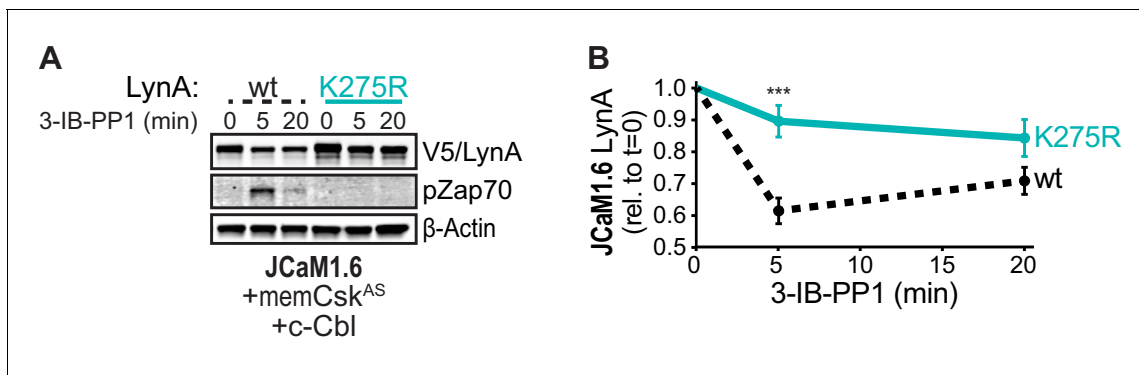


Figure 6—figure supplement 1. Catalytically dead LynA^{K275R} is not targeted for rapid degradation. **(A)** Immunoblots showing ectopic expression of wild-type (wt, black dotted) and active-site-mutated (K275R, cyan) His₆V5-tagged LynA protein coexpressed in JCaM1.6 cells with memCsk^{AS} and c-Cbl and treated with 3-IB-PP1. pZap70 shows the impaired function of LynA^{K275R}; β-Actin is shown as a loading control. **(B)** Quantification of V5-tagged LynA protein corrected for total protein content (TPS), reported relative to the steady-state level for each LynA variant. SEM, n = 3. Sig. from ANOVA₂ with Sidak's multiple comparison test: [K275R vs. wt] ***p=0.0008. Refer to **Figure 6—figure supplement 1—source data 1**. DOI: <https://doi.org/10.7554/eLife.46043.031>

