**Supplementary note**

**Hsp70 and Hsc70**

In humans, the inducible Hsp70 and the cognate Hsc70 proteins belong to a same family of proteins, and are encoded by distinct genes with some homologies (e.g. human Hsc70 demonstrated up to 80% homologies with human Hsp7A1). In many cell types, Hsc70 is constitutively expressed and insensitive to stress, whereas Hsp70 is highly sensitive to stress. In Xenopus Laevis erythropoiesis, hsp70 gene is constitutively transcribed at earlier stages of differentiation and heat shock-dependent at later stages (orthochromatic erythroblasts) (RS Winning and LW Browder, 128 (1):111-120, 1988 Dev Biol). To date, the expression and the role of Hsp70 are unknown in human erythropoiesis.

**Supplementary methods**

**Reagents**

Antibodies used included those targeting GATA-1 (N1 and C20 for immunoblot; N6 for confocal immunofluorescence microscopy and immunoprecipitation), Lamin B (M20), Hsp27 (M20) and Actin (I19) from Santa Cruz, Bcl-X\textsubscript{L} (610211) from BD Biosciences, GPA (555569) from BD Pharmingen, Hsp70 (SPA-812), Hsp90 (SPA-830) and Hsc70 (SPA-815) from Stressgen Laboratories, activated caspase-3 from Cell signaling (#9661) for confocal microscopy and from Neomarkers (RB-1197) for immunoblotting. TO-PRO-3 (T-3605) and Orange Sytox (S11368) were from Molecular Probes, anti-rat-cy3 (712-166-153) and rabbit-cy5 (111-175-144) from Jackson ImmunoResearch, and rat immunoglobulin G (R 5005) from Sigma. Leptomycin B was a gift from Dr. A Benmerah, (Cochin Institute, Paris).

**In vitro generation of erythroid cells.**

Umbilical cord blood units from normal full-term deliveries were obtained, after informed mothers’ consent, from the Obstetrics Unit of Hôpital Necker-Enfants Malades. CD36\textsuperscript{+} erythroid progenitors, generated from 7 days IL-6 (100 ng/ml) +IL-3 (10 ng/ml) +SCF (100 ng/ml)-cultured CD34\textsuperscript{+} progenitors isolated from cord blood (Miltenyi CD34 Progenitor Cell Isolation Kit), were cultured in the presence of IL-3 (10 ng/ml) + SCF (100 ng/ml) + Epo (2U/ml) in IMDM (Gibco cell culture) supplemented with 15% BIT 9500 (Stem Cell
Technologies) as described earlier [1]. After seven days of culture, apoptosis was induced by cytokines starvation (-Epo) during two (h2) or five (h5) hours.

**Immunoblot analyses.**

Cells were lysed in Laemmli buffer (whole cell lysates) or lysed 5 min on ice in a lysis buffer (0.2% NP40, 20mM Hepes pH7.9, 10mM KCl, 1mM EDTA, 10% glycerol, 1 mM orthovanadate, 1mM PMSF, 1 mM DTT, and 10µg/ml of aprotinin/leupeptin/pepstatin) before centrifugation (2 min, 14,000 rpm) and supernatant collection (cytoplasmic extracts). Remaining pellets were lysed (350mM NaCl; 20% glycerol; 20mM Hepes pH 7.9; 10mM KCl; 1mM EDTA; 1mM orthovanadate, 1mM PMSF, 1mM DTT, and 10µg/ml of aprotinin/leupeptin/pepstatin), incubated for 30 min on ice and centrifuged for 20 min at 14,000 rpm before collecting the supernatant (nuclear extracts).

Whole lysates of $5 \times 10^5$ cells or $50\mu g$ of proteins of nuclear or cytoplasmic extracts were resolved on 12% acrylamide gels and analysed by immunoblotting. Antigens were visualized by chemiluminescence using SuperSignal West Dura (Pierce).

**Generation of Hsp70 mutants**

Recombinant Hsp70 was generated using the pET-16b vector system (Novagen) with induction of protein expression and subsequent Ni$^{2+}$-affinity-purification according to the manufacturer’s protocol. Mutants of Hsp70 lacking either the ATP (ΔABD) or the peptide binding (ΔPBD) domain were generated through restriction enzyme cleavage and subsequent ligation of full-length Hsp70 cDNA in pET-16b vector. ΔABD was generated through BglII digestion, creating an in-frame deletion of amino acids 119-426 comprising the ATPase domain. ΔPBD was generated through SmaI digestion, creating an in-frame deletion of amino acids 437-617 comprising the peptide-binding domain. After purification, all proteins had their His-tag removed and were subsequently buffer-exchanged to D-PBS.

