

**LCK-mediated RIPK3 Activation Controls Double-Positive Thymocyte Proliferation
and Restrains Thymic Lymphoma by Regulating the PP2A-ERK Axis**

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KEYWORDS

RIPK3, Thymic lymphoma, Double-positive (DP) thymocytes, LCK, PP2A, ERK

Supporting Information Figure Legends

Supporting information 1. Analysis of thymus- and spleen-derived immune cells from RIPK3-deficient mice.

A) The expression level of RIPK3 protein in the thymus (left panel) and spleen (right panel). RIPK3 protein level measured by Western blot analysis.

B) Thymi from *Ripk3*^{+/+} and *Ripk3*^{-/-} mice are shown.

C) Populations of dendritic cells (CD11c⁺) and macrophages (F4/80⁺) in thymic T cells. (n=7 for each group).

D) Spleen from *Ripk3*^{+/+} and *Ripk3*^{-/-} mice is shown (left) and its weight is shown in the graph (right). (n=5 for each group).

E) Representative H&E images of spleen from both *Ripk3*^{+/+} and *Ripk3*^{-/-} mice. (R: red pulp. W: white pulp).

F) Splenic T cell populations from *Ripk3*^{+/+} and *Ripk3*^{-/-} mice. Average of the splenic T cell subsets and the absolute cell number by the indicated subsets are shown in each graph. (n=7 for each group).

G) Populations of naïve (CD62L^{hi}CD44^{lo}) and effector T cell (CD62L^{lo}CD44^{hi}) in the splenic T cells. (n=7 for each group).

H) Populations of dendritic cells (CD11c⁺) and macrophages (CD11c⁻F4/80^{lo}, CD11c⁻F4/80^{hi}) in splenic T cells. (n=7 for each group).

Statistical analyses were performed using the two-tailed unpaired Student *t*-test. P values below 0.05 were considered significant in the following manner.

Supporting information 2. RIPK1, RIPK3 and MLKL expression levels in various tissues and different cell types.

A) Total thymocytes from *Ripk3*^{+/+} (n=6) and *Ripk3*^{-/-} (n=6) mice were treated with TNF- α (50 ng/ml), z-VAD (20 μ M) and Smac mimetic (200 nM) for 5 h and 24 h. Dead cells were identified by Annexin V and PI staining.

B-D) Distribution of *Ripk1* (B), *Ripk3* (C) and *Mlkl* (D) gene expression in various immune

cell from BioGPS website.

E, F) Expression levels of MLKL in the indicated cell lines. MLKL protein level was measured by Western blot analysis (E) and Flow Cytometry (F).

Supporting information 3. Loss of RIPK3 does not impact the population and proliferation of T cells from spleen and peripheral lymph nodes in carcinogen-induced mouse model.

A) The ratio of thymus weight from control mice and ENU-injected mice were shown in the graph.

B) Spleen (left), peripheral lymph node (pLN; middle) and mesenteric lymph node (mLN; right) of each genotype is shown.

C-H) T cell populations of spleen (C), pLN (E) and mLN (G) from the indicated genotype, displayed as 2D plots of relative fluorescence of the indicated marker. Average of the T cell subsets are shown in each graph. Relative fluorescence of Ki-67 in CD4⁺ and CD8⁺ T cells. (C, D; Spleen, E, F; pLN, G, H; mLN). *Ripk3*^{+/+} and *Ripk3*^{-/-} littermates were analyzed at 60 days after three-time injections of ENU (n=10 -12 for each group).

Statistical analyses were performed using the two-tailed unpaired Student *t*-test. P values below 0.05 were considered significant in the following manner.

Supporting information 4. Tumor progression and incidence rate according to RIPK3 expression in thymic lymphoma-bearing Trp53-deficient mice

A, B) Western blotting analysis of RIPK3 expression level in tissues lysates from *p53*^{-/-} background thymic lymphoma.

C) Tumors found in various organs and their frequency in *Ripk3*^{+/+}*p53*^{-/-} and *Ripk3*^{-/-}*p53*^{-/-} animals.

Supporting information 5. Protein Interacting domains between LCK and RIPK3

A) Tandem affinity purification of the tagged RIPK3 protein and interacting proteins using

streptavidin resin followed by calmodulin resin.