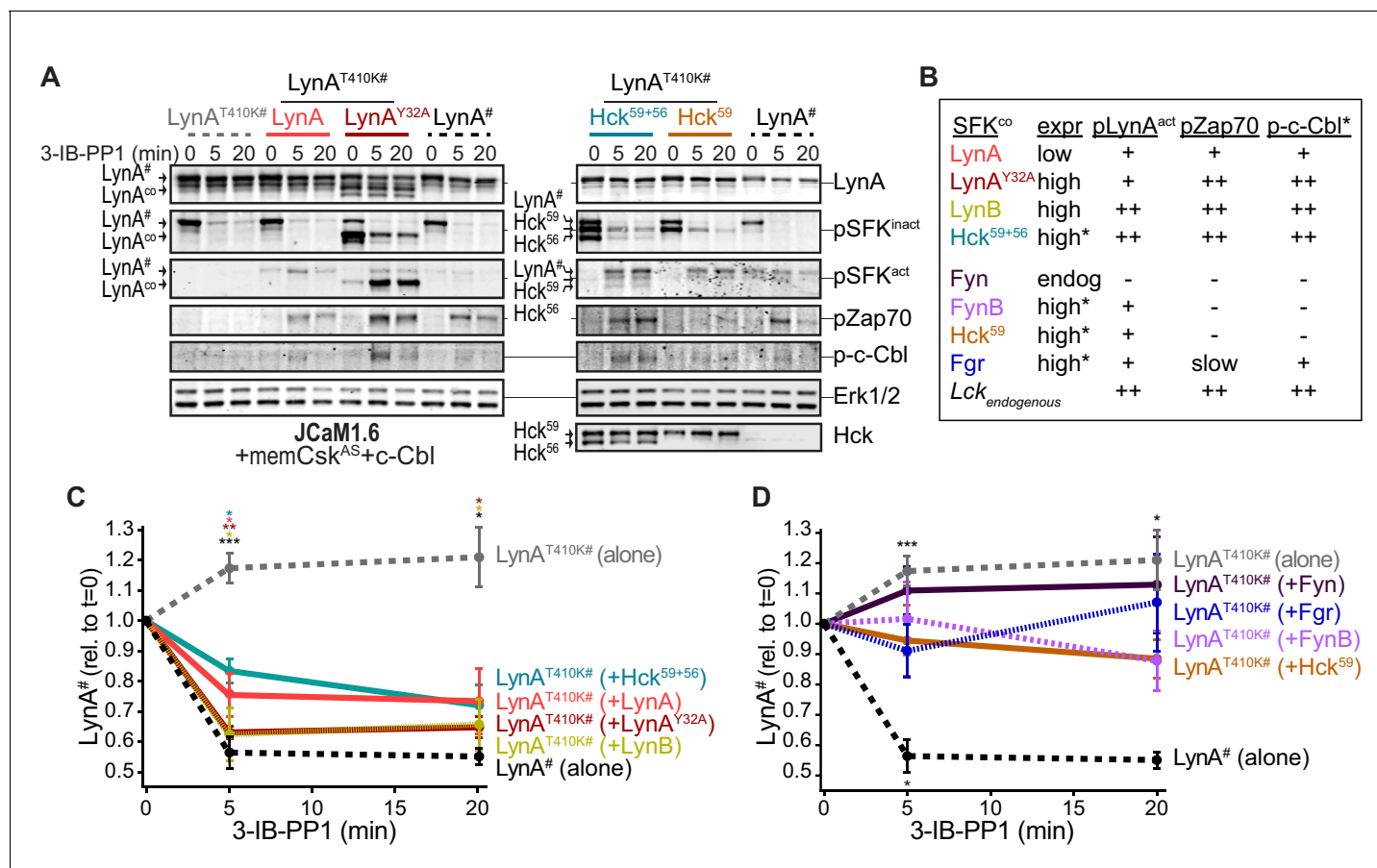


Figure 7. Cotransfected LynA, LynB, and Hck⁵⁶ can induce rapid degradation of LynA^{T410K} in trans. (A) Representative immunoblots showing levels of His₆V5-tagged LynA^{T410K} (#) cotransfected with empty vector or active SFK (co) in memCsk^{AS}- and c-Cbl-expressing JCaM1.6 cells. Refer to **Figure 7—figure supplement 1** for an illustration of the experimental layout. Cotransfected SFKs include untagged LynA, untagged LynA^{Y32A}, His₆V5-tagged LynB, the long and short isoforms of Hck (Hck⁵⁹⁺⁵⁶), the long isoform of Hck (Hck⁵⁹), FynT (Fyn), FynB, and Fgr. FynT is also expressed endogenously. His₆V5-tagged LynA^{T410K} (or wild-type LynA[#] in the positive control condition) was quantified from a total LynA blot (top panel). Colors correspond to later figure panels. Coexpressed SFKs are visualized via total SFK and pSFK^{inact} (inhibitory-tail phosphorylated SFK, corresponding to Src^{PY530}) blots as indicated. pSFK^{act} (activation-loop phosphorylated, corresponding to Src^{PY416}), pZap70, and p-c-Cbl blots are used to show 3-IB-PP1-induced activity of the cotransfected SFK; Erk1/2 is shown as a loading control. Refer to **Figure 7—figure supplement 2A** for immunoblots of LynB, Fgr, and Fyn cotransfections. When cotransfected with LynA^{T410K}, untagged wild-type LynA was expressed at very low levels, but its presence is inferred based on induction of Zap70 and c-Cbl phosphorylation with 3-IB-PP1 treatment. (B) Comparison of the expression (expr) levels of cotransfected SFKs (SFK^{co}) and their ability to induce phosphorylation of the LynA^{T410K} activation loop (pLynA^{act}, the highest band in the pSFK^{act} blots), Zap70^{PY319} and c-Cbl^{PY731} (p-c-Cbl) following 3-IB-PP1 treatment. If expression could not be directly compared with the wild-type LynA in the positive-control sample, pSFK^{inact} and/or pSFK^{act} blots were compared, with the caveat that the antibodies may not bind different SFKs equally well (marked with asterisks). Despite transfection, Fyn was expressed at endogenous levels (endog). Refer to **Figure 7—figure supplement 2B** for blots showing the effects of activating endogenous Lck. (C–D) Quantification of His₆V5-tagged wild-type LynA alone (black dotted) or His₆V5-tagged LynA^{T410K} alone (gray dotted) or cotransfected with the kinase-active SFKs. All LynA quantifications (#) are corrected for total protein content (TPS) and reported relative to the steady-state level for each transfection condition. SEM, n = 5 for LynA^{T410K} and LynA alone, SEM, n = 4 for all others. Sig. from ANOVA₂-Tukey: [LynA^{T410K} vs. LynA black asterisks], [LynA^{T410K} vs. LynA^{T410K}+LynA, coral asterisks], [LynA^{T410K} vs. LynA^{T410K}+LynA^{Y32A}, dark orange asterisks], [LynA^{T410K} vs. LynA^{T410K}+LynB, gold asterisks], [LynA^{T410K} vs. LynA^{T410K}+Hck⁵⁹⁺⁵⁶, teal asterisks], [Lyn vs. LynA^{T410K}+Fyn, dark purple asterisk]; ***p=0.0007, **p=0.0017, *p=0.0180–0.0466. Other pairs ns. (C) Quantification of His₆V5-tagged LynA^{T410K} cotransfected with SFKs that induce degradation of LynA^{T410K}. These include: untagged wild-type LynA (coral), untagged LynA^{Y32A} (dark orange), His₆V5-tagged LynB (gold dotted), and Hck⁵⁹⁺⁵⁶ (teal). (D) Quantification of His₆V5-tagged LynA^{T410K} cotransfected with SFKs that do not induce degradation of LynA^{T410K}: Hck⁵⁹ (tan), Fgr (blue dotted), Fyn (purple), and FynB (light purple). Refer to **Figure 7—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.034>

