A novel role for survivin in erythroblast enucleation

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Online Supplementary Design and Methods

Cell culture

Mouse erythroleukemia (MEL, line F4-12B2) and K562 (human erythroleukemia) cell lines were cultured in RPMI medium with 2 mM L-glutamine, 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. K562 cells were differentiated into erythroid cells by the addition of cytosine arabinoside to a final concentration of 1 µM.

Immunofluorescence

Cells were washed and layered onto cover slips treated with VECTABOND (Vector Laboratories) and incubated at 37°C for 5-10 min. The adherent cells were then washed once in PBS and fixed in 3.75% paraformaldehyde/PBS and permeabilized by 0.1% Triton-X-100/PBS. After 1 h of blocking in 3% BSA/PBS, the cover slips were incubated at room temperature for 1 h with primary antibodies (survivin at 1:50, lamin B at 1:100, INCENP at 1:50, clathrin at 1:200, EPS15 at 1:200 and aurora B kinase at 1:200), washed and incubated for 1 h in secondary antibodies (1:200 dilution). Actin was visualized by staining with rhodamine phalloidin (1:40 dilution). After a final washing, the cover slips were mounted with anti-fade reagent. Confocal images were acquired with a Leica SP-5 spectral 2-photon confocal microscope and deblurred by Leica software.

Analysis of confocal images

Images were analyzed for co-localization by the Co-Localisation Threshold pluggin (available from the Wright Cell Imaging Facility at University Health Network Research). This software quantifies the degree of co-localization between two 8- or 16-bit images or stacks using Costes co-localization quantification. Details of the effectiveness and statistical significance of this software have been reported elsewhere.[1] We used this software to determine the overlap between pixels in different channels (reported as percentage volume overlap) throughout the entire z stack. While calculating the threshold, zero-zero pixels are excluded. We analyzed the percentage volume overlap between survivin and either aurora B, INCENP, EPS15, clathrin or actin for 14 individual enucleating cells (individual points) and plotted the values (Figure 1F). Linear regression was used to determine the best fit (colored lines). The mean percentage volumes of survivin co-localized with aurora B kinase, INCENP, EPS15, clathrin and actin were calculated to be: 19.5% (range

11.7-27.5), 17.8% (9.1-26.5), 56.75% (48.5-64.9), 37.2% (28.5-45.9) and 60.6% (52.7-68.5), respectively (98% Confidence Interval). Note that the extent of survivin co-localization with EPS15, actin and clathrin is 2-3 fold greater than the extent of co-localization of survivin with aurora B kinase or INCENP. Also, the percentage volumes calculated above were compared by Student's t-test and the corresponding *P* values for their comparisons are listed in Figure 1G. This analysis reveals that there is a significant difference between co-localization of survivin with EPS15 and aurora B (*P*=4.86E-6). Similarly, there is a significant difference between co-localizations of survivin with clathrin and survivin with actin and that of survivin and aurora B (*P*=0.0012 and *P*=8.67E-6, respectively).

Immunoprecipitations

Extracts from undifferentiated K562 cells or from K562 cells differentiated for 72 h were prepared in co-IP lysis buffer (25 mM Tris pH 7.5, 10 mM MgCl2, 100 mM NaCl, 0.75% NP-40, 10% glycerol, 1 mM DTT and protease inhibitors, Sigma) and then pre-cleared by the addition of Protein G Sepharose Fast Flow beads (GE healthcare). Cleared lysates were incubated with antibodies and beads overnight at 4°C and the protein bound beads are washed 2 times in wash buffer I (25 mM Tris pH 7.5, 10 mM MgCl2, 100 mM NaCl and 0.75% NP-40) and II (50 mM Tris pH 7.5, 500 mM NaCl, 0.1% NP-40 and 0.05% sodium deoxycholate), and once with wash buffer III (50 mM Tris pH 7.5, 0.1% NP-40 and 0.05% sodium deoxycholate). Washed bound proteins were denatured from beads by LDS sample buffer (Invitrogen) and loaded onto 12% PAGE.

