Journal: Nature Medicine

Article Title:	The activin receptor IIa ligand trap Sotatercept corrects ineffective erythropoiesis in beta-thalassemia
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Supplementary Figure 1	[TITLE OF FIGURE (descriptive title used in legend)]
Supplementary Table 1	[TITLE OF TABLE (descriptive title)]
Supplementary Methods (or Discussion or Data or Note)	[NO DESCRIPTIVE TITLE NECESSARY]
Supplementary Video 1 (or Audio or Spreadsheet)	[CAPTION TO BE POSTED ONLINE (include title and explanation of contents)]

The activin receptor IIa ligand trap Sotatercept corrects ineffective erythropoiesis in beta-thalassemia

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental figure 1: (**a-h**) *RAP-011 effects on hematological parameters in wild-type mice*. C57BL/6 mice were treated with RAP-011 (10 mg/Kg twice a week) or PBS for 30 days. Evaluation of (**a**) red blood cell counts (RBC), (**b**) hematocrit level, (**c**) percentage and (**d**) absolute numbers of reticulocytes, (**e**) hemoglobin, (**f**) mean corpuscular volume (MCV), (**g**) mean corpuscular hemoglobin (MCH) and (**h**) MCH concentration (MCHC). (**i-l**) *RAP-011 effects on bone marrow and spleen cellularity and erythroblasts numbers in wild-type mice 30 days post-treatment*. C57BL/6 mice were treated with RAP-011 or PBS. (**i,j**) spleen and bone marrow cellularity. (**k,l**) Absolute numbers of Ter-119^{+} cells in spleen and bone marrow. (**m-o**) *RAP-011 effects on erythroblast differentiation in thalassemic mice*. Hbb$^{th1/th1}$ mice were treated 5-30 days with RAP-011 or PBS and cell differentiation was evaluated by flow cytometry. The absolute numbers of different erythroblast populations (ProE, proerythroblast; Ery.A, basophilic erythroblast; Ery.B, late basophilic and polychromatic erythroblast; Ery.C, orthochromatic erythroblast) on (**m**) spleen and (**n**) bone marrow are shown. (**o**) An index of ineffective erythropoiesis was established by calculating the ratio of Ery.B and Ery.C populations. (**p**) *Erythroid differentiation in C57BL/6 mice treated twice a week for 30 days with 10 mg/kg RAP-011 or PBS.* Bone marrow and spleen were harvested and erythroblast differentiation was evaluated by CD71/Ter-119 staining and FSC distribution. The absolute numbers of erythroblast populations are shown. *p<0.05, N=5 per group. Data represent means ± S.E.M.

Supplemental figure 2: (**a**) *RAP-011 reduces ineffective erythropoiesis in Hbb$^{th1/th1}$ thalassemic mice.* (**a**) lactate dehydrogenase (LDH) levels in thalassemic mice treated for up to 60 days with 10 mg/kg RAP-011 or PBS. (**b-d**) *RAP-011 effects on bilirubin and LDH levels in wild-type mice.* Biochemical analysis of sera from wild-type mice treated for 30 days with 10 mg/kg RAP-011 or PBS: (**b**) direct bilirubin, (**c**) total bilirubin and (**d**) LDH. (**e-g**) *RAP-011 effects on iron homeostasis parameters in wild-type mice.* Biochemical analysis of iron homeostasis parameters of sera from wild-type mice treated for 30 days with RAP-011 or PBS: (**e**) Systemic iron levels, (**f**) transferrin levels and (**g**) transferrin saturation. (**h-j**) *RAP-011 effects on GDF15 expression.* Hbb$^{th1/th1}$ mice were treated with PBS or RAP-011 for 30 days. *Gdf15* mRNA levels measured by qPCR in (**h**) spleen or (**i**) bone marrow samples. (**j**) *RAP-011 has no effect on GDF15 signaling*. CAGA-Luc/GFP C2C12 cells were treated with GDF15 (100 ng/ml) or with vehicle in the presence of RAP-011 (10 µg/ml) or a specific GDF15 antibody (1 µg/ml). Luciferase activity was normalized to GFP fluorescence; values are expressed as relative luminescence units per relative fluorescence units (RLU/RFU). (**k-m**) *RAP-011 effects on globin chains expression 30 days post-treatment*. Hbb$^{th1/th1}$ mice were treated with PBS or RAP-011 for 30 days. Adult (Hbb-b2) (**k**) or embryonic (Hbb-bh1 and Hbb-Ey) (**l,m**) globin mRNAs expression was evaluated by qPCR in purified immature (CD71^{+}) erythroblasts from wild-type and Hbb$^{th1/th1}$ mice. All data are expressed as the mean ± S.E.M. *P<0.05, **P<0.01, ns=not significant; N=5 per group for each of three independent experiments performed.

Supplemental figure 3: (**a-b**) *retrospective analysis of dataset GSE34125 showing the expression levels of several TGF-β superfamily.* Expression of TGF-β superfamily (**a**) ligands and (**b**) receptors on CD71^{+}Ter-119^{+}FSC^{high} matched erythroblasts from E14.5 fetal livers of wild-type and Hbb$^{Th3/Th3}$ embryos. Data were obtained at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and analyzed using GEO2R software. **(c-f)** *TGF-β superfamily gene expression analysis in spleen and bone marrow samples from wild-type and Hbb$^{th1/th1}$ mice.* mRNA levels of (**c**) *Acvr2a*, (**d**) *Acvr2b*, (**e**) *Acvr1b*

(ALK4) and (**f**) *Tgfbr1* (ALK5) were evaluated by qPCR in spleen and bone marrow purifed erythroblasts derived from wild-type and thalassemic mice. (**g-i**) *Gene expression analysis from spleen and bone marrow-derived cultured erythroblasts from wild-type and* $Hbb^{th1/th1}$ *mice.* (**g**) *Acvr2a*, (**h**) *Acvr2b and* (**i**) *Acvr1b* (ALK4) mRNA levels evaluated by qPCR. All data are expressed as the mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.005; ns = not significant; N=5 per group for each independent experiment.

