XPO1 regulates erythroid differentiation and is a new target for the treatment of β-thalassemia

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Supplemental Materials

Supplemental Figures

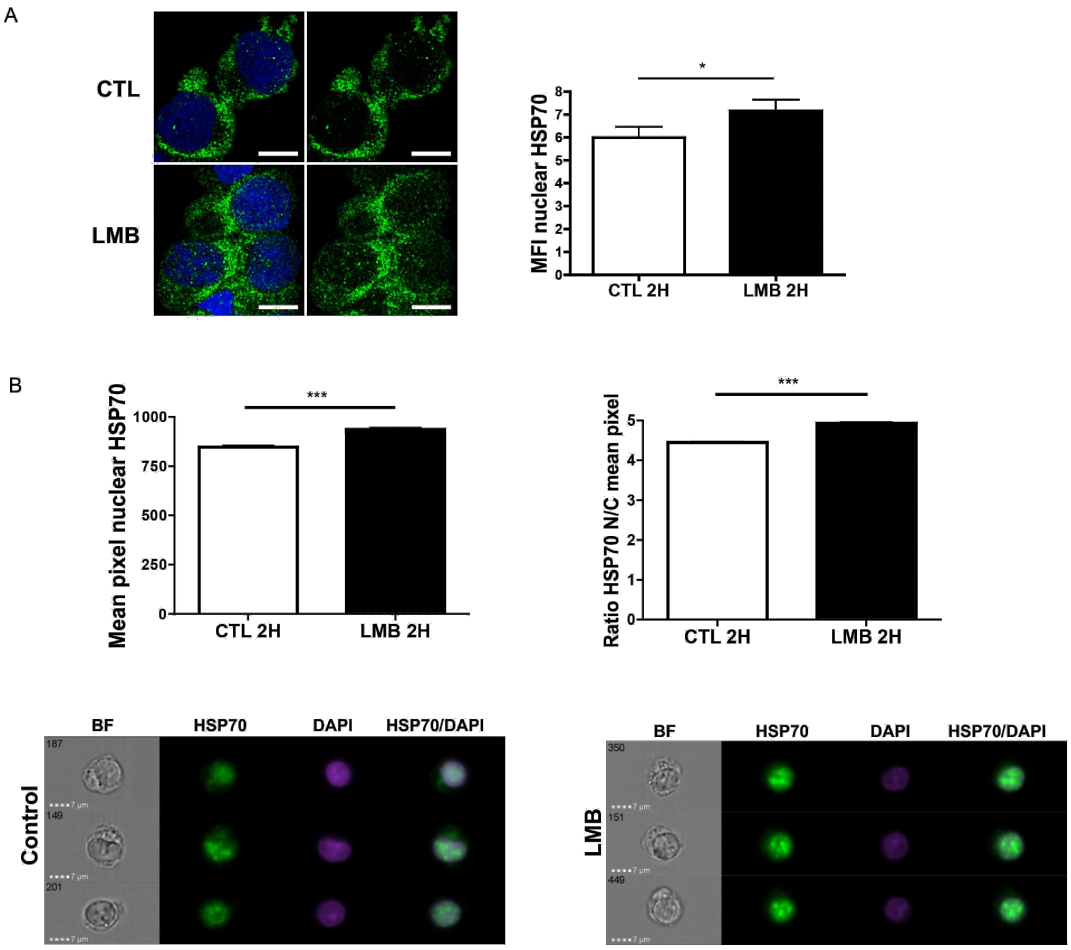

Figure S1. LMB treatment induces an increase in HSP70 nuclear expression in human erythroid progenitors. Erythroid progenitors from cord blood at day 3 of $CD36^{+}$ culture were treated for 2 hours with 20nM Leptomycin B (LMB), an inhibitor of nuclear export, or with ethanol (control). HSP70 nuclear expression was analyzed by confocal microscopy and by ImageStream. **(A) Left:** Representative confocal microscopy analysis of HSP70 expression (x63 oil objective, scale bar= 10µm). **Right:** Quantification of HSP70 nuclear expression (MFI) normalized with volume. Data are presented in histograms as mean ± SEM for 30 cells from one experiment (MFI nuclear HSP70 5.985±0.47 (CTL 2H) versus 7.156±0.48 (LMB 2H), $P<0.05$). *P* values were determined by paired T-test, $*P<0.05$. **(B)** These data were confirmed by ImageStream technology from two independent experiments, using two different anti-HSP70 antibodies. **Upper panel:** quantification of HSP70 nuclear expression

