Differential Proteomic Analysis of Human Erythroblasts Undergoing Apoptosis Induced by Epo-Withdrawal

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Abstract

The availability of Erythropoietin (Epo) is essential for the survival of erythroid progenitors. Here we study the effects of Epo removal on primary human erythroblasts grown from peripheral blood CD34+ cells. The erythroblasts died rapidly from apoptosis, even in the presence of SCF, and within 24 hours of Epo withdrawal 60% of the cells were Annexin V positive. Other classical hallmarks of apoptosis were also observed, including cytochrome c release into the cytosol, loss of mitochondrial membrane potential, Bax translocation to the mitochondria and caspase activation. We adopted a 2D DIGE approach to compare the proteomes of erythroblasts maintained for 12 hours in the presence or absence of Epo. Proteomic comparisons demonstrated significant and reproducible alterations in the abundance of proteins between the two growth conditions, with 18 and 31 proteins exhibiting altered abundance in presence or absence of Epo, respectively. We observed that Epo withdrawal induced the proteolysis of the multi-functional proteins Hsp90 alpha, Hsp90 beta, SET, 14-3-3 beta, 14-3-3 gamma, 14-3-3 epsilon, and RPSA, thereby targeting multiple signaling pathways and cellular processes simultaneously. We also observed that 14 proteins were differentially phosphorylated and confirmed the phosphorylation of the Hsp90 alpha and Hsp90 beta proteolytic fragments in apoptotic cells using Nano LC mass spectrometry. Our analysis of the global changes occurring in the proteome of primary human erythroblasts in response to Epo removal has increased the repertoire of proteins affected by Epo withdrawal and identified proteins whose aberrant regulation may contribute to ineffective erythropoiesis.

Introduction

Red blood cell production in the bone marrow is maintained by a delicate balance between erythroid cell proliferation, differentiation and apoptosis. This process is regulated by Erythropoietin (Epo), Stem Cell Factor (SCF) and glucocorticoids [1,2]. Epo is a 34 kD glycoprotein produced primarily by the kidney and its production increases under hypoxic conditions [3]. It is essential for erythropoiesis [4] and the availability of Epo is known to facilitate the survival of erythroblasts during the Epo-dependent stage of erythropoiesis [5]. Epo acts by binding to its cognate receptor, the single transmembrane erythropoietin receptor (EpoR) [6]. EpoR lacks kinase activity but Epo binding triggers the activation of the Janus family protein tyrosine kinase 2 (JAK2) [7], which in turn phosphorylates tyrosine residues in EpoR, creating docking sites for intracellular signalling proteins such as phosphatidylinositol 3-kinase [8], SHP1 [9] and STAT5 [10]. These events lead to the activation of multiple signal transduction pathways and specific gene expression that result in the survival, proliferation, and differentiation of erythroblasts [11].

During homeostatic bone marrow erythropoiesis 16% of the erythroblasts die of apoptosis but this level of apoptosis is reduced by increased Epo [12]. Determining the molecular mechanisms behind the action of Epo is essential for our understanding of erythropoiesis in the bone marrow, thereby helping to efficiently reproduce erythropoiesis in vitro. It is also important for the development of novel erythropoiesis-stimulating agents and for understanding Epo’s cytoprotective action on other cell types [13]. It is also relevant to human disease since apoptotic mechanisms are implicated in the development of anaemia in myelodysplasia [12]. Understanding how Epo withdrawal induces apoptosis may also help improve apoptosis-inducing treatments of erythroid and non-erythroid leukaemia and identify the signalling pathways important for leukemic progression of specific leukemic clones.

Several molecular pathways involved in the induction of apoptosis in response to Epo withdrawal have been identified. For instance, studies on mice have shown that Epo inhibits pro-apoptotic Bim [14] and Bad [15] and induces anti-apoptotic SERPINA-3G and TRB3 [16]. In primary human erythroblasts, Epo inhibits pro-apoptotic GSK3 beta [17]. In addition, chaperone proteins play an important role in human erythroblast cell survival with Hsp70 preventing the transcription factor Gata-1 from being cleaved by Caspase 3 [18] and inhibiting the nuclear
import of Apoptosis-inducing Factor (AIF) [19]. Another chaperone protein Mortalin has also been identified as a mediator of Epo signalling [20].