Co-immunoprecipitations in primary human erythroblasts were performed using µMACS Protein A/G Microbeads and µ Columns purchased from Miltenyi Biotec. Primary erythroblast lysates were prepared in a high salt lysis buffer [500mM NaCl, 1% Igepal CA 630 (NP-40), 50 mM Tris HCl (pH 8.0), Complete protease inhibitor cocktail-EDTA free (Roche Diagnostics)], pre-cleared, incubated with antibody and magnetic beads for 30 min to 1 h and captured using µMACS separation unit. After washing 4-5 times with high salt lysis buffer and a low salt wash buffer (1% Igepal CA 630 (NP-40), 50 mM Tris HCl, pH 8.0), the immunoprecipitated proteins were recovered using SDS gel loading buffer (50mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 0.005% bromophenol blue, 10% glycerol). For immunoprecipitation assays, EPS15 (rabbit antibody from Covance) at a dilution of 1:250, clathrin heavy chain (X22) at 1:250 dilution and normal

isotype IgGs (Abcam) were used.

Enucleation assays of primary murine erythroblasts

For enucleation assays of fetal liver erythroblasts, cells were collected from E12.5-14.5 wild-type C57Bl/6 embryos and subjected to Ter119 depletion (Stem Cell Technologies) such that mature erythroblasts and reticulocytes are removed. Enucleation assays were then performed as described.[1] To knock-out the survivin gene, fetal liver cells were collected from survivin fl/fl embryos[2,3] and immature fetal erythroblasts were then incubated with 1.6 μM TAT-Cre,[4] TAT-Scr (TAT fused to a scrambled sequence; a gift from Dr. Michael Thirman), or no TAT protein in serum free medium for 1 h at 37°C. After this TAT protein treatment, the experiment was performed as described above for wild-type cells. The percentages of reticulocytes were quantified at 48 h by gating on the Syto 64^{low} and Ter119-PE (BD Biosciences) positive populations. Student's t-test (paired, two-tail distribution) was used to evaluate the statistical significance of the differences in enucleation.

Enucleation assays of primary human erythroblasts

Human primary CD34$^+$ early hematopoietic cells were cultured *in vitro* towards erythroid lineage as described.[5] Knockdown experiments were performed as previously reported.[6] Survivin (L-003459-00), EPS15 (L-004005-00) or a non-targeting control (D-001810-10) ON-TARGETplus SMARTpool siRNA were purchased from Dharmacon, Thermo Scientific.

Electron microscopy

Terminal erythroblasts derived from fetal liver progenitors after 48 h of differentiation were processed and sectioned as previously described.[6] Images were acquired using a JEOL 1200EX electron microscope (JEOL, USA, Inc., Peabody, MA, USA) and analyzed with GatanDigitalMicrograph software.

Vectors

Human full-length survivin cDNA was cloned into pcDNA3, pcDNA3-HA (Invitrogen) and pEF1αBiotag such that the epitope tags were present at the C-terminal end of the protein.

In vivo *biotinylation and survivin complex purification*

In vivo biotinylation of survivin was performed as described by de Boer *et al.*[7] with minor modifications. pEF1αBiotag-survivin (neomycin resistant) and pEF1αBirA (puromycin resistant) vectors were transfected into MEL cells and individual clones were double selected with 300 μg/mL G418 (Invitrogen) and 10 ng/mL puromycin (Sigma). Similarly, control clones were generated by selecting for MEL cells harboring empty pEF1αBiotag and pEF1αBirA vectors. Lysates of 1x10^9 exponentially growing stable cells were passed through streptavidin mutein matrix columns (Roche Applied Sciences) and bound proteins were purified according to the manufacturer's protocol. Eluted proteins were concentrated in an Ultracel YM-10 Centricon filter (Millipore) and then passed through a Superdex s200 column to further purify the complexes (see below). Peak survivin containing fractions were pooled, concentrated, and subjected to mass spectrometry. The protein complexes were digested with trypsin, loaded onto a Zorbax 300SB-C18 reversed phase column and then analyzed by electrospray tandem mass spectrometry (LC–MS/MS) on an Agilent XCT ion trap.[8] Mascot and Spectrum Mill were used to identify proteins based on MS/MS spectra using NCBInr and MSDB databases for mouse proteins.