normalized with volume (mean pixel nuclear HSP70 846.5±4.97 (CTL 2H) versus 937.2±5.11 (LMB 2H), $P<0.0001$) and HSP70 N/C ratio (mean pixel HSP70 N/C ratio 4.45±0.008 (CTL 2H) versus 4.93±0.009 (LMB 2H), $P<0.0001$). Data are presented in histograms, as mean ± SEM. *P* values were determined by paired T-test: *** $P<0.0001$. **Lower panel**: three representative ImageStream images with brigthfield, HSP70 in green, DAPI in purple and merge (scale bar=7μm).

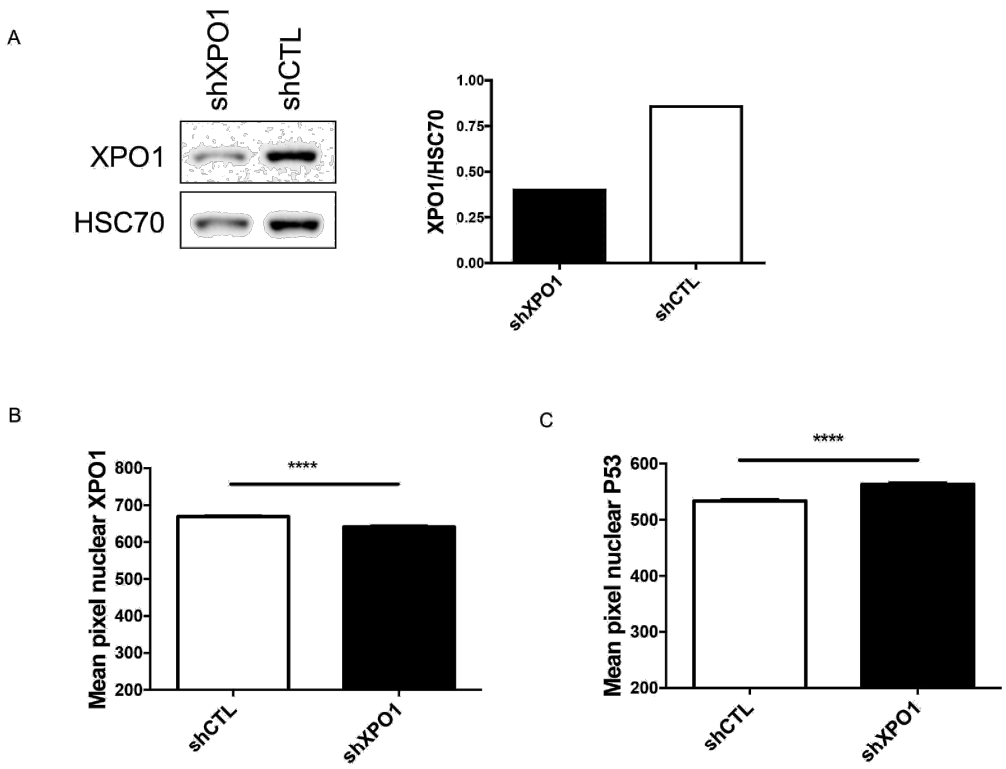

Figure S2. Analyze of shRNA XPO1 repression by immunoblot and ImageStream and efficiency in regulating nuclear export of the well-known XPO1 target P53. Erythroid progenitors derived from β-TM erythroblasts, analyzed two days after transduction with a shRNA specific to XPO1 or shCTL (day 4 of $CD36^+$ cell culture)**. (A)** XPO1 immunoblot from total fractions (30µg) of erythroid progenitors. HSC70 was used as loading control. Graph shows optical relative quantity values of XPO1 protein normalized to that of HSC70 from one experiment (XPO1/HSC70 quantity 0.398 (shXPO1) versus 0.858 (shCTL)), data are presented in histograms. (**B)** XPO1 expression normalized to volume (mean pixel) analyzed by ImageStream (mean pixel nuclear XPO1 669.3±1.964 (shCTL) versus 641.4±2.153 (shXPO1), P<0.0001). (**C**) P53 nuclear expression normalized to volume (mean pixel) analyzed by ImageStream (mean pixel nuclear P53 