Supplemental figure 4: *mRNA levels for TGF-β family members in spleen and bone marrow erythroblast samples of wild-type and* $Hbb^{th1/th1}$ *mice.* (**a**) *Inha* (activin A), (**b**) *Inhb* (activin B) and (**c**) *Gdf15* mRNA levels in spleen and bone marrow erythroblasts from wild-type and thalassemic mice. (**d**) *RAP-011 effects on ActIIa ligands expression*. Thalassemic or wild-type erythroblasts were either cultured in the presence of RAP-011 (10 μg/ml) or PBS for 48h. mRNA levels for activin A (*Inha*), activin B (*Inhb*), GDF11 and ActRIIa (*Acvr2a*) evaluated by qPCR. (**e-g**) *RAP-011 traps GDF11.* (**e**) Immunoblotting analysis of bone marrow from thalassemic mice treated with PBS and RAP-011 show a significant decrease of GDF11 protein levels. Recombinant protein was used as a positive control. (**f**) Immunohistochemical analysis of GDF11 pro-peptide and mature form in bone marrow from wild-type and thalassemic mice. (**g**) Intracellular flow cytometric analysis of splenic erythroblasts treated with 10 μg/ml RAP-011 or PBS for 48 hours and stained using specific antibodies against activin A, activin B and GDF11 (proform and mature). Quantification of the staining is shown in the bar graph and expressed as mean fluorescence intensity (MFI). Right panel demonstrates a representative FACS histogram where gray lines indicate isotype antibody control, black and red represent PBS or RAP-011 treated erythroblasts (respectively). Data represent means ± S.E.M. All data are expressed as the mean ± S.E.M.*P<0.05, ns=not significant; N=5 per group for each independent experiment.

Supplemental figure 5: *GDF11 expression of thalassemic mice and thalassemia subjects.* (**a**) Confocal micrograph showing a representative section of spleen white pulp (WP) and red pulp (RP) from $Hbb^{th1/th1}$ mice. GDF11 protein expression is shown in green, CD71 in red and nuclei (DAPI) are shown in blue. (**b**) *GDF11 in overexpressed in GPA^+ cells from thalassemic subjects.* Confocal micrograph showing a representative cytospin of cultured $CD36^+$ erythroblasts from thalassemic subjects and cord blood-derived erythroblasts from healthy donors. GDF11 protein expression is shown in green. GPA^+ erythroblasts are marked in red and nuclei (DAPI) are shown in blue. **(c,d**) Flow cytometric quantification of Fas and FasL-positive $CD71^-Ter\text{-}119^-$ populations harvested from (**c**) spleen or (**d**) bone marrow of $Hbb^{th1/th1}$ mice treated twice weekly with 10 mg/kg RAP-011 or PBS. (**e**) *GDF11 inactivation promotes spontaneous apoptosis of erythroblasts.* Cultured erythroblasts from thalassemic mice were transfected with siRNA against a scrambled control (gray), *Inhb* (blue) or *Gdf11* (red). The histograms represent annexin V binding. (**f-h**) *Recombinant GDF11 activates the Smad2/3 but not the Smad1/5 pathway*. (**f**) Growing doses of GDF11 (0-100 ng/ml) induces Smad2/3 signaling in C2C12 cells as demonstrated by CAGA-Luc/GFP but not Smad 1/5 signaling as demonstrated by BRE-Luc/GFP reporter vectors. BMP2 (20 ng/ml) and TGF-β1 (5 ng/ml) were used as positive controls. **(g)** The Alk4/Alk5 inhibitors SB505124 and SB431542 abrogate the signaling induced by GDF11 in C2C12 cells. (**h**) RAP-011 blocks GDF11 and GDF8 Smad2/3 signaling. CAGA-Luc/GFP C2C12 cells were treated with GDF11 (100 ng/ml), GDF8 (100 ng/ml) or vehicle in the presence of RAP-011 (10 μg/ml) or specific neutralizing antibodies (1 μg/ml). Luciferase activity was normalized to GFP fluorescence; values are expressed as relative luminescence units per relative fluorescence units (RLU/RFU). All data are expressed as the mean ± S.E.M. *P<0.05, **P<0.01, ns=not significant; N=3 per group for each of three independent experiments performed.

Supplemental figure 6: (**a-b**) *GDF11 neutralization induces terminal maturation of erythroblasts* (**a**) Erythroblast cultures derived from the spleens of thalassemic mice were treated with neutralizing

antibodies against GDF11 or GDF8 for 48h. Erythroblast differentiation was evaluated by flow cytometry through CD71/Ter-119 staining and FSC distribution into immature (Ter-119^{+}/CD71^{+}) and mature (Ter-119^{+}/CD71^{-}) erythroblasts. The percentages of different erythroblast populations (immature/mature) are shown. (**b**) Morphology of erythroblasts treated with neutralizing antibodies against GDF11 or GDF8 for 48h. (**c-d**) *Recombinant GDF11 induces Smad signaling in thalassemic but not on wild-type erythroblasts.* (**c**) spleen and bone marrow-derived erythroblast culture from wild-type and Hbb$^{th1/th1}$ mice under increasing concentrations of recombinant GDF11 (0-500 ng/ml) were assayed for Smad2 phosphorylation (red) and CD71 (blue) staining by confocal immunofluorescence. (**d**) Western blot analysis on bone marrow-derived erythroblasts.