B) Amino acid sequence of human LCK (upper panel) and human RIPK3 (lower panel).

C, D) Western blot analysis after immunoprecipitation of human RIPK3 and human LCK in HEK293T cells. HEK293T cells were transfected with GST-LCK and/or Flag-RIPK3 expression constructs. Cells were harvested at 24h after transfection and cell lysates were immunoprecipitated with GST antibody (left) or RIPK3 antibody (right) (C). HEK293T cells were transfected with GST-LCK and/or Flag-RIPK3 expression constructs. Cells lysates were immunoprecipitated with RIPK3 antibody (left) or phosphor-Tyrosine antibody (right) (D).

Supporting information 6. Protein Interacting domains between RIPK3 and PP2A

A) Venn diagram represents the overlap of proteins and unique proteins identified by LC/MS/MS among TAP-purified samples as indicated. Total 503 proteins were identified as specific RIPK3 binding proteins.

B) Amino acid sequence of human RIPK3 (upper panel) and human PPP2R2A (lower panel).

C) In vitro kinase activity of RIPK3 toward PPP2R2A with ³²P-labeled ATP.

D) HEK293T cells were transfected with GFP-PPP2R2A and/or Flag-RIPK3 expression constructs. Cells were harvested at 24h after transfection.

Supporting information 7. Treatment of PP2A inhibitor or activator alters ERK phosphorylation status in thymic tumorigenesis.

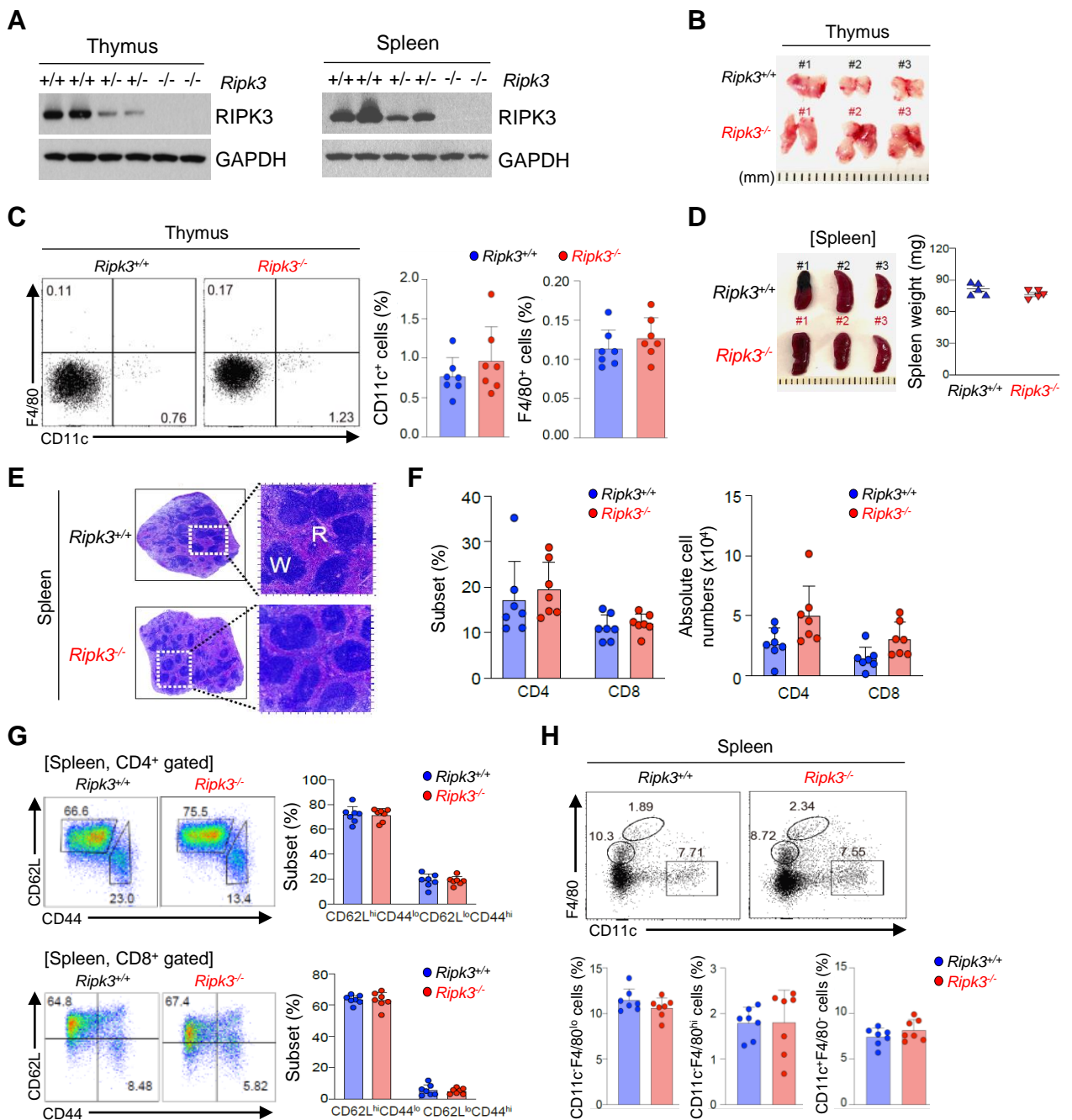
A) Phosphorylation of ERK increased in response to PMA (1μM) and ionomycin (3μM) stimulation, which was further increased by LB-100 treatment.