**Cell fractionation and immunoprecipitation**

Protein-protein interactions between GATA-1 and Hsp70 were determined by immunoprecipitation (IP) experiments. Cell fractionation was performed by incubating the cells in ice-cold buffer [250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA, 1mM orthovanadate, 1mM PMSF, 1mM DTT, and 10µg/ml of aprotinin, leupeptin, pepstatin (pH 7.4)], then by homogenization in a Potter-Thomas
homogenizer. Nuclei were pelleted via a 10 min, 3,200 rpm spin and the supernatant (cytoplasm) was collected [2]. Nuclei and whole cells (obtained from 10^7 cells) were lysed on ice in an IP buffer (1% NP40, 150mM NaCl, 5mM EDTA, 65mM Tris HCl pH 8, 50mM Hepes, 3% glycerol, 1mM orthovanadate, 1mM PMSF, 1mM DTT, and 10µg/ml of aprotinin, leupeptin, pepstatin) for 20 min, then spun at 14,000 rpm for 30 min and the supernatant was collected. Nuclei or whole cell lysates were incubated on ice for 1.5 hours with either an anti-GATA-1 (2 µg) or anti-Hsp70 (1/100) antibody in the presence of 50 µl Protein G Microbeads (Miltenyi). The immune complex was immobilized to a µColumn, isolated according to the manufacturer’s instructions and analyzed by immunoblotting.

**In vitro GATA-1 cleavage assays**

GATA-1 and PARP cDNAs subcloned in PET21 plasmid in frame with amino-terminal T7 tag (GATA-1-PET, PARP-PET) (kindly provided by Dr De Maria and A Zeuner, Roma, Italy) were transcribed by using the In Vitro Translation Kit (L4610) from Promega in the presence of [35S]-methionine (Amersham Biosciences). Two µl of GATA-1 or PARP translation reaction were preincubated with either human recombinant Hsp70 (NSP-555) (0 to 1µg) or Hsp90 (SPP-770) (1µg) from Stressgen Laboratories and Hsp70 mutants (ΔABD or peptide ΔPBD) in a stochiometric manner (~1µg HSP70) for 40 minutes at room temperature in buffer (25mM Hepes, 0.1% Chaps, 5mM DTT, 1mM EDTA, PH7.5) in a final volume of 15µl, then incubated for 1h at 37°C with 70ng recombinant caspase-3 in a final buffer volume of 25µl. Reaction products were run on 10% acrylamide gel, and GATA-1 and PARP products were analyzed by autoradiography of dried gel.

**Hsp70 siRNAs.**

All siRNAs were obtained from Qiagen, and included a non-silencing Alexa Fluor 488 labeled siRNA, a specific siRNA for inducible Hsp70 (CTG GCC TTT CCA GGT GAT CAA); a scramble siRNA (CAG TAA TTG CAC CCG TCG TGT). Stealth siRNA for Hsp70 (ACU UCG UGG AGG AGU UCA AGA GAA A) and a control stealth siRNA (Block-iT, manufacturer’s confidential sequence) were obtained from Invitrogen, life technologies.

**Transfection of HeLa cells.**

HeLa cells were infected with viral particles containing GFP-tagged GATA-1 vector [3] and GFP-positive cells were selected by cell sorting using a Coulter EPICS EPS (Beckman...
Coulter). Twenty-four hours after seeding, these cells were transfected with either wild type or mutated Hsp70 constructs (subcloned in pcDNA3 vector) using the Superfect transfection reagent (Qiagen, Valencia, CA) following the manufacturer’s instructions, and analyzed 48 hours later.

Transfection of hematopoietic progenitors.

2.10^6 of five days IL-6 (100 ng/ml) +IL-3 (10 ng/ml) +SCF (100 ng/ml)-cultured CD34^+ cells were transfected in a Human CD34 Cell Nucleofector buffer with 10µg of siRNA using a Nucleofector (Amaxa Biosystems) according to the manufacturer’s protocol. Cells were then cultured for 1 day with IL-6+IL-3+SCF. Thereafter, dead cells were removed with Dead Cell Removal Kit (Miltenyi). Transfection efficiency of the non-silencing Alexa Fluor 488 labeled siRNA was examined by FACS analysis (>90% of viable cells) (data not shown). Remaining alive cells were cultured with Epo+IL-3 in serum-free medium.

Production of retroviral particles and infection of haematopoietic progenitors.