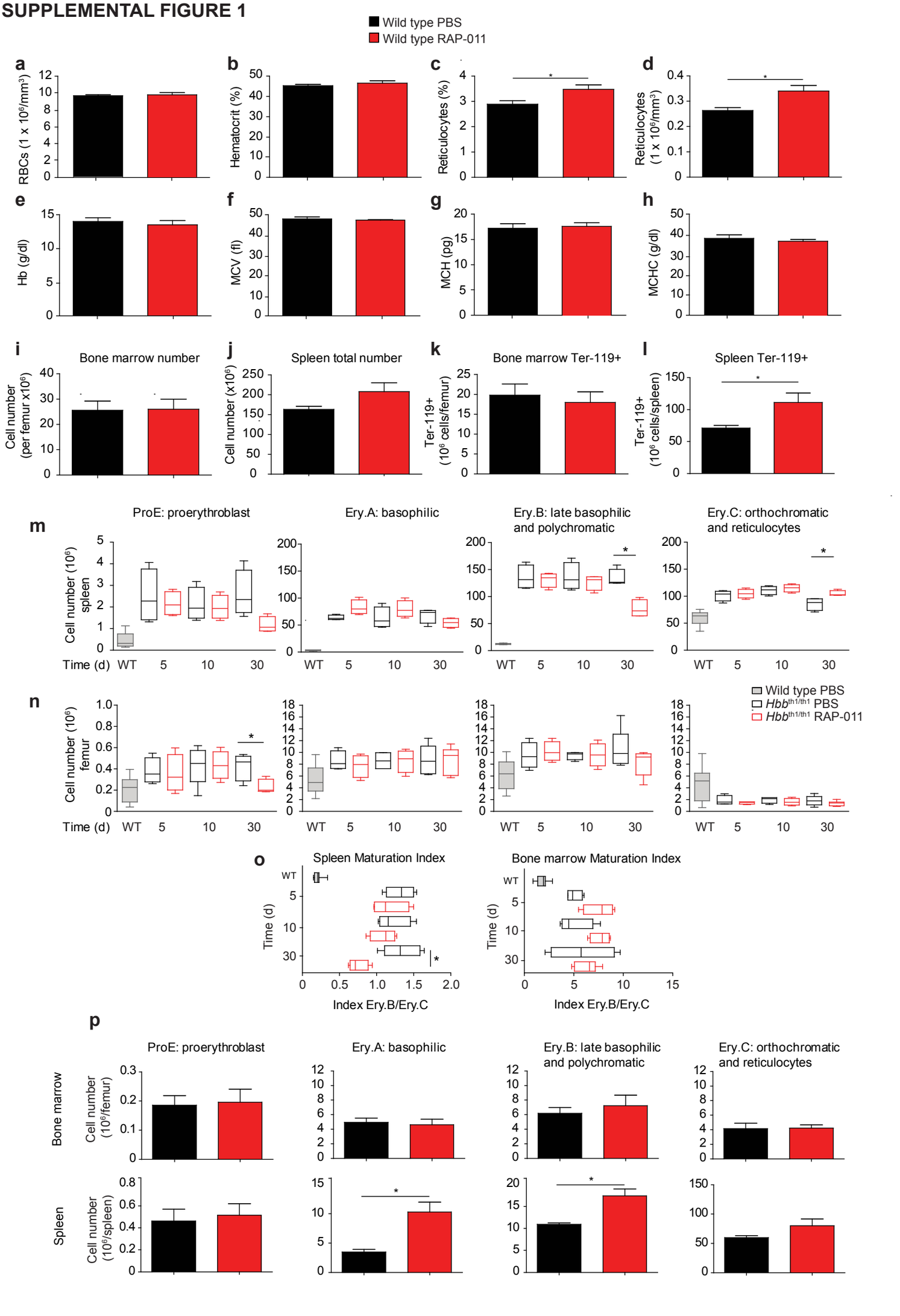
Supplemental figure 7: (**a-b**) *Recombinant GDF11 promotes thalassemic erythroblasts terminal maturation arrest.* (**a**) Erythroblast differentiation was evaluated in samples treated with recombinant GDF11 (0-500 ng/ml) for 7 days by flow cytometry through CD71/Ter-119 staining into immature (Ter-119^{+}/CD71^{+}) and mature (Ter-119^{+}/CD71^{-}) erythroblasts. (**d**) Percentages of mature and immature erythroblast populations are shown. (**c,d**) *Effect of recombinant GDF11 on cell proliferation and survival.* Spleen and bone marrow-derived erythroblast culture from wild-type and Hbb$^{th1/th1}$ mice under increasing concentrations of recombinant GDF11 (0-500 ng/ml) were assayed for (**c**) ATP content by a luminescence-based assay and (d) cell counts. All data are expressed as the mean ± S.E.M.*P<0.05, ns=not significant; N=5 per group for each independent experiment.

Supplemental figure 8: *A model illustrating the proposed role of ActRIIa signaling in ineffective erythropoiesis of thalassemia*. Increased GDF11 levels induce Smad2 activation leading to (i) early erythroblasts accumulation through the impairment of Fas/Fas-L expression and to (ii) terminal maturation arrest by promoting oxidative stress (left side of figure). RAP-011, an ActRIIa trap, sequesters GDF11 (and potentially other ActRIIa ligands) impairing ActRIIa activation and Smad2/3

phosphorylation. This results in reduced α-globin aggregates/oxidative stress therefore promoting terminal erythroid maturation and in activation of Fas/Fas-L pathway resulting in apoptosis of early erythroblasts (right side of figure). Under these conditions, reversion of ineffective erythropoiesis results in improvement of anemia of thalassemia.