B) Representative images of thymus from LB-100 or PBS injected mice (upper panel). LB-100 did not significantly change the thymus phenotype. Spleen, mesenteric lymph node and peripheral lymph node (lower) of each group were shown. (n=4 for each group)

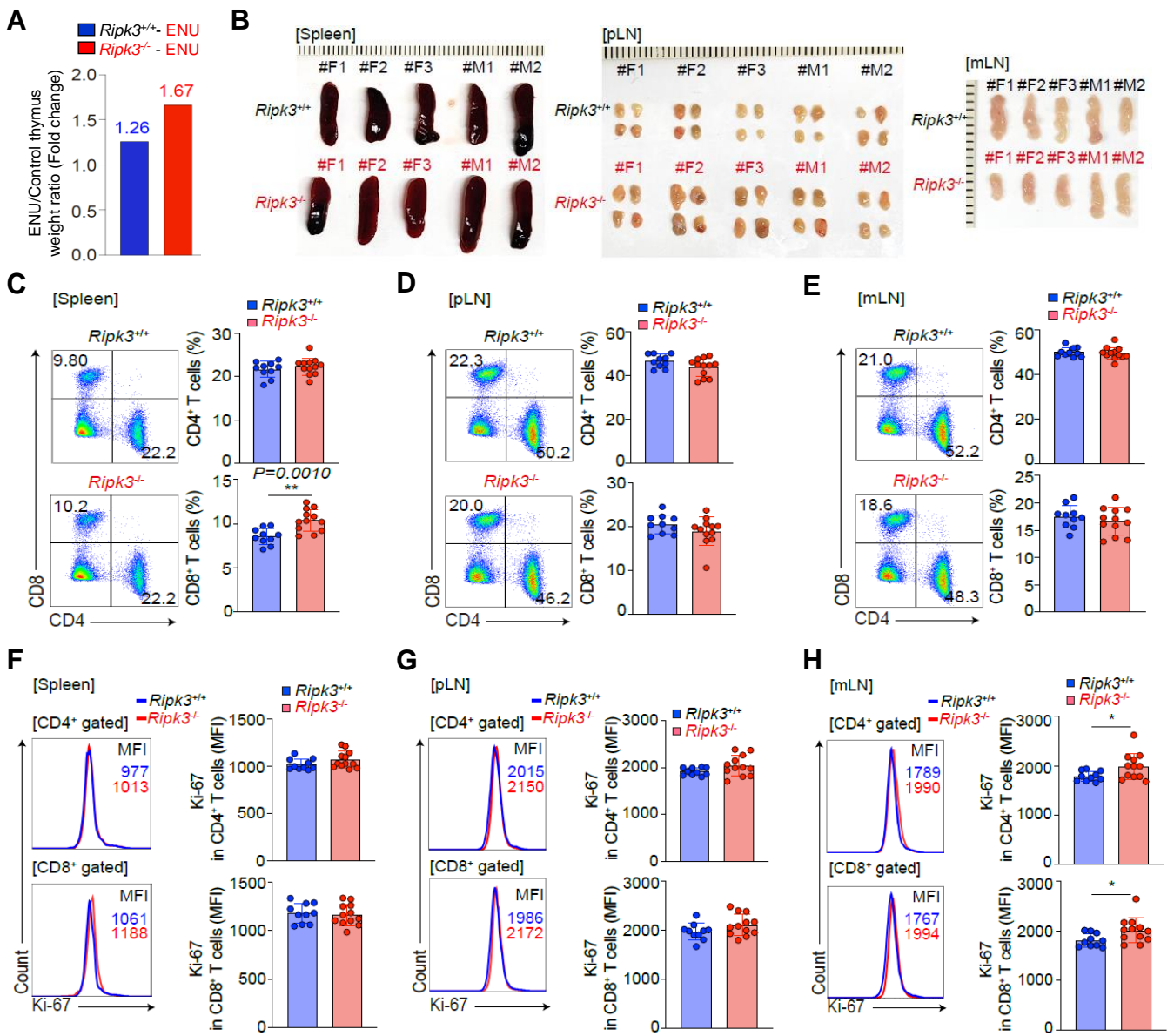
C, D) Western blotting analysis of PPP2R2A and PPP2CA expression level in thymus tissues lysates from ENU-injected mice. The PP2A expression pattern was not affected by injection of LB-100 or SMAP.