To further our understanding of how Epo withdrawal induces apoptosis, we adopted a 2 Dimensional fluorescence difference gel electrophoresis (2D DIGE) proteomics approach coupled with mass spectrometry to compare the proteomes of expanding erythroblasts with that of erythroblasts undergoing apoptosis due to Epo removal. Using this methodology we identified in an unbiased fashion, novel key reproducible alterations in the proteome of primary human erythroblasts +/−Epo. In particular, our results highlight that within 12 hours of Epo withdrawal, several multi-functional proteins are cleaved, including SET, RPSA, Hsp90 and 14-3-3- proteins. The proteolysis of proteins pivotal to many pro-survival cellular signalling cascades may be vital to ensure that the cell enters apoptotic cell death, and interestingly, aberrant regulation of these proteins is already known to occur in human diseases.

Materials and Methods

Erythroid Cell Culture

Waste peripheral blood from anonymous donors was provided with written informed consent for research use given in accordance with the Declaration of Helsinki (NHSBT, Filton, Bristol). The research into the mechanisms of erythropoiesis was reviewed and approved by the Southmead Research Ethics committee 08/05/2008 REC Number 08/H0102/26. Mononuclear cells (PBMCs) isolated from waste peripheral blood were washed in PBS, and the CD34+ cells isolated using anti-CD34-conjugated, BD Pharmingen, 555714) and anti-CD71 (RPE or APC-conjugated, BD Pharmingen, 555357). To measure apoptotic cell death, cultured erythroblasts were labelled with Annexin V- FITC, together with Propidium Iodide according to the manufacturer’s instructions (Arcus Biologicals). To measure mitochondrial membrane potential (ΔΨm), tetramethylrhodamine ethyl ester perchlorate (TMRE, Sigma) was used. Erythroblasts were washed in PBS, resuspended in PBS containing 25 nM TMRE. To quantify caspase activation by flow cytometry, Caspase–3, Caspase-8 and Caspase 9 detection kits were used according to the manufacturer’s instructions (Calbiochem). Fluorescent signals were measured using a Coulter EPICS XLMCL flow cytometer (Beckman Coulter, High Wycombe, UK) or a FACS CantoII-F60 machine (BD Biosciences). All data was analysed using the Flowjo 7.2.5 software (Flowjo, Ashland, OR, USA).

Cytospins

2.5x10⁶ cells were cytospun onto glass slides, fixed in methanol and stained with May Grünwald/Giemsa stains according to the manufacturer’s protocol. Images were taken with an Olympus CX31 microscope coupled to an Olympus LC20 camera using a 50x (0.75NA) lens and processed using Adobe Photoshop 9.0 (Adobe).

Subcellular Fractionation to Detect Cytochrome C Release into the Cytosol

Cytochrome c release into the cytosol was assessed as previously described [23]. Cells (2x10⁶) were washed in PBS, resuspended in 50 μl of buffer (140 mM mannitol, 46 mM sucrose, 50 mM KCl, 1 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, 5 mM Tris, pH 7.4) supplemented with a mixture of protease inhibitors (Complete Mini-EDTA Free, Roche) and digitonin at a final concentration of 40 mg/ml. Cells were permeabilised on ice for 10 min and centrifuged at 12,000g for 10 min at 4°C. Supernatant and pellet fractions were subjected to Western blot analysis.

Western Blotting

5x10⁶ cells were lysed for 10 min on ice in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 10 mM Na₃VO₄, 2 mM PMSF and protease inhibitors, Calbiochem). Protein concentration determination by Lowry assay (Bio-Rad). Lysates were separated by SDS-PAGE and immunoblotted. Primary antibodies were used (with catalog numbers in brackets) were Caspase 8 (1C12, 9746), Caspase 9 (9502), cleaved Caspase 3 (9664), Hsp90beta (5087) and Lamin A/C (2032) from Cell Signalling Technology; Hsp90alpha (mAb 9D2, SPA-840) from Enzo/Stressgen; Actin (sc-1616 rabbit), RPSA (Laminin-R (16), sc-10157) and SET (IP2P2A, sc-5655) from Santa Cruz; Cytochrome C (Clone 7H8,2C12, 556443) from BD Pharmingen; Bax (anti-Bax NT, 06–499), 14-3-3 beta (anti-Bax NT, 06–499), 14-3-3 gamma (clone CG31-2B6, 556443) from BD Pharmingen; and Lamin A/C (2032) from Cell Signalling Technology.