Nature Medicine: doi:10.1038/nm.3468

SUPPLEMENTAL FIGURE 1



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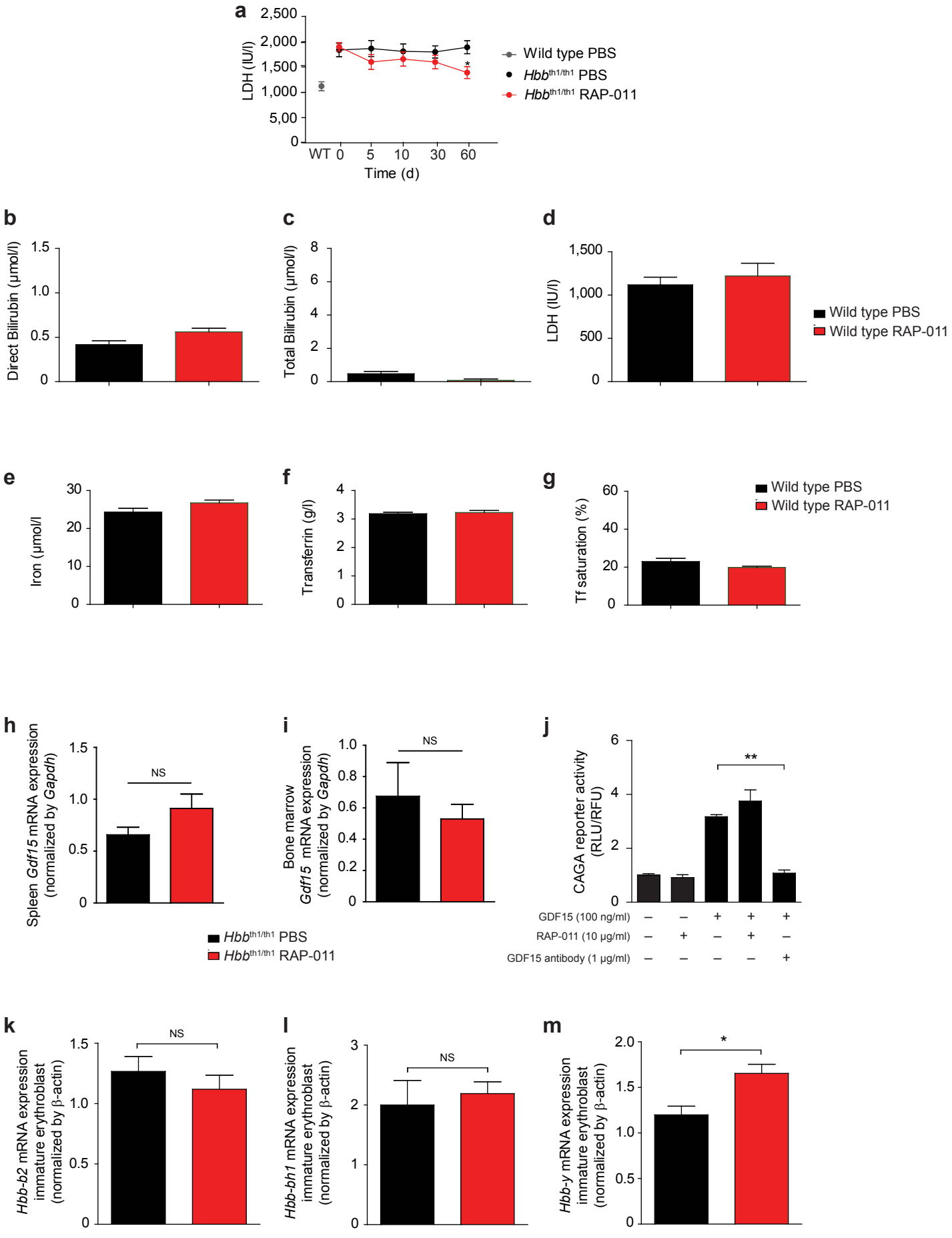
SUPPLEMENTAL FIGURE 2