Gel chromatography

In order to compare the approximate size of survivin protein complexes formed by biotinylated and endogenous forms of survivin, and also to separate the supernumerary survivin complexes from other proteins, we performed gel chromatography with a Superdex s200 column (GE Healthcare). The column was equilibrated with the co-IP lysis buffer lacking detergent and enzymes (25 mM Tris pH 7.5, 10 mM MgCl$_2$, 100 mM NaCl, 10% glycerol, 1 mM DTT and 50 μg/mL PMSF (Sigma) and then 2 mL of protein (2-2.3 mg) was loaded onto the column at a flow rate of 0.3 mL/min. The eluted proteins were collected for 2 min per tube, and 60 tubes were collected per sample. The OD$_{280}$ profile of the protein flow-through is shown in *Online Supplementary Figure S1C and D*). The eluted fractions were analyzed by SDS-PAGE and either stained with silver (*Online Supplementary Figure S2A and B*) or analyzed by a Western blot for survivin/INCENP (Figure 2B).

Flow cytometry

All acquisitions were performed on an LSRII flow cytometer (BD). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Animals

Survivin floxed mice were obtained from E Conway and A Winoto.[3] Animal studies were approved by the Northwestern University IACUC. Three primers (Adv17, Adv25 and Adv28) were used to genotype the wild-type, floxed and Cre-deleted survivin allele, as previously described.[2]

References

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| A2L	B8L	A2S	B8S
- EPS15 (Epidermal growth factor receptor pathway substrate-15)	No peptides returned	- Insulin degrading enzyme	- Insulin degrading enzyme
		- Heat shock 70 protein	- 78 kDa glucose regulated protein
- Clathrin		- 78 kDa glucose regulated protein	- Triose phosphate isomerase
- Insulin degrading enzyme			- Cyclin B1 Hydrocephalus inducing protein
- Ubiquitin			