Immunofluorescence Microscopy

1.5-2x10⁶ erythroblasts were left to adhere on poly-L-lysine coated coverslips (mol wt 70,000-150,000, 0.01% w/v solution; Sigma) for 30 min at 37°C, 5% CO₂ before fixation using 4% formaldehyde (TAAB Laboratories Ltd, Aldermaston, England, UK) in PBS for 15 min. For some experiments, 100 nM MitoTracker® Red CMXRos (Invitrogen) was included. Cells were washed in PBS and then permeabilised with 0.2% (w/v)
Triton X-100 in PBS for 5 min or ice-cold methanol for 1 min. Cells were washed in PBS, blocked for 20 min in PBS-4% BSA and incubated for 1 hour in primary antibodies diluted in PBS-1% BSA. After further washes in PBS, cells were incubated for 1 hour with secondary antibodies in PBS-1% BSA. After 3 X 5 min washes in PBS, cells were stained with Hoechst (2 mg/ml, Invitrogen) for 5 min, washed and mounted over MOWIOL 4-88 (Calbiochem) containing 0.6%, 1,4-diazabicyclo-(2.2.2)octane (DABCO, Sigma) as an anti-photobleaching agent. Confocal microscopy was performed using a Leica AOBS SP2 confocal microscope (x63/1.4 oil-immersion objective). A serial Z stack at 0.5 μm intervals was taken and a projected image produced using Leica software. The primary antibodies used include Bak-NT and Bax-NT (Upstate Cell signalling), and BRIC256 (Glycophorin A). The secondary antibodies were Goat anti-Mouse or anti-Rabbit, Alexa 488 or Alexa 594 (Invitrogen).

Sample Preparation for 2D-DIGE

Cell pellets (4.5-8 X 10^6 erythroid progenitors per pellet) were resuspended in 2D lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS), sonicated in a water bath for 15 min and incubated for 2 hour at room temperature with intermittent vortexing. Solubilised samples were then precipitated using a 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer’s instructions and the resulting pellets were resuspended to a concentration of between 5 and 10 mg/ml in DIGE lysis buffer (30 mM Tris, pH 8.5, 7 M Urea, 2 M Thiourea, 4% (w/v) CHAPS). 50 μg of each sample was labeled for DIGE analysis using fluorescent cyanine dyes according to the manufacturer’s guidelines (GE-Healthcare). In brief, samples were labeled using Cy3 or Cy5 N-hydroxysuccinimide (NHS) ester DIGE dyes freshly dissolved in anhydrous dimethylformamide by mixing 50 μm protein with 1 μL CyDye (400 pmol/μL). An internal standard was generated by pooling all samples in the experiment and labelling with a third dye, Cy2. In each case, the labelling reaction was allowed to proceed on ice in the dark for 30 min. The reaction was terminated by the addition of 10 nmol lysine and subsequent incubation on ice for the dark for an additional 10 min.

2D Gel Electrophoresis

Each Cy3- and Cy5-labelled sample pair was mixed with an aliquot of the Cy2-labelled internal standard and Destreak rehydration solution (GE Healthcare) containing 0.5% (v/v) IPG Buffer pH3-11NL added to give a total volume of 450 μL. This was loaded onto a 24 cm Immobiline DryStrip gel (pH 3-11 non-linear) by passive rehydration for a minimum of 12hour. Following rehydration, the DryStrip gel was transferred to an Ettan IPGPhor 3 system (GE Healthcare) and isoelectric focusing performed according to the manufacturer’s instructions (in brief, by applying 500 Volts for 1 hour, increasing to 1,000 Volts over 1 hour, and then to 10,000 Volts over 3 hours and held at 10,000V for a further 2.5 hour). After isoelectric focusing, strips were equilibrated in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromphenol blue) containing 1% (w/v) DTT for 15 min at room temperature followed by a second incubation in SDS equilibration buffer containing 2.5% (w/v) iodoacetamide for 15 min at room temperature. After equilibration, strips were applied to 12.5% (w/v) SDS-PAGE gels and run at 5 mA per gel for 1 hour, 8 mA per gel for an additional hour and then at 13 Watts/gel until completion on an Ettan DALT-6 separation unit (GE Healthcare). Each gel was scanned at three separate wavelengths using a Typhoon 9400 variable mode imager (GE Healthcare) to generate Cy3, Cy5 and Cy2 images. Determination of protein spot abundance and analysis of protein expression changes between samples was conducted on DeCyder V6.5 software (GE Healthcare). Spots which were present in all samples and which showed a change in average ratio of +1.3 or -1.3 fold with a t-test of p≤0.05 were chosen for identification by mass spectrometry. Analysis of the DIGE gels using the DeCyder software identified 2437 spots in the master gel; of these, 1569 were reproducibly detected and quantified in all 4 gels used in the experiment. Only spots that were detected in all 4 gels (i.e. in all 4 independent DIGE experiments) were selected for identification by mass spectrometry.