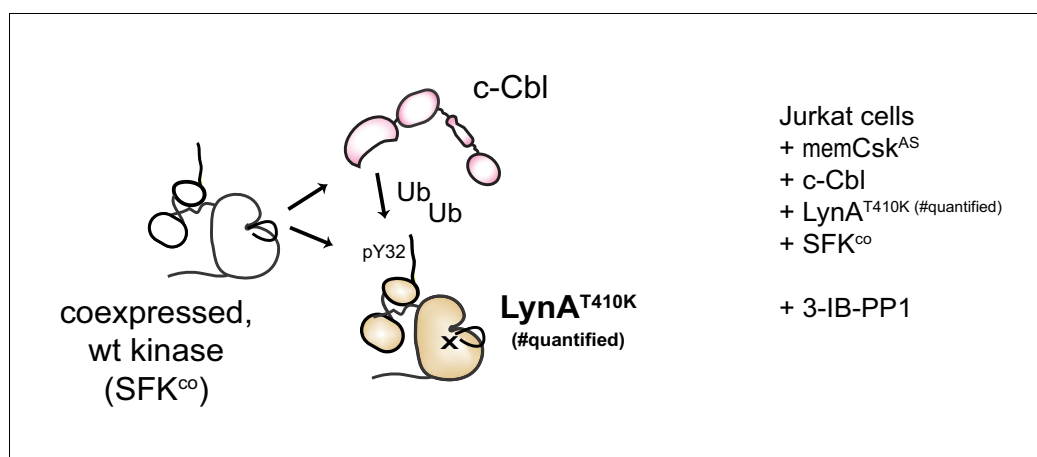


Figure 7—figure supplement 1. Assessing degradation of LynA mediated by SFKs in trans. Experimental layout, showing interactions of a substrate-binding-impaired His₆V5-tagged LynA^{T410K}, which is not on its own degradable, with functionally-competent SFKs cotransfected into JCaM1.6 cells.