Extracts from MEL clone A2 (cells that stably over-expressed biotinylated survivin) or MEL clone B8 (cells that expressed only the biotag ligase) were first purified by passing through streptavidin mutein matrix. Eluates were then passed through an S-200 gel filtration column. Fractions corresponding to the large and small survivin complexes were pooled and subjected to mass spectrometry. Data were analyzed using Mascot and Spectrum Mill software. Top scoring proteins are listed.
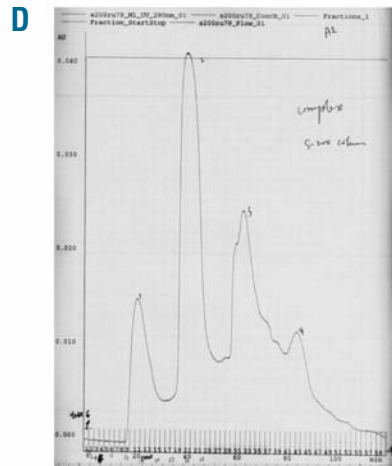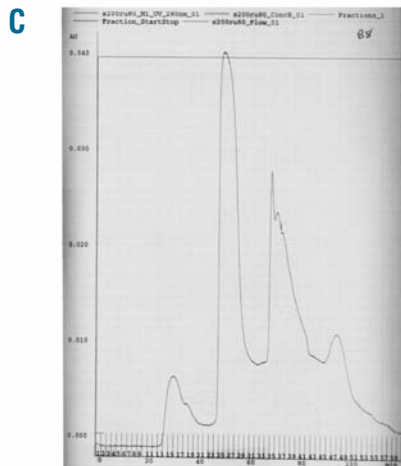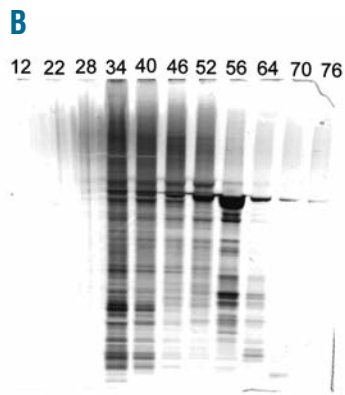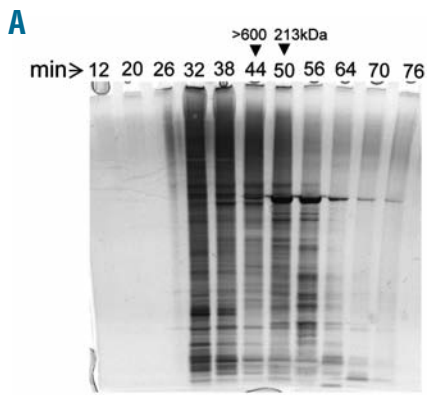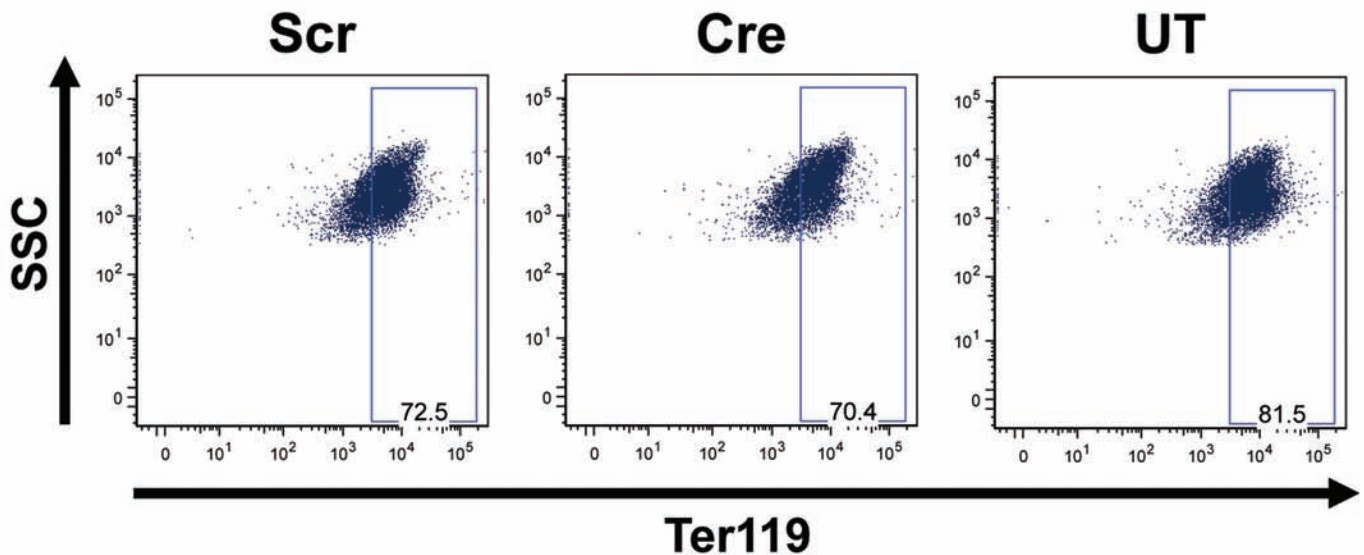
Online Supplementary Figure S1. MEL cell lysates of *in vivo* biotinylated survivin or control over-expressing clones have similar profiles when passed through a S-200 gel filtration column. (A and B) MEL cell lysates of (A) control or (B) survivin over-expressing clones were injected into a Superdex s200 gel filtration chromatography and the eluted proteins at the mentioned time points were loaded onto a 