E) Representative images of thymus from SMAP or PBS injected mice (upper panel). Injection of SMAP decreased thymus size in ENU-injected *Ripk3*^{-/-} mice. Spleen, mesenteric lymph node and peripheral lymph node (lower) of each group were shown. (n=4 - 6 for each group)

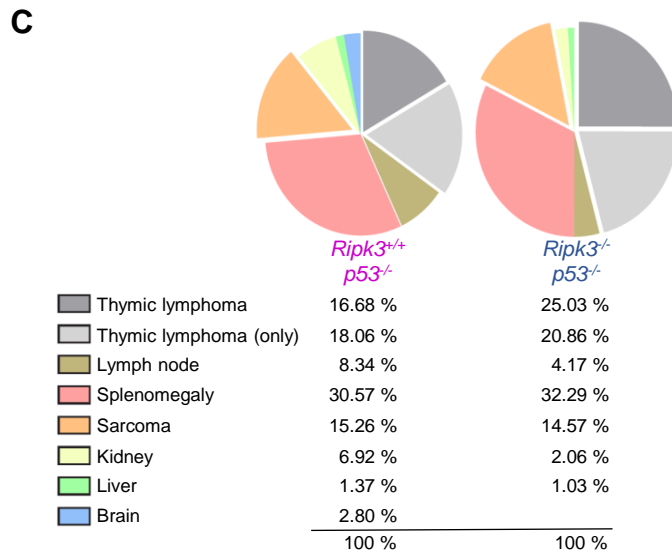
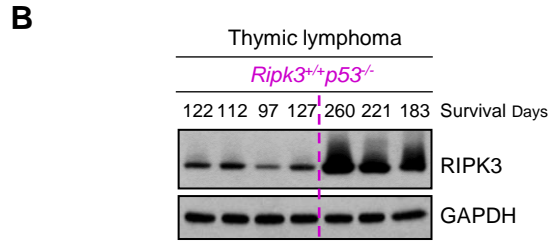
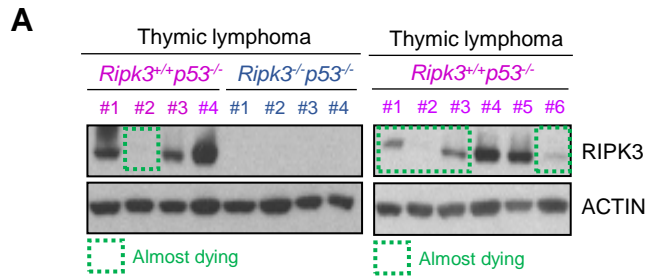
Statistical analyses were performed using the two-tailed unpaired Student *t*-test. P values below 0.05 were considered significant in the following manner: *P<0.05, **P<0.01, ***P<0.001.