Proteolytic Digestion and Mass Spectrometry

For preparative gels, pooled samples were generated by combining 100 μg of each SDL or ESDL sample prior to DIGE labelling. Following 2D-PAGE (as above), the resulting gels were stained using SYPRO® Ruby total protein stain (Invitrogen) and visualised using a Typhoon 9400 variable mode imager (GE Healthcare). Spots selected for Mass spectrometry were picked using the Investigator ProPic Automated 2-D spot picker and digested with trypsin using the ProGest automated digestion unit (both from Digilab UK Ltd). The resulting peptides were then subjected to Mass Spectrometry. Mass spectra were recorded in positive ion mode on an Applied Biosystems 4700 MALDI mass spectrometer. MS spectra were recorded in reflector mode. For MSMS analysis the top 5 most intense, non-tryptic, precursors were selected for fragmentation by collision induced dissociation. Neither baseline subtraction nor smoothing were applied to recorded spectra. MS and MSMS data were analyzed using GPS Explorer 3.5 (Applied Biosystems). MS peaks were filtered with a minimum signal to noise ratio of 35 and to exclude masses derived from trypsin autolysis. MSMS peaks were filtered to exclude peaks with a signal to noise ratio less than 35 over a mass range of 50Dalton to 20Dalton below the precursor mass. The mass spectral data for each spot was subjected to a combined analysis using the Mascot algorithm (Matrix Science) against the NCBI nr Human database. The combined analysis uses the initial MS spectra as a peptide mass fingerprint with supporting sequence data provided by up to 5 MSMS spectra per spot. A maximum number of missed cleavages of 1 and a charge state of +1 were assumed for precursor ions. A precursor tolerance of 100 ppm and an MSMS fragment tolerance of 0.15Dalton were used in the database search. Routinely, samples were analysed with methionine oxidation considered as a variable modification and carbamidomethylation of cysteine as a fixed modification.

Protein Phosphorylation

To analyse changes in protein phosphorylation between control (ESDL) and test (SDL) samples, two 2D preparative gels were prepared as above containing pooled protein (100 μg each) of the 4 ESDL or SDL cultures. These were stained for phosphoproteins using Pro-Q Diamond phosphoprotein stain (Invitrogen) and imaged using a Typhoon 9400 Variable Mode Imager (GE Healthcare). Gels were then stained for total protein using SYPRO® Ruby protein gel stain (Invitrogen) and imaged again. Differences in the pattern of protein phosphorylation were identified using ImageQuant v5.2 software and the corresponding spots were excised from the SYPRO® Ruby stained gel and identified by mass spectrometry (as described above). The 12 hour SDL 2D PAGE gels were reproduced again in duplicate, using 400 μg protein loaded per gel from two further independent experiments. The equivalent spots were then subjected to Nano LC Mass Spectrometry. Briefly, selected spots were excised and subjected to in-gel tryptic digestion using a ProGest automated digestion unit (Digilab UK). The resulting peptides were
Figure 1. Epo removal induces apoptosis of erythroblasts. A) Cytospins of 2 separate erythroblast cultures obtained after 9 days in culture in ESDL medium and of erythroblasts after 24 hours in culture in SDL medium. FL2 fluorescence (x axis) versus cell number (y axis) of cells labelled with the isotype control antibody (dotted grey line) and antibodies against CD117/c-kit, CD71, GPA (BRIC256) and Band 3 (BRIC6) (thick black line). B) Flow cytometry analysis of Annexin V (FL1) and Propidium Iodide (PI, FL3) labelling of erythroblasts kept for 24 hours in ESDL or SDL. In this representative experiment, 90% of the cells kept in ESDL are alive (Annexin V and PI negative) compared to only 28% in SDL. C) Graph showing the average percentage of live erythroblasts kept for 24 hours in ESDL or SDL, normalised to the percentage of live cells in ESDL. After 24 hours, only 37% of the cells in SDL are live (Annexin V/PI negative; +/− 15%, n = 15). D) Flow cytometry analysis of mitochondrial membrane potential (ΔΨm) using TMRE. In this representative experiment, the FL2 fluorescence for erythroblasts cultured for 24 hours in ESDL (thick black line) is overlayed with that of cells cultured for 24 hours in SDL (dotted grey line). The loss of TMRE fluorescence indicates a loss of mitochondrial membrane potential (ΔΨm). E) Western blots against cytochrome c and total Bax were carried out on the cytosolic fraction depleted of all organelles, obtained from erythroblasts kept for 24 hours in ESDL or SDL. Beta actin was used as a loading control. G) Overlay projections of confocal images taken from cells cultured for 24 hours in ESDL or SDL showing that cells cultured for 24 hours in the absence of Epo have lost their plasma membrane integrity and have fragmented nuclei. doi:10.1371/journal.pone.0038356.g001
Fractionated using a Dionex Ultimate 3000 nanoHPLC system. In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Dionex). After washing with 0.5% (v/v) acetonitrile 0.1% (v/v) formic acid peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Dionex) over a 120 min organic gradient with a flow rate of 300 nl min⁻¹. Peptides were ionised by nano-electrospray ionisation at 2.0 kV using a stainless steel emitter with an internal diameter of 30 μm (Thermo Scientific). Tandem mass spectrometry analysis was carried out on a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). The Nano LC was set to analyse the survey scans at 60,000 resolution and the top twenty ions in each duty cycle selected for MSMS in the LTQ linear ion trap. Data was acquired using the Xcalibar v2.1 software (Thermo Scientific). The raw data files were processed using Proteome Discoverer software v1.2 (Thermo Scientific) with searches performed against the SwissProt Human database (54523 entries) using the Mascot search engine v1.9 (Matrix Science) with the following criteria; peptide tolerance = 10 ppm, trypsin as the enzyme, carbamidomethylation of cysteine as a fixed modification and oxidation of methionine and phosphorylation of serine, threonine and tyrosine as variable modifications. Individual ions with Mascot scores higher than 20 were used, making sure the average peptide scores of all identified proteins exceeded 20, a threshold commonly used for confident protein identification from tandem MS data [24]. The reverse database search option was enabled and all data was filtered to satisfy false discovery rate (FDR) of less than 5%.