533,4±2,372 (shCTL) versus 563,0±2,684 (shXPO1), P<0.0001). Data are presented in histograms as mean ± SEM, P values were determined by unpaired T-test: **** P<0.0001. Data are representative of three independent experiments with 2 different shRNA XPO1.

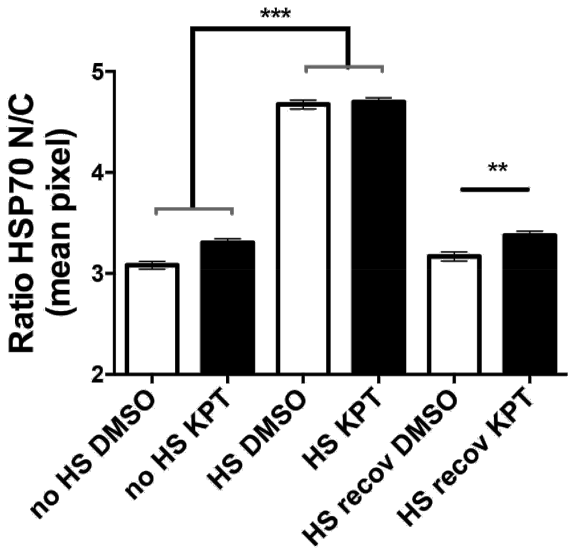

Figure S3. XPO1 regulates HSP70 nuclear export in HeLa cells.

HeLa cells were pre-treated with KPT-251 at 1000nM or DMSO (control) for 24 hours. Cells were stained with anti-HSP70 antibody, DAPI and goat anti-mouse alexa fluor 488 secondary antibody. HSP70 nucleus/cytoplasm (N/C) ratio expression normalized to volume (mean pixel) was analyzed by ImageStream in HeLa cells before heat shock at 43°c (no HS), after heat shock (HS), and after 6 hours of heat shock recovery at 37°c (HS recov). Heat shock induces HSP70 nuclear increase (N/C ratio HSP70 mean pixel 3,081±0,037 (no HS DMSO) and 3,305±0,038 (no HS KPT) compared to 4,673±0,044 (HS DMSO) and 4,698±0,042 (HS KPT) P<0,001). After 6 hours of recovery at 37°c, HSP70 exits the nucleus and this mechanism is delayed when cells have been pre-treated with KPT-251 XPO1 inhibitor (N/C ratio HSP70 mean pixel 3,168±0,045 (HS recov DMSO) versus 3,376±0,044 (HS recov KPT), P<0,01). Data are presented in histograms as mean ± SEM, P values are determined by ANOVA Dunn's multiple comparison test ***P<0.001, **P<0.01. Data are representative of two independent experiments.