Wild-type (WT) and mutated GATA-1 (µGATA-1) cloned in PINCO vector [3] were transfected in the amphotrophic packaging cell-line PLAT-A (kindly provided by Pr Toshio Kitamura, Institute of Medical Science, University of Tokyo, Tokyo, Japan) and supernatants containing viral particles were collected 48 h after transfection. CD34^+ cells exposed to SCF (100 ng/ml), IL-3 (60 ng/ml), IL-6 (20 ng/ml) and TPO (100 ng/ml) were daily infected during 72h, after 24h of culture. After three additional days of culture without TPO, siRNA were transfected 24h later after last infection. Finally, cells were grown in serum-free medium supplemented with IL-3 (0.01 U/ml) and Epo (3 U/ml). GFP-positive cells were sorted 24 h after siRNA transfection using a FACS VANTAGE cell sorter (Becton Dickinson).

Cell permeabilization and labelling for Fluorescence microscopy

5.10^4 cells were washed, spin on slides, acetone fixated, hydrated with cold 1X PBS/1% BSA for 30 minutes, treated with formaldehyde (Sigma) for 15 minutes, then with methanol (Prolabo) for 10 minutes at room temperature. Cells were then permeabilized with 1X PBS/0.2% Triton X100 (Sigma) for 10 minutes at 4°C, washed with 1X PBS/1% BSA and incubated in 3% BSA for 30 min. They were then sequentially incubated with antibodies diluted in 1XPBS/1% BSA/0.1% Tween (Sigma): anti-GATA-1 overnight at 4°C; then anti-rat-Cy3 for 45 minutes at room temperature, then rabbit anti-Hsp70 or anti-caspase-3 for 1 hour at room temperature, then anti-rabbit Cy5 for 45 minutes at room temperature. Nuclei
were stained with TO-PRO-3, and slides were examined with a confocal laser microscope (LSM 510 Carl Zeiss). Fresh normal bone marrow cells were separated by centrifugation on Ficoll, spin on slides and fixed with acetone. Permeabilization and labelling with anti-GATA-1 and anti-Hsp70 antibodies was performed as above. Then, the cells were incubated for 1 hour at room temperature with anti-GPA antibody and DNA was stained with Orange Sytox for 5 min at room temperature before confocal laser microscope observation.

Supplementary figure legends

**Figure 1S. Role of caspases in erythropoiesis regulation.** **A**- Caspase-3 is required for terminal erythroid maturation. Erythropoiesis is a multistep process that involves the sequential formation of proerythroblasts (ProE) and basophilic (Baso), polychromatic (Poly) and orthochromatic (Ortho) erythroblasts leading to red cells (RC) production. During maturation of erythroblasts, caspase-3 is activated and its inhibition results in erythroid differentiation arrest at the proerythroblast stage. **B**- Patterns of caspase-3-induced proteolysis during apoptosis and differentiation of erythroid cells. During both apoptosis and differentiation, caspase-3 cleaves several targets involved in nucleus and chromatin condensation. In contrast, GATA-1 is protected from caspase-3 proteolysis during erythroid differentiation. GPA (glycophorin A), Hb (hemoglobin), Epo (erythropoietin).

**Figure 2S. Caspase 3 is the main caspase activated in the nucleus of erythroblast during erythroid differentiation.**

Immunoblot analysis of caspases 3, 6, 7, 8, 9 activation in nucleus and cytoplasm extracts of CD36+ cells cultured for 7 days in the presence of Epo+IL-3+SCF and then Epo-starved (-Epo) or not (+Epo) for 5 hours. Cleavage fragments of caspase-3, suggesting activation, and the proform of caspase-7 (without cleavage fragments) were detected in the nucleus. Caspase 6, or caspase 8 were not identified in the nucleus.

**Figure 3S. Nuclear colocalization of Hsp70 and GATA-1 in GPA positive cells from fresh normal human bone-marrow.**

Expression analysis by confocal microscopy in normal fresh bone-marrow cells of **a**- GPA (blue), GATA-1 (red) and Hsp70 (green). White hue shows GATA-1 and Hsp70
colocalization. b- GPA (blue), Hsp70 (green), DNA (yellow). c- Morphologic analysis after May Grunwald staining (MGG).

**Figure 4S. Ability of Hsp70 interaction with GATA-1 is not affected by Epo starvation.** CD36⁺ cells cultured for 7 days in the presence of Epo+IL-3+SCF and then Epo-starved (-Epo) or not (+Epo) for 5 hours. Nuclear proteins were immunoprecipitated using an anti-GATA-1 antibody, then immunoblotted for indicated proteins. A 5-fold increase in the input of nuclear extract from cells starved of Epo (h5x5) is compared to non starved cells (d7).