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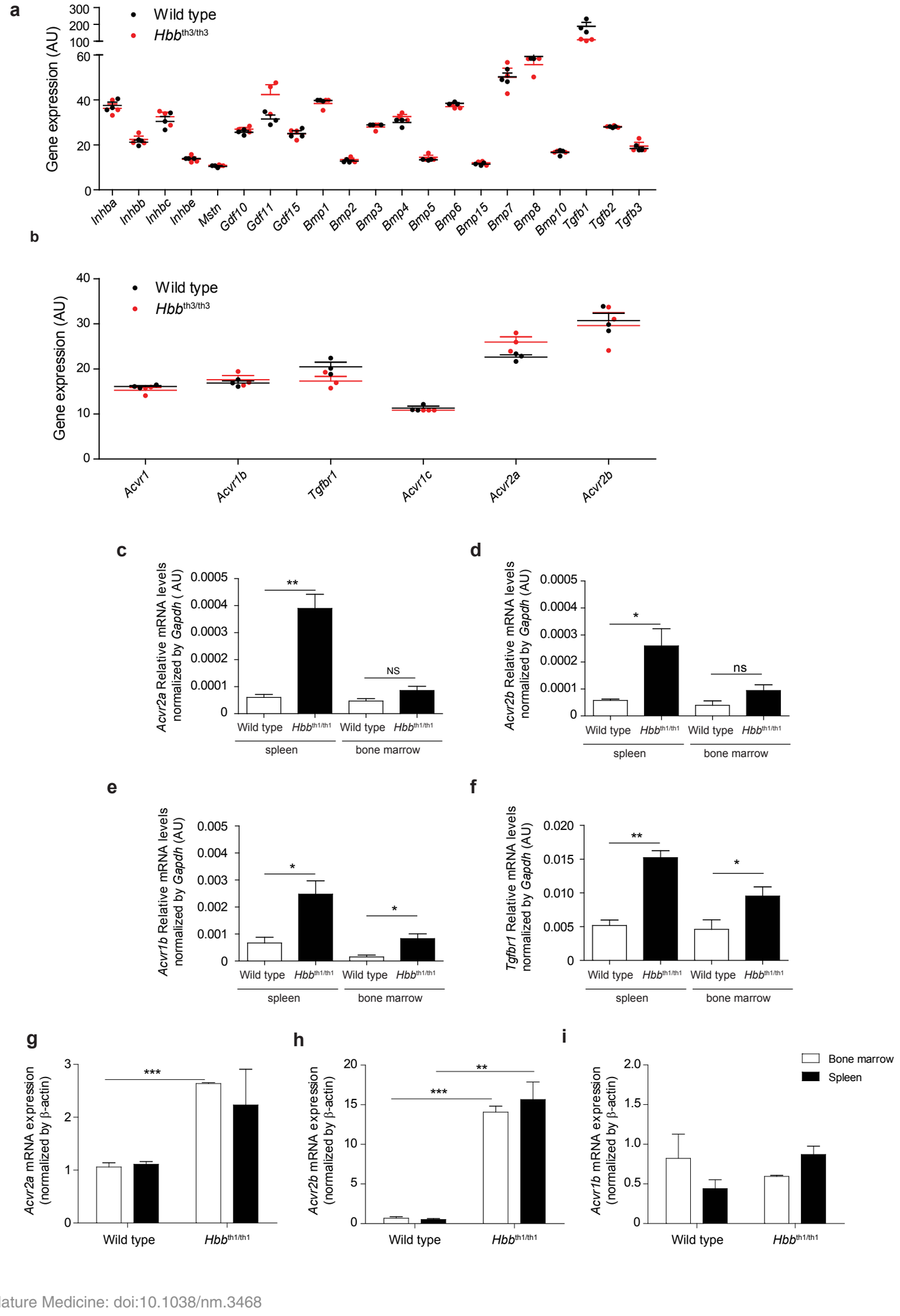

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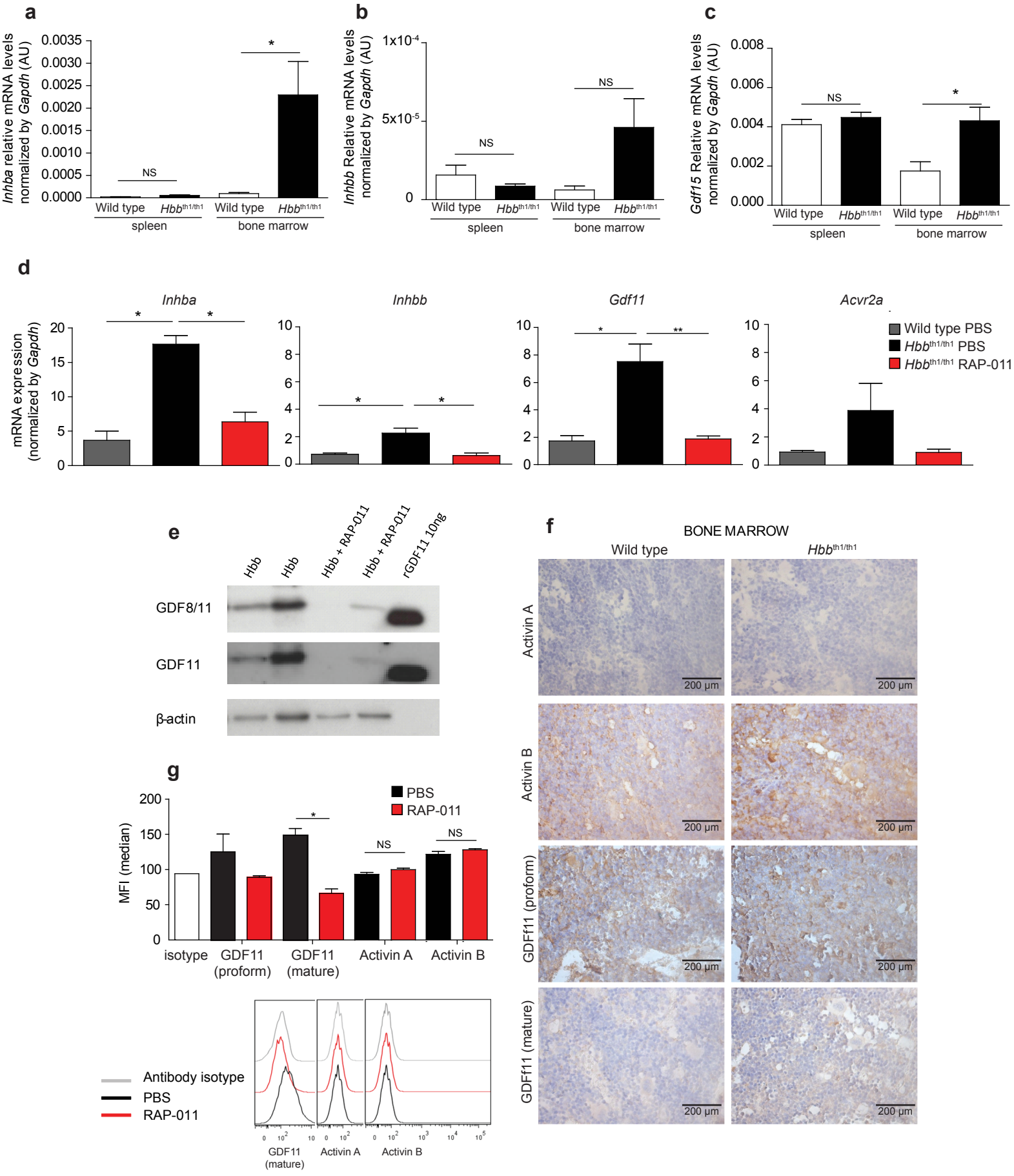