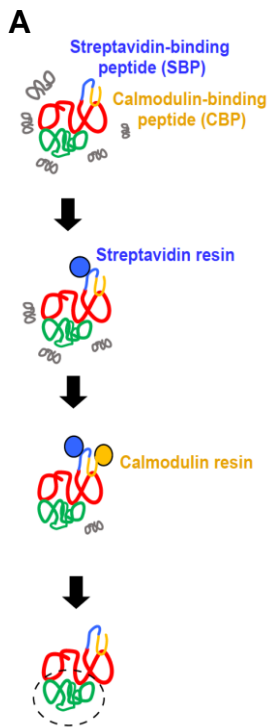
Supporting Information 1



Supporting Information 3



Supporting Information 4



B > Tyrosin-protein kinase LCK isoform a (LCK) [*Homo sapiens* (human)]

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MGC GCS SHPE DDWMENIDVC ENCHYPIVPL DGKGTLLIRN GSEVRDPLVT YEGSNPPASP LQDNLVI LK
SYEP SHDGL GFEKGEOLRI LEQSGEWWKA QSLTTGQEGF IPFN FVAKAN SLEPE WFFK NLSRKDAERQ
LLAPGNTHGS FLIRESESTA GSFSLSVRDF DQNGQEVVKH YKIRNLDNGG FYISE IITFP GLHELVIHYT
NAS IGLCTRL SRPCQTQKPQ KPWWEDWEV PRETLK LVER LGAGQFGEVW MGYINGHTKV AVKSLKQGS M
SPDAFLAEAN LMKQLQHRL VRLYAVVTQE PIYIITEYME NGSLVDFLKT PSGIKLTINK LLDMAAQIAE
GMAFIEERNY IHRDLRAANI LVSDTL SCKI ADFGLARLIE DNEYTAREGA KFPKWTAPE AINYGFTTIK
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RSVLEDDFFTA TEGQYQPQP
  
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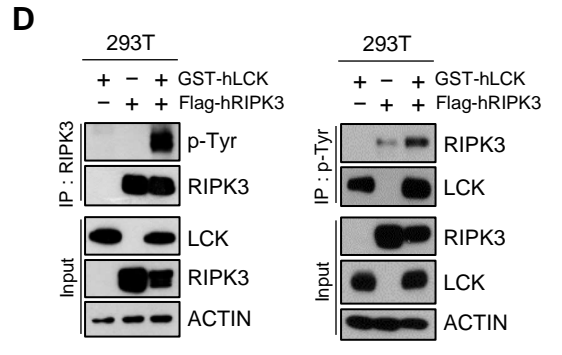
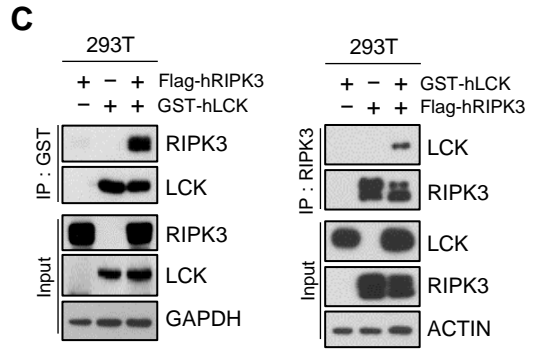
■ : RIPK3 binding sites □ : SH3 domain □ : SH2 domain □ : Kinase domain

> Receptor-interacting serine/threonine-protein kinase 3 (RIPK3) [*Homo sapiens* (human)]