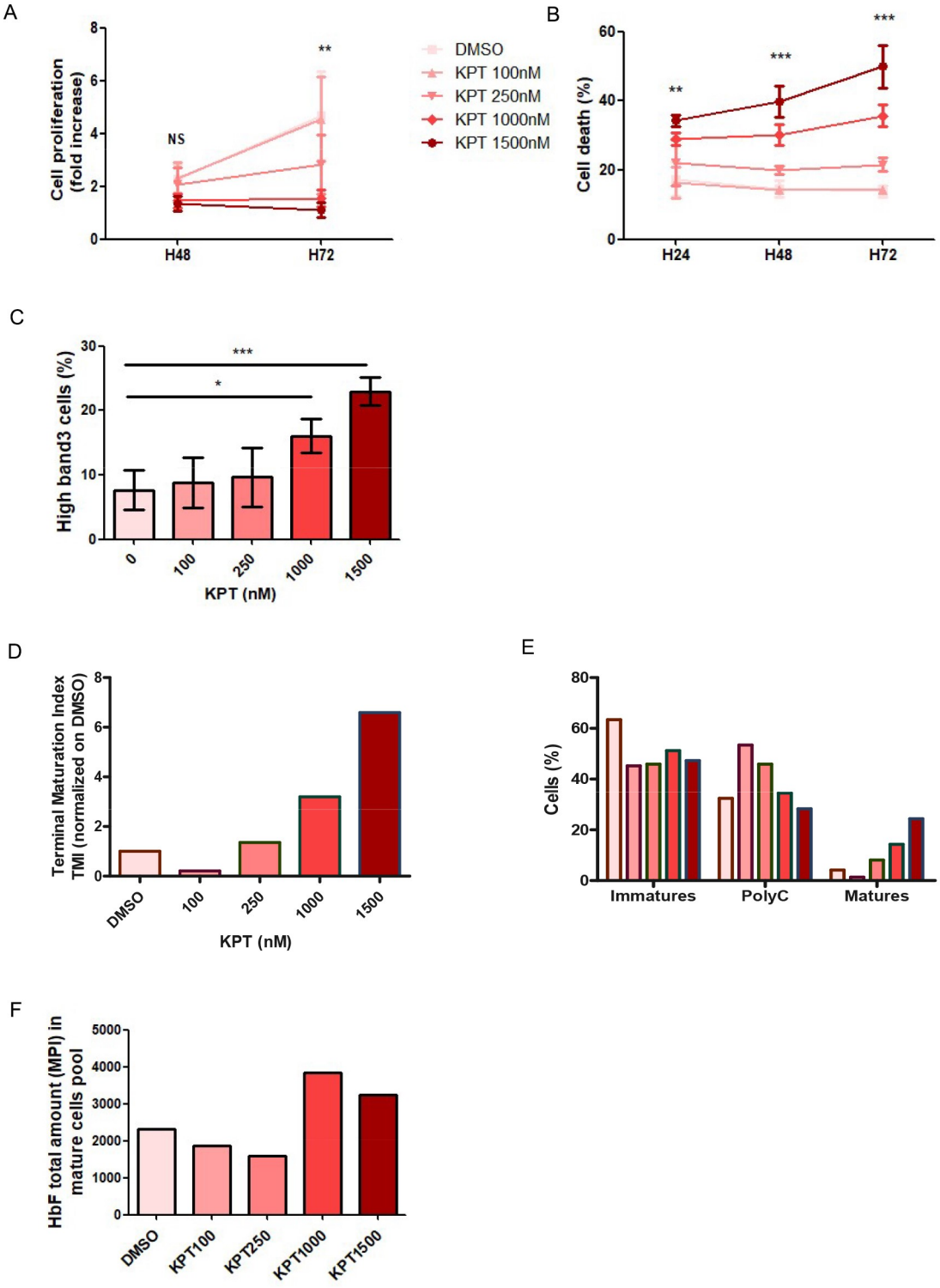
A
Cell proliferation (fold increase)
NS
**
H48
H72
DMSO
KPT 100nM
KPT 250nM
KPT 1000nM
KPT 1500nM
B
Cell death (%)
**
***
***
H24
H48
H72
C
High band3 cells (%)
***
*
0
100
250
1000
1500
KPT (nM)
D
Terminal Maturation Index TMI (normalized on DMSO)
DMSO
100
250
1000
1500
KPT (nM)
E
Cells (%)
Immatures
PolyC
Matures
F
HbF total amount (MPI) in mature cells pool
DMSO
KPT100
KPT250
KPT1000
KPT1500

Figure S4. KPT treatment induces an increase in HbF amount in the pool of β-TM mature cells from the threshold dose of 1000nM.