4-12% gradient SDS-polyacrylamide gel. After performing the electrophoresis, the gels were silver stained using Silver Stain Plus Kit (Bio-Rad). (C and D) OD_{280} profiles of cell lysates of (C) control and (D) survivin over-expressing clones when passed through the S-200 gel filtration column.
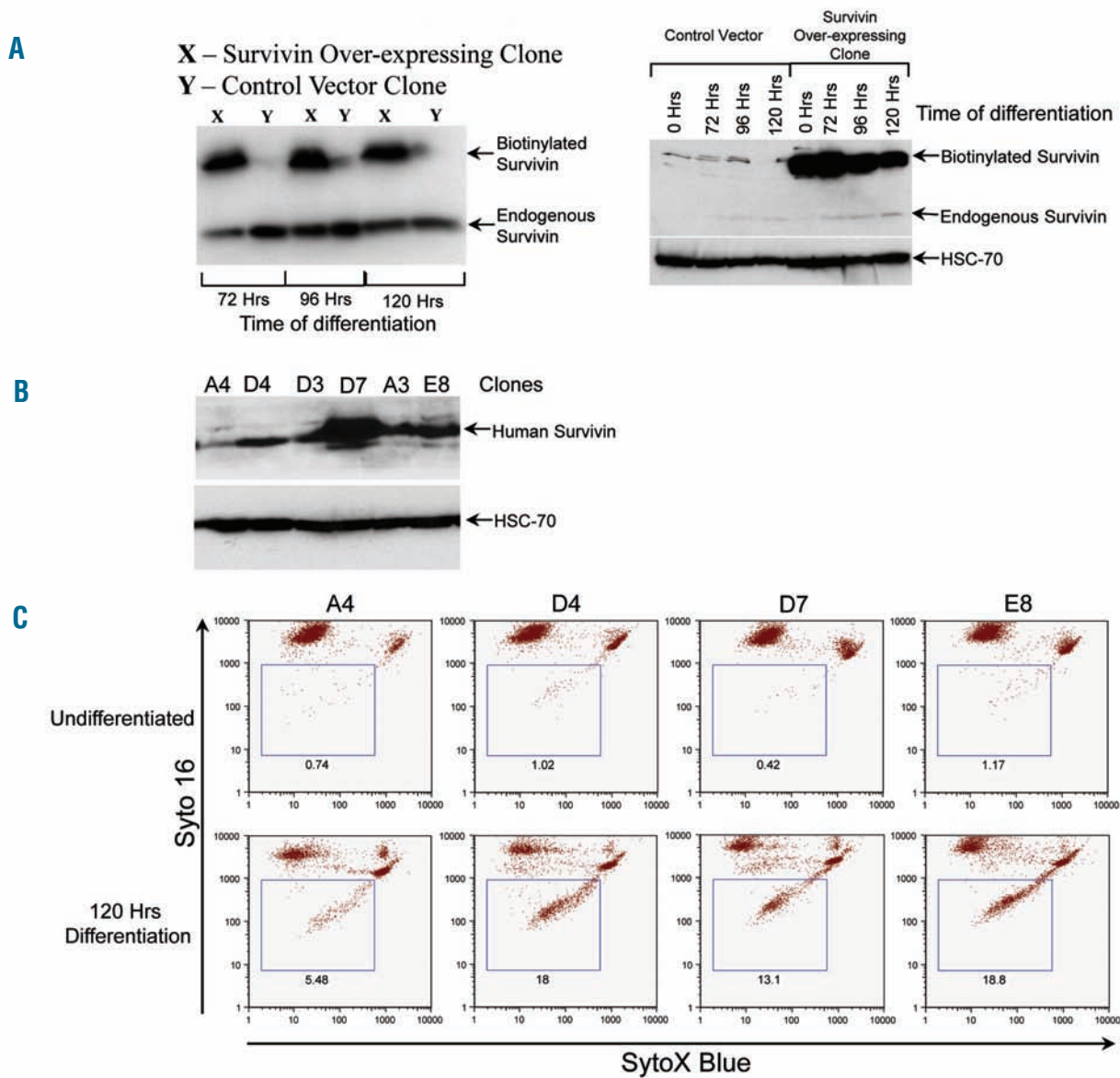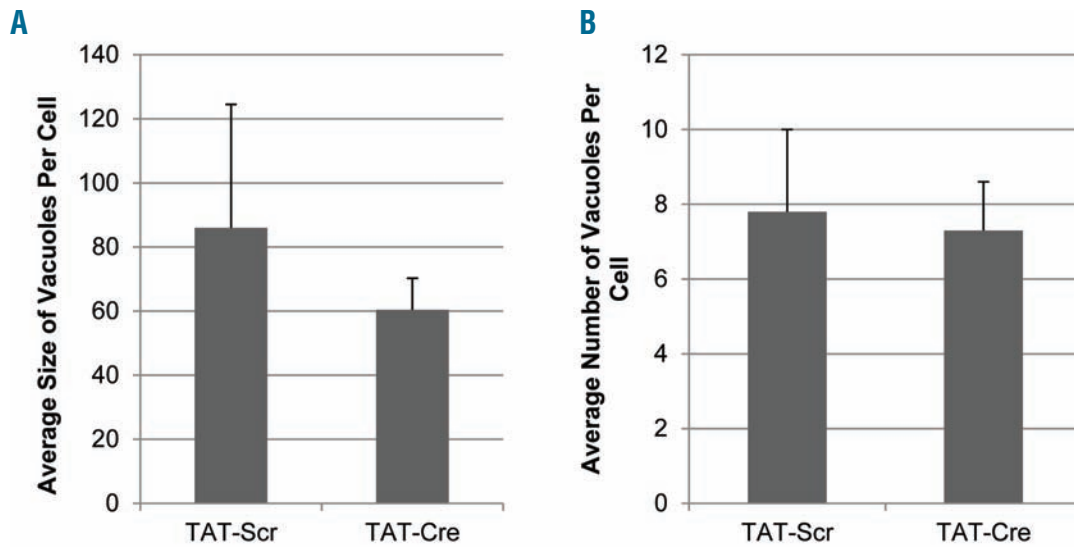
Online Supplementary Figure S2. MEL engineered to over-express survivin undergo enucleation upon differentiation. (A) Multiple MEL cell clones that over-express biotinylated survivin or only express the biotag ligase were differentiated with 2% DMSO for five days. The levels of survivin protein were assayed by Western blot on Days 3, 4 and 5 of differentiation. Endogenous and biotinylated isoforms of survivin are marked for comparison. (B) Cell lysates from multiple untagged human survivin over-expressing MEL cell clones were subjected to Western blotting and immunostaining with anti-survivin and anti-HSC-70 antibodies. (C) Randomly selected survivin over-expressing clones were differentiated in the presence of 