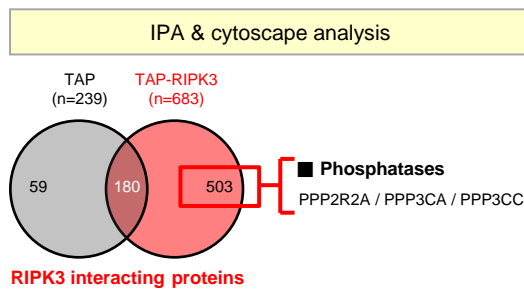
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RDLKPSNLL DPFLHVRLAD FGLSIFOGGS OSGTGSSEPG GILGYLAPEL FVNVNRKAST ASDVYSFGIL
MWAFLAGREV ELPTPESLVY EAVCNRONRF SLAELPOAGF ETPGLEGLKE LMOLCWSSEP KDRPSFOECL
PKTDEVFQMV ENNMNAAVST VKDFLSQLRS SNRRFSIPES GQGGTMDGF RRTIENQHSR NDVMVSEWLN
KLNLEPPSS VPKKCP SLTK RSRAQEEQVP QAWTAGTSSD SMAQPPQTFE TSTFRNQMP S PTSTGT PSG
PRGNQGAERQ GMNWS CRTPE PNPVTGRPLV NIYNCSGVOV GDNNYLTMQQ TTALPTWGLA PSKGKRG LQH
PPVGSQEGP KDPEAWSRPQ GWYNHSGK
  
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■ : LCK binding sites □ : Kinase domain □ : RHIM domain



Supporting Information 5

A**B**

> Receptor-interacting serine/threonine-protein kinase 3 (RIPK3) [*Homo sapiens* (human)]

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MSCVKLWPSG  APAPLVSIEE  LENQELVGKG  GFGTVFRAQH  RKWGYDVAVK  IVNSKAISRE  VKAMASLDNE
EVLRLLEGVIE  KVNWDQDPKP  ALVTKFMENG  SLSGLLQSQC  PRPWLLCRL  LKEVVLGMFY  LHDQNPVLLH
LDLEPSNYLL  DELHVKLAD  FGLSTFQGG  QSGTGSQEG  GILGILFEL  FVNVVKAAS  ASDVYSFGIL
MWAVLAGREV  ELPTEPSLVY  EAVCNRNRNP  SLAELPOAGP  ETPGLEGLKE  LMOLCWSSEP  KDRPSFOECL
EPKTDEVFQMV  ENNMNAAVST  VKDFLSQLRS  SNRRFSIPES  GGGTEMDGF  RRTIENQHSR  NDVMVSEWLN
KLNLEEPSS  VPKKCPSLTK  RSRQQEEQVP  QAWTAGTSSD  SMAQPPQTP  TSTFRNQMP  PTSTGTESPG
PRGNQGAERQ  GMNWSCRTP  PNPVTGRPLV  NIYNCSGVOV  GDNNYLTMQ  TTALPTWGLA  PSGKGRGLQH
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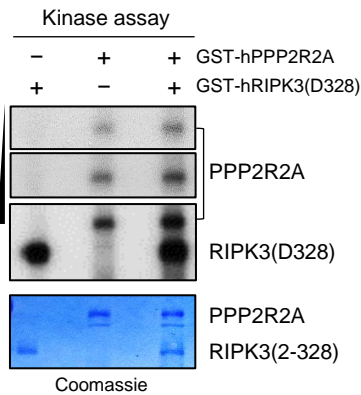
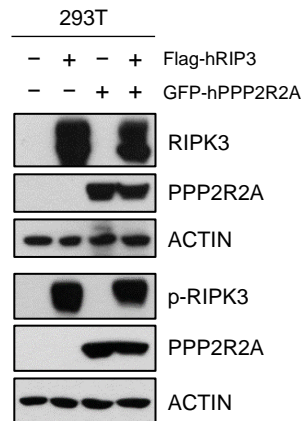
■: PP2A-B α 551 binding sites □: Kinase domain □: RHIM domain

> Serine/threonine-protein phosphatase 2A 55kDa regulatory subunit B alpha isoform 1 (PP2A-B55B α) [*Homo sapiens* (human)]