Erythroblasts derived from β-TM peripheral blood cells were treated at day 4 of CD36+ cell culture with 100nM, 250nM, 1000nM, or 1500nM of KPT-251, or with DMSO (control) for 72 hours.

(A, B) Cell proliferation and cell death curves analysis of β-TM erythroid progenitors, assessed by blue trypan staining respectively at 48, 72 hours and 24, 48, 72 hours of treatment conditions. Data are presented as mean ± SD. P values are determined by ANOVA Dunnett's multiple comparison test **P<0.01***P<0.0001, NS= not significant, n=3 independent experiments (3 different patients). **(C)** Percentage of high Band3 cell population under the different treatment conditions, analyzed by flow cytometry, after 72 hours of treatment. Data are presented as mean percentage ± SD (n=3 independent experiments). P values are determined by ANOVA Dunnett's multiple comparison test, *P<0.05, ***P<0.0001.

(D, E) Terminal maturation index and proportions (%) of immature, polychromatophilic (PolyC), and mature (orthochromatic erythroblasts + reticulocytes) cells after 72 hours of treatment (n=1 experiment). **(F).** HbF total amount (MPI) in the pool of mature cells (High Band3 population) was calculated by multiplying the mean concentration of HbF in mature cells (determined by ImageStream HbF Mean Pixel Intensity (MPI) in gated high band3 cells) by the absolute number of mature cells (n=1 experiment).

Supplemental Material and methods

BioLayer interferometry

Protein-protein interaction experiments were conducted by BioLayer Interferometry (BLI) with an OctetRed instrument (ForteBio, Menlo Park, CA, USA). The wild type HSP70, mutant S400A-HSP70 and XPO1-GST were produced with a rabbit reticulocyte lysate according to the manufacturer's protocol (Abnova). The ligand (XPO1-GST) was immobilised onto anti-GST biosensors (FortéBio, Menlo Park, CA, USA) at a concentration of 100nM and dipped into black Greiner 96-well microplates agitated at 1,000 rpm and filled with a solution of 200 µL containing the analyte (HSP70) at 25°C. Sensorgrams were corrected for baseline drift by subtracting a control sensor exposed to running buffer only (ForteBio, Data analysis software version 7.1.).

Protein sequence analysis

The Nuclear Export Sequence (NES) in HSP70 protein sequence was identified with two software packages: NES Finder 0.2 http://research.nki.nl/fornerodlab/NES-Finder.htm and NetNES 1.1 Server http://www.cbs.dtu.dk/services/NetNES/. NCBI Reference Sequence for *Homo sapiens* proteins HSP70 (HSPA1A): NP_005336.3

Viral transduction:

Lentiviral production

Lentivirus production was performed as previously described for pLKO.1-CMV-tGFP lentiviral vector shRNA specific for XPO1 NM_003400, clone TRCN0000154386, clone TRCN0000150975 and shRNA scrambled (shCTL): pLKO.1-puro-CMV-GFP non target shRNA (Sigma Aldrich)[3]. Three clones of shXPO1 lentiviral vectors were tested and the clone with the highest degree of XPO1 repression was selected for the experiments.

Infection of erythroid cells

For shXPO1 and shCTL transduction, $CD34^+$ cells isolated from β-TM peripheral blood mononuclear cells were cultured for 7 days in $CD34^+$ media and then cultured for 2 days in CD36+ media in the presence of EPO, as described above. They were then infected by lentiviruses. After 24 hours, cells were extensively washed in PBS and cultured in $CD36^+$ media for another 24 hours. GFP^+ cells were then purified by flow cytometry cell sorting and labeled with anti HSP70, anti-XPO1 and DAPI for ImageStream analysis, or lysed for protein extraction and immunoblot.