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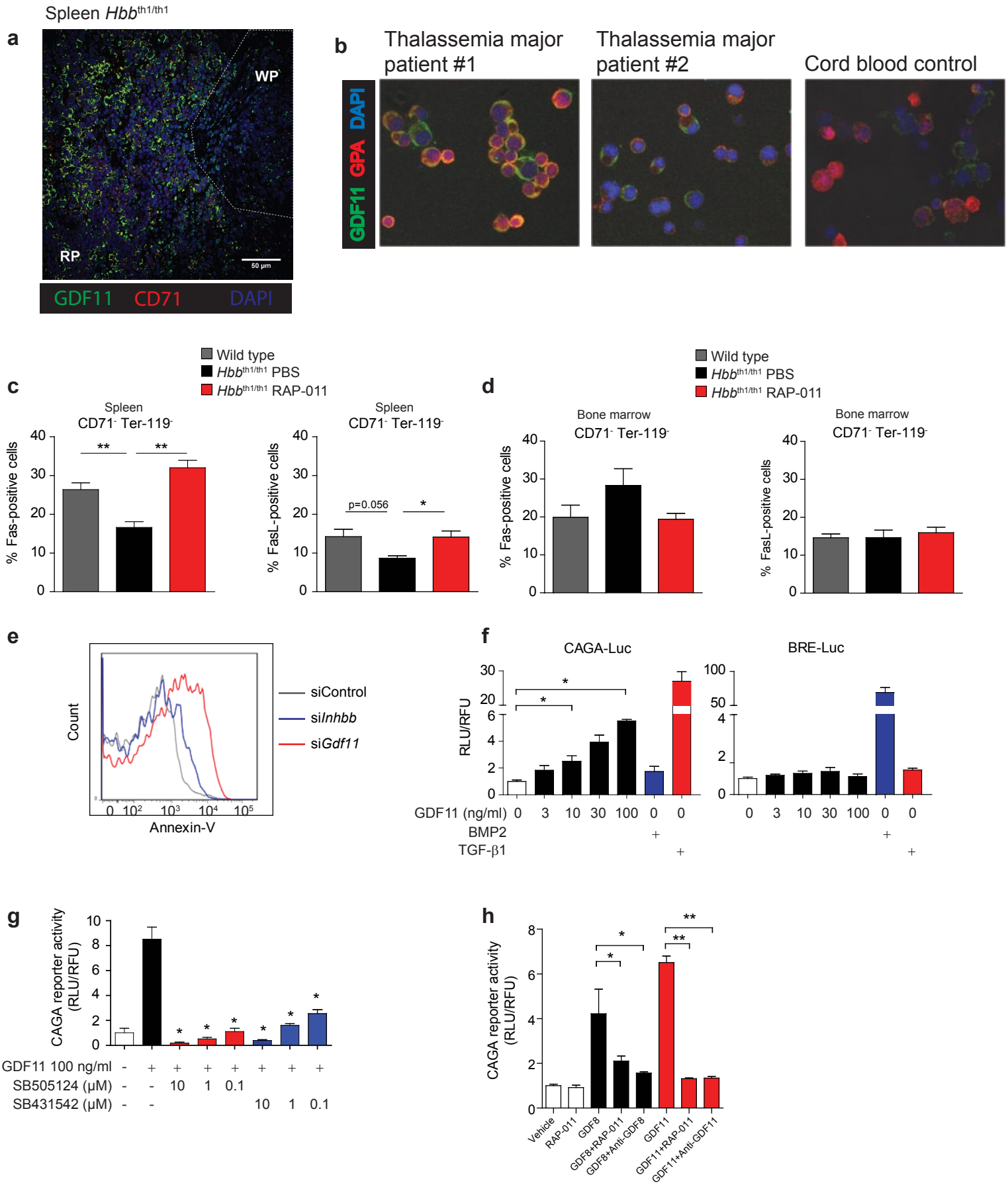