**Figure 5S. Before caspase activation, decrease in nuclear Hsp70 content is not associated with GATA-1 degradation.** After 5 days of culture in the presence of IL-3+IL-6+SCF, CD34⁺ progenitors were transfected with 10 µg of a siRNA targeting Hsp70 (siRNA Hsp70) or a scrambled siRNA (siRNA control). Two days after transfection, cells were diluted to 4.10⁵ cells/ml, and then cultured in the presence Epo+IL-3 for 2 days. Immunoblot analysis of indicated proteins in nuclear and cytoplasmic extracts is shown (see also figure 3b, day 2).

**Figure 6S. Decreased Hsp70 content induces death of erythroblasts undergoing differentiation.** After 5 days of culture in the presence of IL-3+IL-6+SCF, CD34⁺ progenitors were transfected with 10 µg of an antisense targeting Hsp70 or a control antisense. Two days after transfection, cells were diluted to 4.10⁵ cells/ml, then cultured in the presence Epo+IL-3 for indicated times (days). a- Growth curves. b- Percentage of cell death. c-Percentage of mature cells. d- Percentage of mature cells exhibiting morphological features of apoptosis. Results are the mean +/- SD of 3 independent experiments. * p < 0.05. e- Immunoblot analysis of Hsp70 in whole cell lysate at day 2 of culture (d2). The non-inducible Hsc70 is used as loading control.

**Figure 7S. Decreased Hsp70 content induces death of erythroblasts undergoing differentiation even in the presence of SCF.** After 5 days of culture in the presence of IL-3+IL-6+SCF, CD34⁺ progenitors were transfected with 10 µg of an siRNA stealth targeting Hsp70 or a control siRNA stealth. Two days after transfection, cells were diluted to 4.10⁵ cells/ml, then cultured in the presence Epo+IL-3 and in the presence of SCF for indicated times (days). a- Growth curves. b-
Percentage of cell death. c- Percentage of mature cells. d- Percentage of mature cells exhibiting morphological features of apoptosis. Results are the mean +/- SD of 3 independent experiments. * p < 0.05. e- Immunoblot analysis of Hsp70 in whole cell lysate at day 2 of culture (d2). The non-inducible Hsc70 is used as loading control.

Figure 8S. Model of GATA-1 protection from caspase-3-induced cleavage by Hsp70 during erythroid differentiation.

Supplementary references


Fig. 1S

CASPASE-3

Erythroid progenitors

ProE

Erythroblasts

Baso

Poly

Ortho

RC

Caspase-6

ACINUS

LAMINE B

NUCLEUS

CONDENSATION

GATA-1

DNA

CONDENSATION

LIFE (Bcl-xL)

DIFFERENTIATION

(GPA, Hb)

Differntiation

(+Epo)

APOPTOSIS

(-Epo; Fas-L)

CASPASE-3

Active

ACINUS

GATA-1

DIFFERENTIATION

(Life (Bcl-xL))

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Fig. 2S
Fig. 3S

Supplementary Information
**Fig. 4S**

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**IP GATA-1**

**Input**
Fig. 5S
**Fig. 6S**

- **a.** Number of cells x 10^6/mL over time (d2, d4).
- **b.** Trypan blue + cells (%) over time (d2, d4).
- **c.** Differentiated cells (%) over time (d2, d4).
- **d.** Differentiated dead cells (%) over time (d2, d4).
- **e.** Western blot analysis showing ASHsp70 and Control conditions for Hsc70 and Hsp70 proteins at d2.
**Fig. 7S**

- **a**
  - Graph showing the number of cells ($10^6/\text{ml}$) over time (d2 to d4).

- **b**
  - Graph showing the percentage of trypan blue + cells over time (d2 to d4).

- **c**
  - Graph showing the percentage of differentiated cells over time (d2 to d4).

- **d**
  - Graph showing the percentage of differentiated dead cells over time (d2 to d4).

- **e**
  - Bar graph comparing Hsc70 and Hsp70 levels under SiRNA Hsp70 and control conditions.

Legend:
- Control siRNA stealth
- Hsp 70 siRNA stealth

**Supplementary Information**

[Link to Supplementary Information](www.nature.com/nature)
**Fig. 8S**

**APOPTOSIS**

**SURVIVAL and MATURATION**

**-Epo**

- Hsp70
- casp-3
- GATA-1

**+Epo**

- Hsp70
- casp-3

Cytoplasm

Nucleus

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