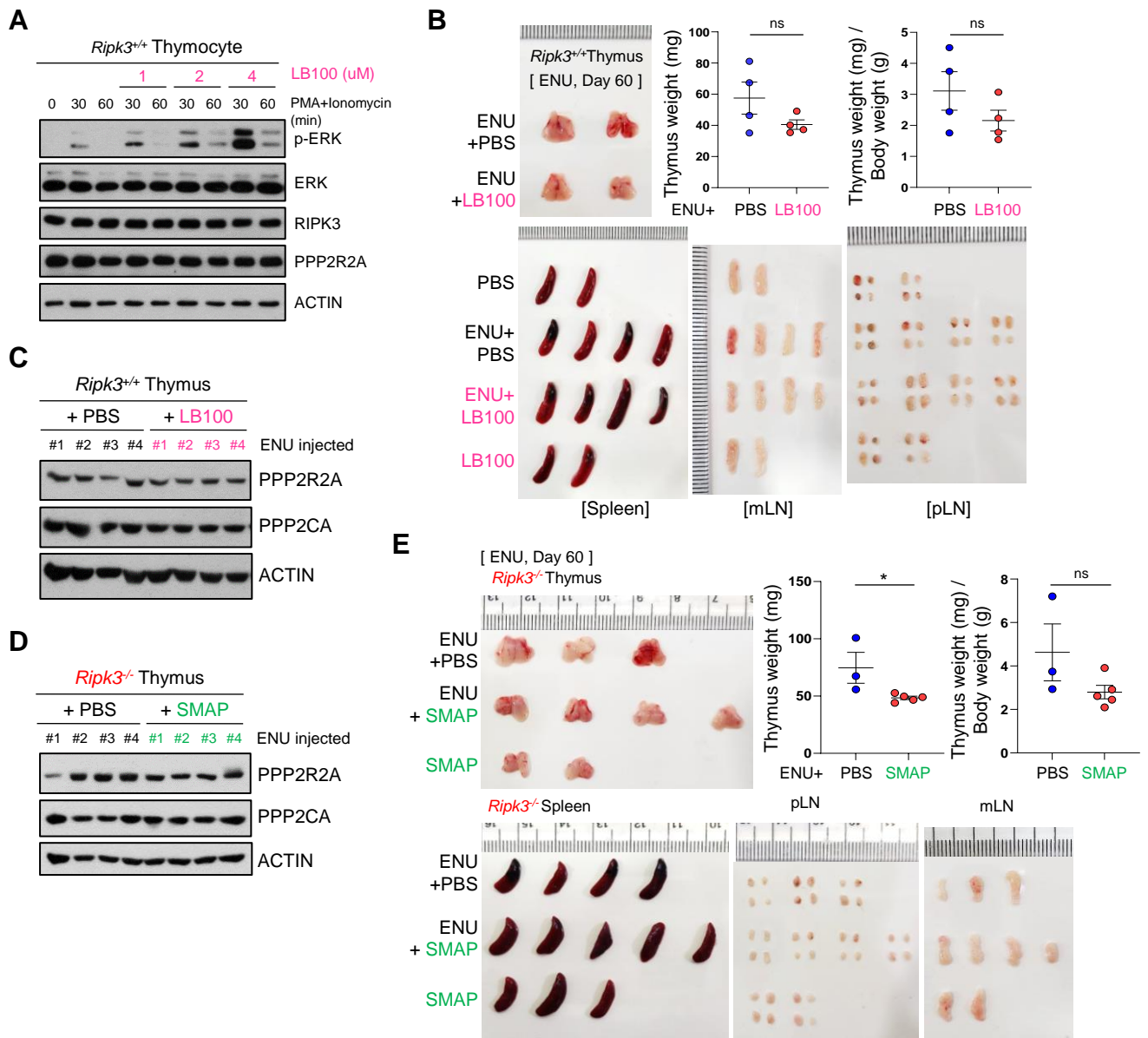
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GRYRDPTTVT  TLRVPVFRPM  DLMVEASPRR  IFANAHTYHI  NSISINSDYE  TYLSADDLRI  NLWHLEITDR
SFNIVDIKPA  NMEELTEVIT  AAEFHNSCN  TFVYSSSKGT  IRLCDMRASA  LCDRHSLKFE  EPEDPSNRSF
FSEIISSISD  VKFSHSGRYM  MTRDYLSVKI  WDLNMENRPV  ETYQVHEYLR  SKLCSLYEND  CIFDKFECCW
NGSDSVVMTG  SYNFFFRMFD  RNTKRDITLE  ASRENNKPR  VLKPRKVCAS  GKRKKDEISV  DSLDFNKKIL
HTAWHPKENI  IAVATTNNLY  IFQDKVN
  
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■: RIPK3 binding sites

C**D**

Supporting Information 6



Supporting Information 7