Confocal analysis:

The cells were washed, spun onto slides, and fixed with acetone. Next the cells were permeabilized with methanol for 10min at -20°c, washed with 0,05% tween 1X TBS (TTBS) for 5min and incubated in 10% BSA TTBS for 1 hour. They were then incubated with the primary antibodies overnight at 4°c: rabbit anti-GATA-1, mouse anti-HSP70, or rabbit anti-CRM1 (XPO1) and then incubated with secondary antibodies for 2 hours at 37°c: anti-mouse alexafluor 488 or alexafluor 568 (Life technologies), and anti-rabbit alexafluor 647 (Jackson Immunoresearch). All antibodies were diluted in 3% BSA TTBS. Nuclei were stained with DAPI, and the slides were examined with a confocal microscope (LSM 700 Carl Zeiss). Image analysis and figures were performed using ImageJ software (v1.49v) and FigureJ plugin (v1.10b). For each independent experiment, 6-8 fields were collected per slide. For each field, DAPI-positive staining was used to define the nuclear region of interest (ROI), and HSP70 to define the cytoplasmic ROI. Bright field images were also used to demarcate each cellular compartment and detect viable cells. Each of these ROI masks was then applied, by the analysis software, to obtain quantitative MFI data. On average, 25 cells were analyzed per experiment.

Duolink assay

To analyze the HSP70-XPO1 interaction, we used the Duolink II technology (Olink Bioscience), which is an in-situ proximity ligation assay technology. Slides were incubated with primary antibodies (mouse anti-HSP70, or mouse anti-GATA-1 as negative control (Enogene E10-30047), with rabbit anti-XPO1) and with secondary antibodies conjugated with oligonucleotides (PLA probe Minus anti-rabbit And PLA probe PLUS anti-mouse). Ligation and amplification reactions were performed according to manufacturer's instructions.

Imaging flow cytometry (ImageStream)

$CD36^+$ culture cells from cord blood or β-TM patient peripheral blood ($1x10^6$) were fixed and permeabilized using the transcription factor Staining Buffer Set from Ebioscience (Affymetrix Santa Clara, CA, USA). Staining was performed using primary antibodies against HSP70 (3A3 sc-32239 mouse Santa Cruz, and ADI-SPA-812 rabbit Enzo life technologies), GATA-1 N-terminal (ab173816 rabbit Abcam) or XPO1 (CRM1 H-300 sc-5595 rabbit Santa Cruz) for 1 hour at 4°c. Goat anti-mouse alexafluor 488, donkey anti-mouse alexafluor 568 and goat anti-rabbit alexafluor 647 antibodies were used for secondary staining for 1 hour at 4°c. For HbF staining, $CD36^+$ cells were fixed with 0.05% cold glutaraldehyde, permeabilized with 0.1% Triton X-100 and then stained with PE anti-HbF (clone 2D12, BD Biosciences) and APC anti-Band3 (clone REA368, Miltenyi Biotec) antibodies for 30 min. After washing, cells were stained with DAPI in a total volume of 50µl and image acquisition was performed. Samples were run on an ImageStream ISX mkII (Amnis Corp, Millipore, Seattle, WA) and a 60x magnification was used for all acquisitions. Data were acquired using the INSPIRE software (Amnis Corp) and analyzed using the IDEAS^{TM} software (version 6.2 Amnis Corp). On average, 30,000-50,000 events were collected in all experiments. Single stain controls were run for each fluorochrome used and spectral compensation was performed. Cells were

gated for single cell using the area and aspect ratio of the brightfield image, gated for focused cells using the gradient RMS feature and viable cells were selected on the positive expression of DAPI. HbF was then quantified in Band3^{high} subset. Nuclear and cytoplasmic masks were created to study localization and expression of HSP70, GATA-1 and XPO1. Results were expressed as mean pixel intensity value, which is the intensity normalized to surface area. The nuclear translocation of HSP70 was calculated using the 'Similarity' feature, (log transformed Pearson's Correlation Coefficient between the pixel values of two image pairs), which provides a measure of the degree of co-localization of two stains (HSP70 and DAPI in this case). High similarity scores reflect a positive correlation between the images (corresponding to a predominant nuclear distribution of HSP70). Low similarity scores reflect no correlation between the images (corresponding with a predominant cytoplasmic distribution of HSP70).