DOI: <https://doi.org/10.7554/eLife.46043.035>

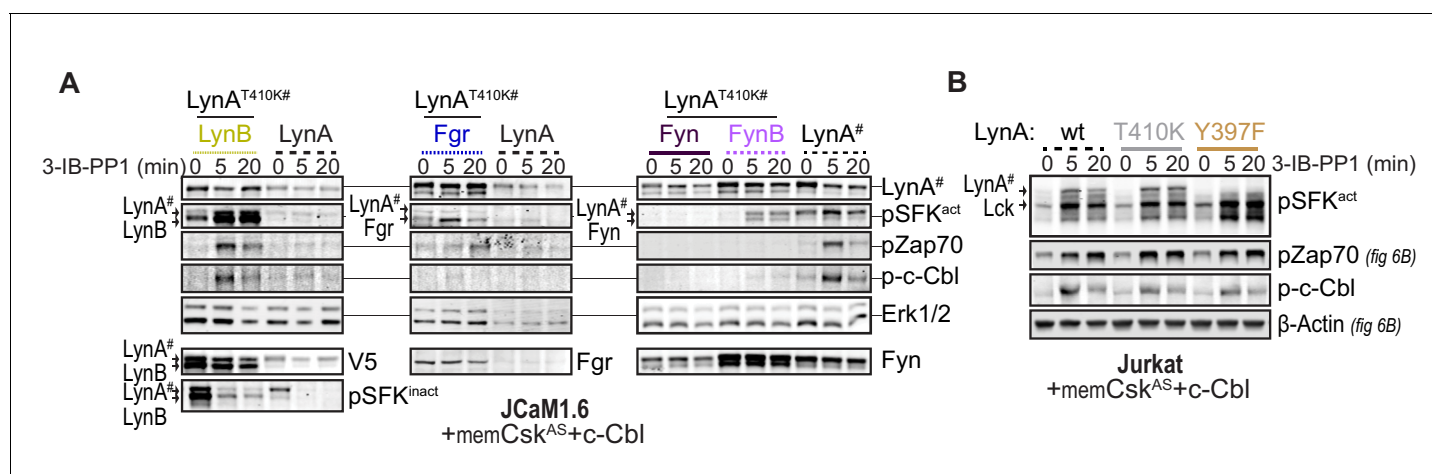


Figure 7—figure supplement 2. Additional immunoblots from cotransfection experiments. (A) Immunoblots showing cotransfected LynB, Fgr, and Fyn and 3-IB-PP1-mediated phosphorylation events. (B) Immunoblots from Lck-expressing Jurkat cells showing phosphorylation of activation-loop Y397 of LynA^{T410K}, interdomain B tyrosine 319 of Zap70, and tyrosine 731 of c-Cbl due to the activity of endogenous Lck in cells cotransfected with kinase-dead LynA constructs.