2% DMSO for five days and the percentages of enucleated cells were determined by flow cytometry. The percentages of Syto 16^{low}/SytoX^{low} cells, which represent enucleated MEL cells, are shown below the gate. Note that while survivin over-expression is required for a clone to enucleate, the extent of enucleation is not directly proportional to the level of survivin protein expression.
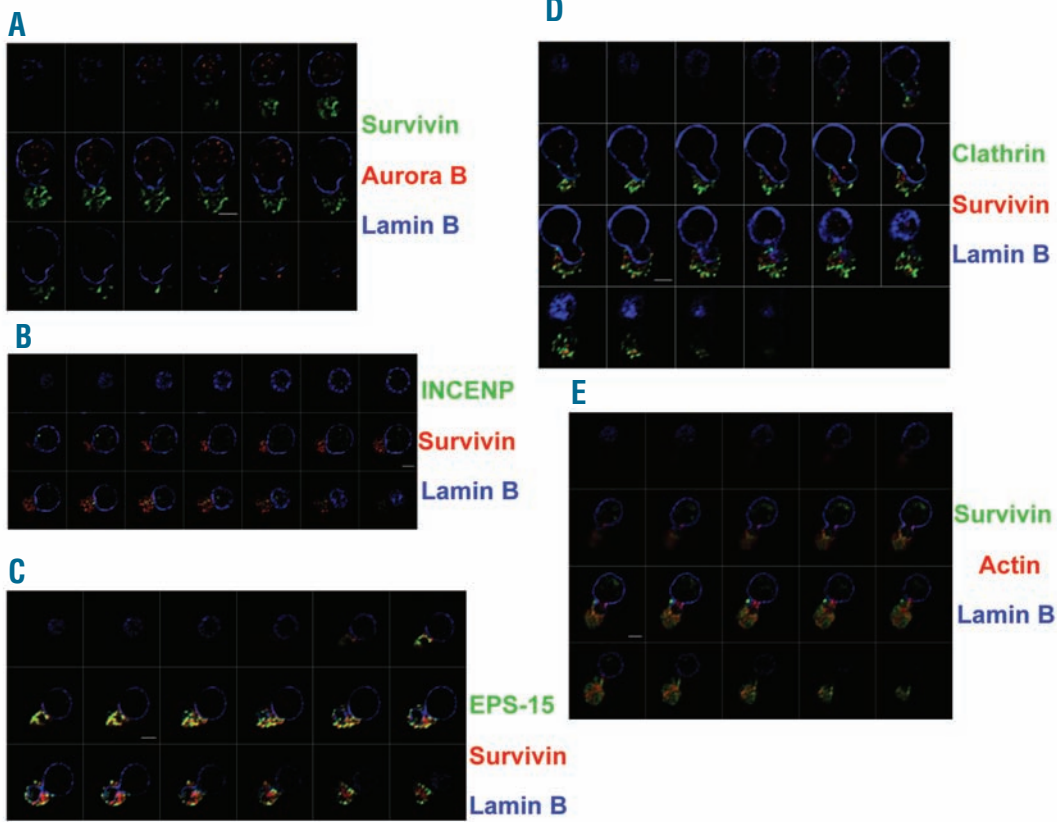
Online Supplementary Figure S3. Survivin co-localizes with EPS-15, clathrin and actin but not aurora B kinase and INCENP in enucleating erythroblasts. Human primary erythroid cells were immunostained for survivin and lamin B, along with (A) aurora B kinase, (B) INCENP, (C) EPS-15 or clathrin, while actin (C) was visualized by staining with rhodamine phalloidin. Cells were imaged by confocal microscopy. Series of z sections, scanning the cell shown from top to bottom at a step size of 0.3 μm. In these images, the green, red and blue channels were merged. Scale bar: 2 μm.

Online Supplementary Figure S4. Loss of survivin in late erythroblasts did not affect differentiation. Enucleation assays of survivin fl/fl mouse fetal liver erythroblasts were performed in the presence of TAT-Scr or TAT-Cre as in Figure 5 and the percentage of Ter119 positive erythroblasts quantified by flow cytometry at a 48 h time point. There is no significant difference between TAT-Scr and TATCre conditions. Figure is representative of 3 independent experiments.

Online Supplementary Figure S5. Loss of survivin leads to a reduction in the average size of vacuoles in late erythroblasts. Late erythroblasts, derived from survivin fl/fl fetal liver Ter119⁻ progenitors and treated with TAT-Scr or TAT-Cre, were cytospun and benzidine stained after 48 h of culture. Using ImageJ software, we evaluated (A) the size and (B) number of vacuoles in each cell that are visible by 1000X magnification in a light microscope. Bar graphs show the mean and standard deviation of 3 independent experiments. While there is a trend towards decreased average vacuole size per cell with TAT-Cre, there is no change in the average number of vacuoles per cell between TAT-Scr and TAT-Cre conditions.