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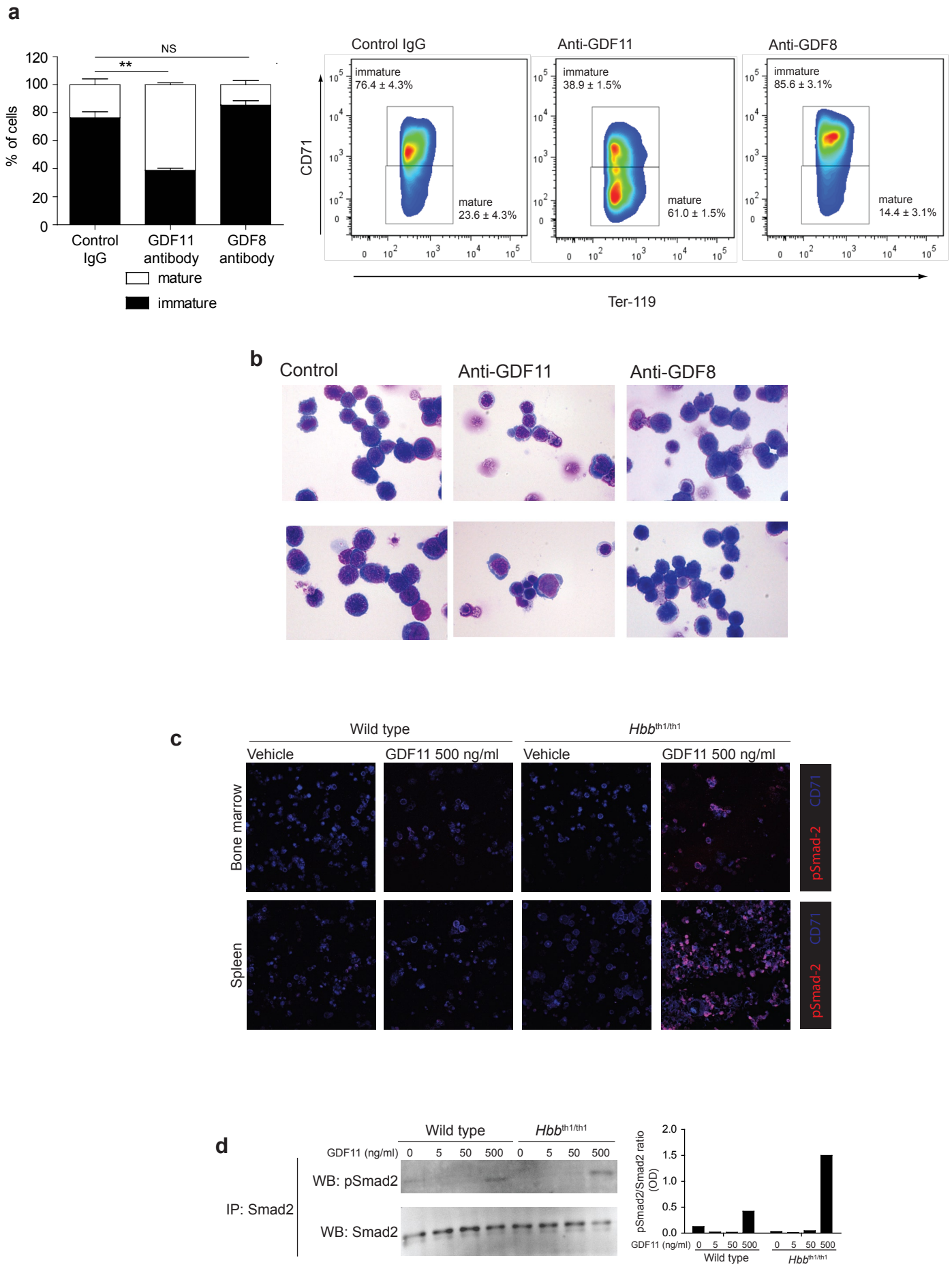

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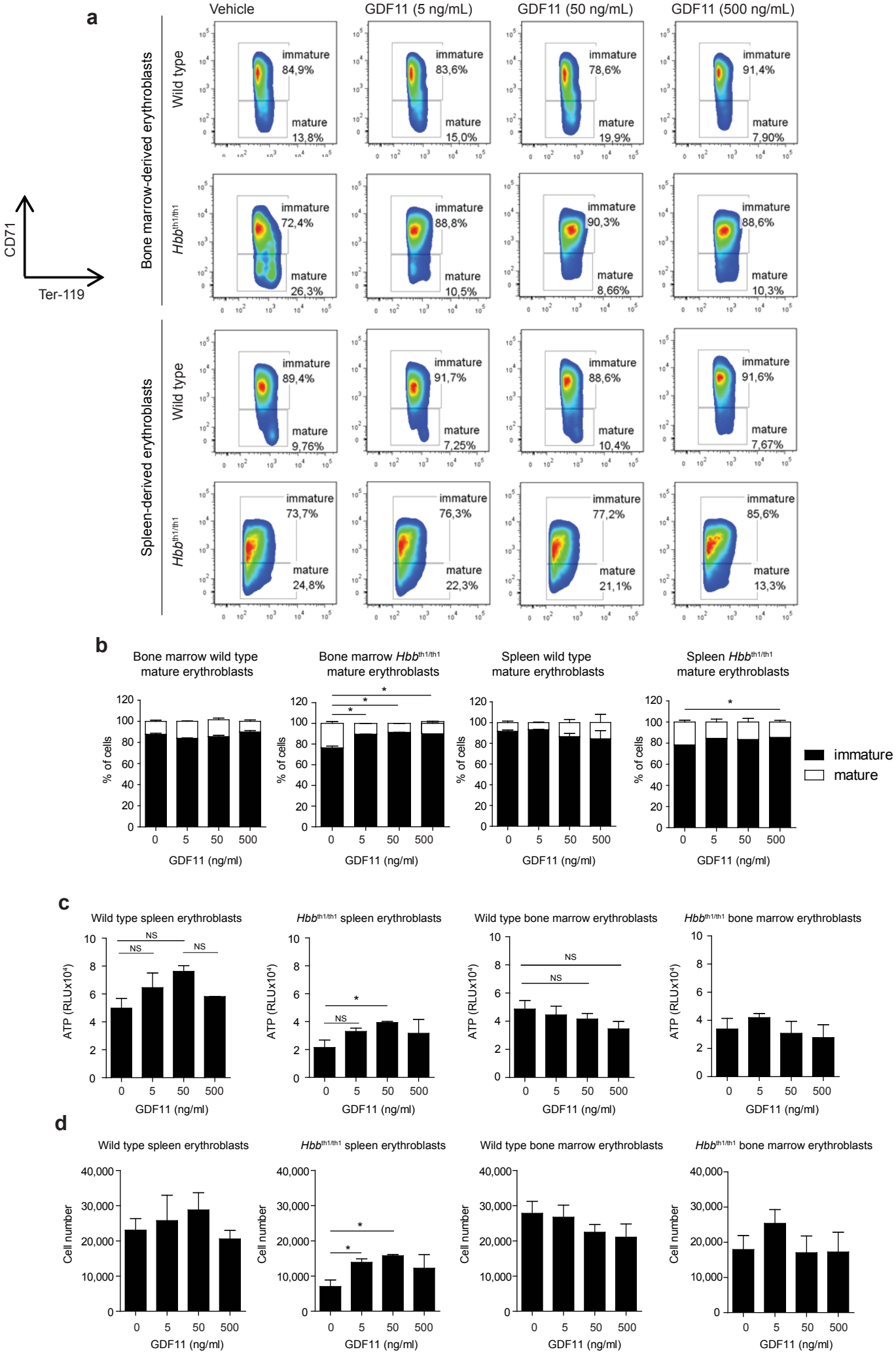