DOI: <https://doi.org/10.7554/eLife.46043.036>

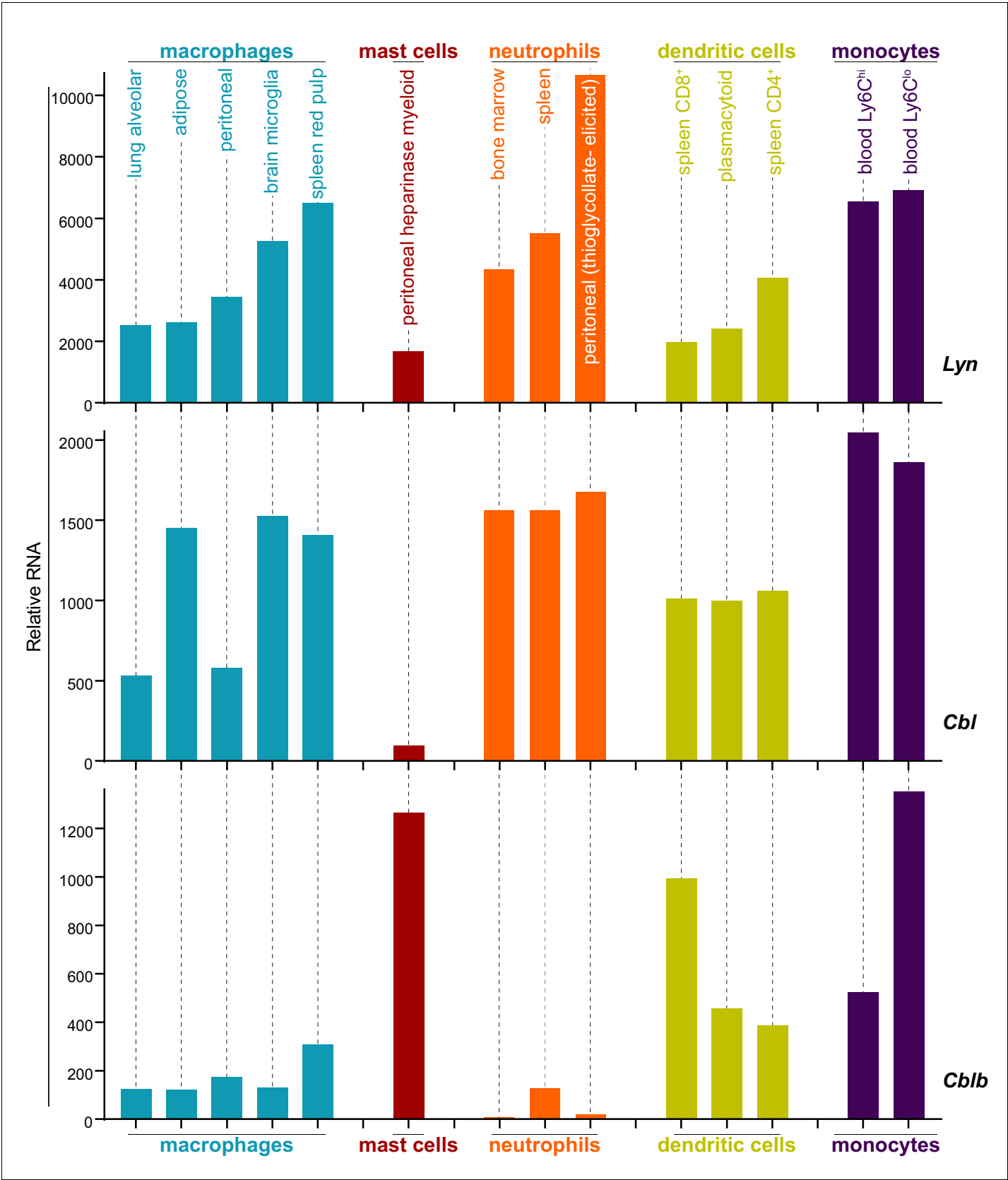


Figure 8. Expression levels of *Lyn*, *Cbl*, and *Cblb* are regulated differentially in myeloid cells. Mouse RNAseq data derived from the Immunological Genome Project (ImmGen, <http://rstats.immgen.org/Skyline/skyline.html>, **Heng and Painter, 2008**; **Dwyer et al., 2016**). *Lyn*, *Cbl*, and *Cblb* mRNA expression levels are shown for macrophages, mast cells, neutrophils, dendritic cells, and monocytes. Refer to **Figure 8—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.038>