Co Immunoprecipitation experiments

Human CD36+ erythroid progenitors at day 4 of culture, or UT7 cell lines, where washed three times with PBS at 4°c, then lysed in buffer: EDTA 1mM, NaCl150mM, Tris HCl 25mM, glycerol 10%. Antiproteases, antiphosphatases and detergent (final concentration 0.1% NP40) were added extemporaneously. Lysate was centrifuged at 20 000 G for 15min at 4°c to remove debris. Preclearing was performed: 15µL of Protein G Sepharose 4 Fast Flow beads (Sigma Aldrich) were added to the lysate and incubated for 1h at 4°c under rotating wheel. After centrifugation (1min at 2000G), supernatant was divided in two conditions for incubation with 2µG of antibodies: either mouse anti-human HSP70 antibody W27 (Santa Cruz sc-24) or mouse IgG control, for 1h at 4°c under rotating wheel. Then, lysates were respectively incubated with 15µL of Protein G Sepharose for 90min at 4°c under rotating wheel. Beads were washed 3 times with PBS at 4°c. Protein complexes were eluted by adding

4X Laemmli buffer followed by heating at 95°c for 5 min. Beta-mercaptoethanol was added (1/10dilution) and the whole eluate was reheated for 1min and centrifugated.

Cell fractionation and Immunoblot

Total protein fractions were extracted from erythroid progenitors using laemmli buffer without 2-mercaptoethanol and bromophenol blue. Separate cytoplasmic and nuclear protein fractions were extracted from erythroid progenitors using NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific), following manufacturer's protocol. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo scientific), protein extracts were reduced by addition of 4X complete laemmli (with 2-mercaptoethanol and bromophenol blue) and by heating for 5min at 95°C. Proteins were resolved on 14% acrylamide gel and analyzed by immunoblotting. Antibodies used included rabbit anti-CRM1 (Calbiochem ST1100), rat anti-GATA-1 (clone N1 sc-266 Santa Cruz Biotechnology) , rabbit anti-HSP70 (SPA-812 Stressgen Biotechnologies), mouse anti-HSP90 (SPA-830 Stressgen Biotechnologies), mouse anti-HDAC2 (3F3, Millipore), rat anti-HSC70 (1B5, Stressgen Biotechnologies).

Heat shock treatment and recovery on HeLa cell line pretreated with KPT-251

We pretreated HeLa cells 24 hours with KPT-251 at 1000nM or with DMSO (control). For heat shock, cells were incubated 1 hour at 43°c, in media pre-warmed at 43°c. For recovery after heat shock, media of heat shock was replaced by media pre-warmed at 37°c and cells were incubated at 37°c for 6 hours. Cells were then washed with PBS 1X at 4°c, scratched, and stained with anti-HSP70 antibody, DAPI and goat anti-mouse alexafluor 488 secondary antibody. We then analyzed HSP70 nucleo-cytoplasmic concentrations (mean pixel HSP70)

by ImageStream for the conditions before heat shock, just after heat shock, and after the 6 hours recovery phase at 37°c.

GATA-1 ChIP-seq analysis

GATA-1 ChIP-seq data was from Pinello et al [14]: ChIP-seq analyses of GATA-1, performed using chromatin prepared from primary human pro erythroblasts. Following antibodies were used: GATA-1 (ab11852; Abcam) and rabbit IgG (12-370; Millipore). Data were extracted and analyzed using Galaxy web-based genome analysis plateform. Raw ChIP-seqsequence reads (GATA-1 and control) were initially processed by FASTQGROOMER for conversion to GALAXY format, then by FASTQC for quality control. Reads were then aligned to the Feb 2009 reference human genome assembly (GRCh37/hg19) by using Bowtie2 with the « full parameter list » setting. Using SAMTOOLS, the aligned sequence reads were then processed. GATA-1 binding peaks were detected by using the MACS software with a *P* value cutoff for peak detection at 1^{e-05}. Peaks were annotated using NEBULATOOLS.