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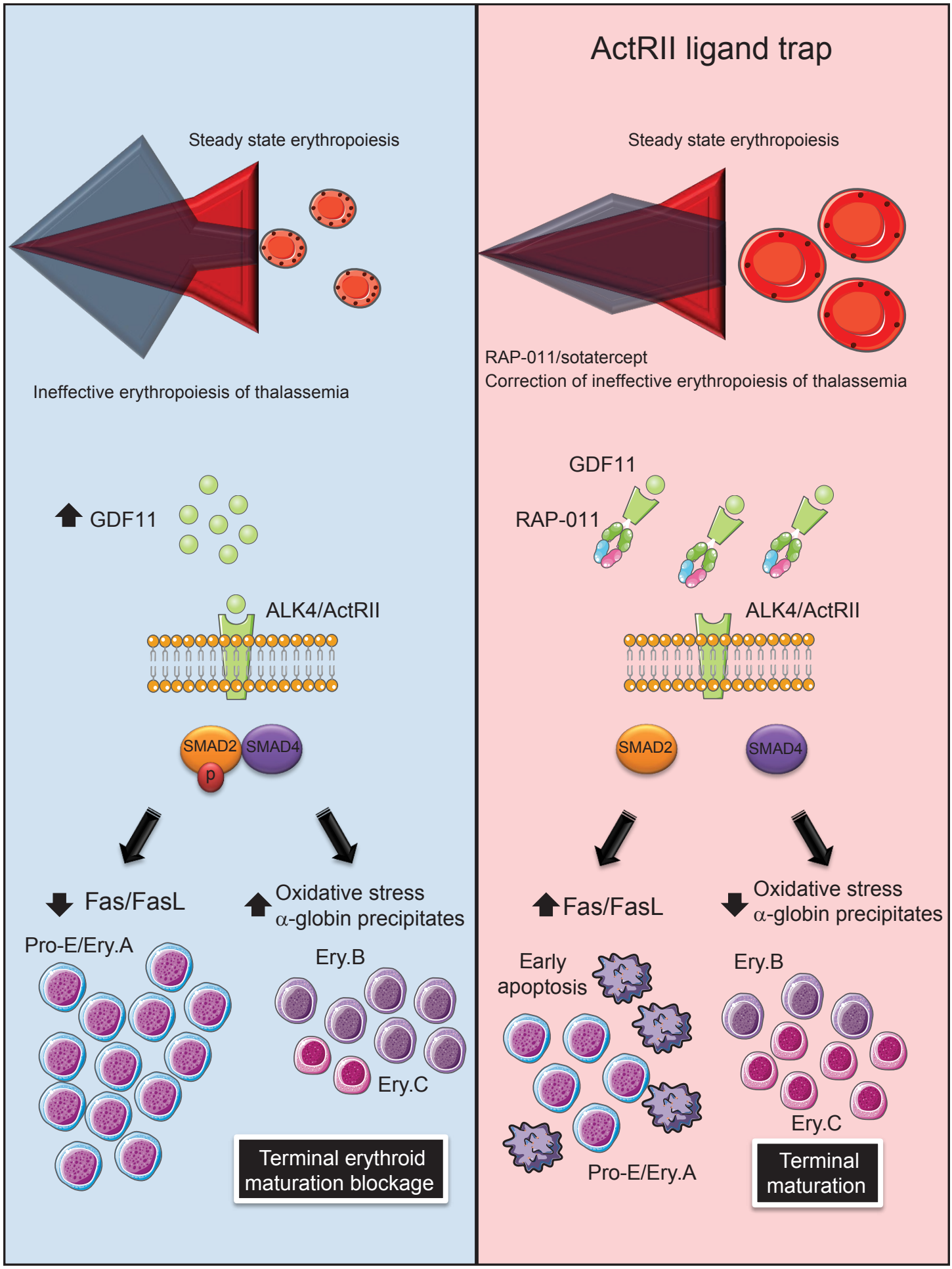
SUPPLEMENTAL FIGURE 8



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