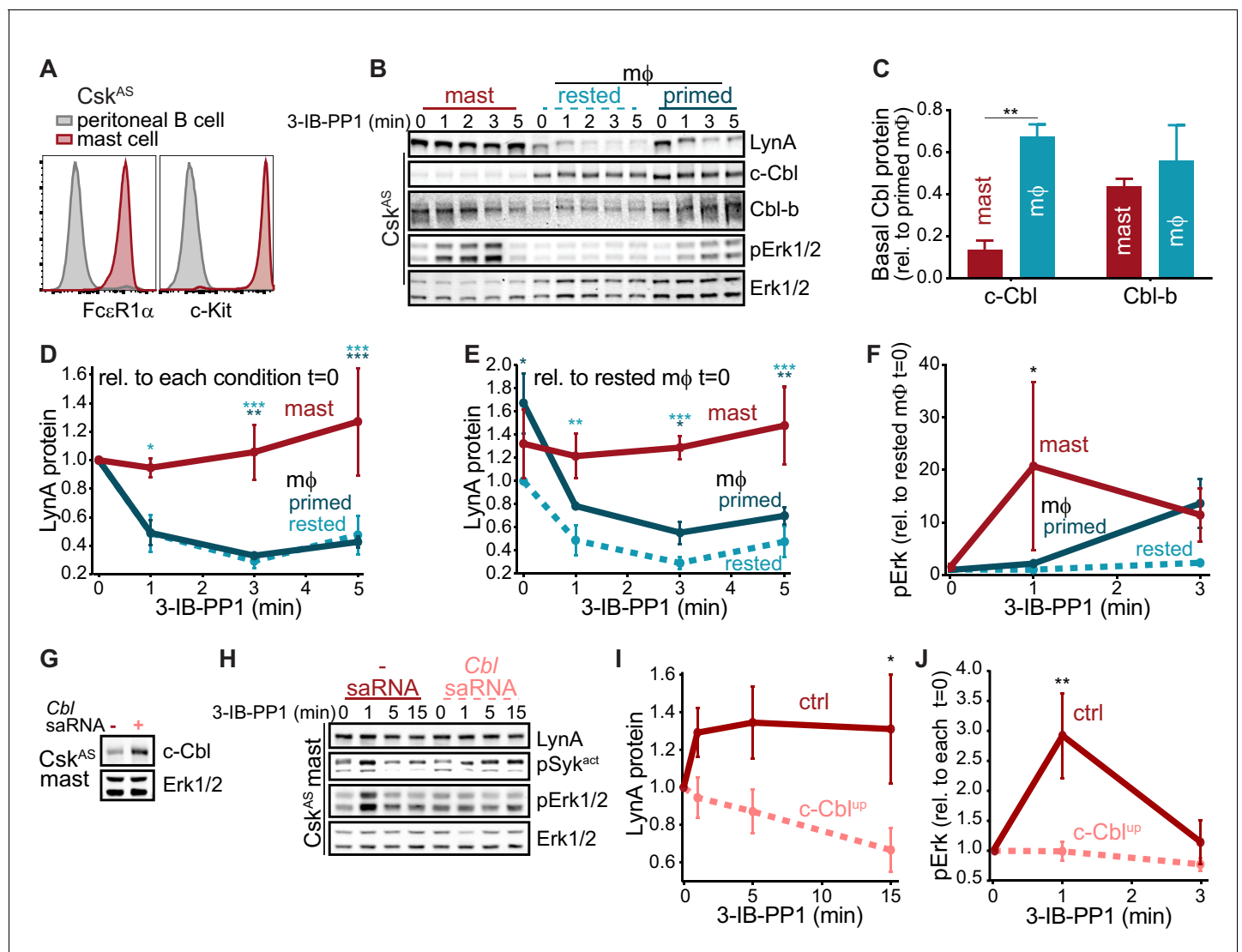


Figure 9. Differential expression of c-Cbl tunes LynA protein levels and SFK-mediated downstream signaling in macrophages and mast cells. (A) Flow cytometry showing surface expression of the mast-cell markers FcεR1α and c-Kit (CD117) in *Csk^{AS}* bone-marrow-derived mast cells (red) compared to peritoneal B cells (gray). (B) Immunoblots of *Csk^{AS}* mast cells and BMDMs (mφ) after resting or IFN-γ priming, showing the levels of LynA, c-Cbl, Cbl-b, and pErk1/2 during 3-IB-PP1 treatment; total Erk1/2 is shown as a loading control. (C–F) Quantification of c-Cbl, Cbl-b, LynA, and pErk in mast cells (red), IFN-γ-primed mφs (dark teal), and rested mφs (light teal). (C) Quantification of steady-state c-Cbl and Cbl-b protein in mast cells and rested mφs, corrected for total protein content (TPS) and reported relative to levels in primed mφs. SEM, n = 3. Sig. from one-way ANOVA with Sidak's multiple comparison test: [mast vs. rested mφs] **p=0.0035. Others pairs ns. (D–E) Quantification of LynA in mast cells and mφs during 3-IB-PP1 treatment, corrected for total protein content (TPS) and reported relative to the steady-state level of LynA in each cell type (D) or relative to the steady-state level of LynA in rested mφs (E). SEM, n = 3 for mast cells and primed mφs, n = 4 for rested mφs. Sig. from ANOVA₂-Tukey: [mast vs. rested mφs, light teal asterisks], [mast vs. primed mφs, dark teal asterisks]; ***p=0.0004–0.0008, **p=0.0025–0.0088, *P=0.0134–0.0466. Other pairs ns. (F) Quantification of pErk1/2 induction during 3-IB-PP1 treatment, corrected for total Erk1/2 expression and reported relative to the steady-state level of pErk1/2 in rested mφs. SEM, n = 3 for mast cells, n = 4 for primed mφs, n = 5 for rested mφs. Sig. from ANOVA₂-Sidak: [mast vs. rested mφs, asterisk]; *p=0.0330. Other pairs ns. (G) Immunoblots showing steady-state expression of c-Cbl in *Csk^{AS}* mast cells transfected in the presence or absence of *Cbl*-specific saRNA; Erk1/2 is shown as a loading control. (H) Immunoblots showing levels of LynA protein, pSyk, and pErk1/2 during 3-IB-PP1 treatment of *Cbl*-saRNA-transfected or mock-transfected mast cells. (I–J) Quantification of LynA and pErk in mast cells with (pink) and without (red) saRNA-induced upregulation of c-Cbl protein. SEM, n = 3. (I) Quantification of LynA protein during 3-IB-PP1 treatment, corrected for total protein content (TPS) and reported relative to the steady-state level for each transfection condition. SEM, n = 3. Sig. from mixed-effects ANOVA analysis: *p=0.0229. (J) Quantification of pErk1/2 induction during 3-IB-PP1 treatment in saRNA-treated and mock-treated mast cells, corrected for total Erk1/2 expression and reported relative to the steady-state level for each condition. SEM, n = 3 for mast cells. Sig. from ANOVA₂-Sidak: **p=0.0045. Refer to **Figure 9—source data 1**.

DOI: <https://doi.org/10.7554/eLife.46043.040>