Results

Apoptosis Occurs in Response to Epo Withdrawal in Cultured Primary Human Erythroblasts

Primary human erythroblasts were cultured from CD34⁺ cells isolated from human peripheral blood in presence of Epo, SCF, Dexamethasone and lipids (ESDL) [21]. This expansion medium

![Figure 2. Caspase activation after Epo removal.](image-url) Western blotting (A) of total cell lysates from one independent culture, harvested after 6 hour, 12 hour and 24 hour in ESDL (+Epo) and SDL (-Epo) using an antibody against cleaved caspase 3, caspase 9, caspase 8, and hsc70 was used as a loading control. 20 μg of protein lysate was loaded in each well (B) Flow Cytometry analysis of active caspase 8, active caspase 3 and active caspase 9 for ESDL (thick black line) grown cells and SDL (dotted grey line) after 24 hours. Active caspase 9 was detected on a separate culture from the caspase 3 and caspase 9 and using a different flow cytometer.

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(ESDL) allows CD34\(^+\) cells to expand and differentiate to the pro-erythroblast stage, whilst limiting pro-erythroblast terminal differentiation. During culture in ESDL, expanding erythroblasts become progressively GPA positive but maintain low or no expression of the early to late differentiation marker band 3 (Figure S1A). Importantly, throughout the ESDL culture conditions, the cells remained highly sensitive to Epo withdrawal (Figure S1B). The day 9 time point of expansion was chosen as this maximised the number of cells for our 2D DIGE experiments but limited the degree of spontaneous differentiation. At day 9 the majority of cells had the morphology of pro-erythroblasts (Figure 1A; counting 40 fields of view from each of two representative cultures; 2–4% pro-erythroblasts, 79–80% pro-erythroblasts, and 16–19% basophilic erythroblasts) and are c-kit\(^+\) positive, CD71\(^{high}\), GPA\(^{low/med}\) and band 3\(^{low/neg}\) (Figure 1B and Figure S1A). The cells are also Fas positive but Fas ligand negative, which is consistent with them being immature erythroblasts [25] (Figure S2).

To monitor i) specific alterations in Epo signalling that cannot be compensated by SCF or dexamethasone and ii) the effect of Epo withdrawal on cellular processes, day 9 cells were either maintained in ESDL (+Epo) or SDL (no Epo). Apoptosis and loss of mitochondrial membrane potential was measured by flow cytometry using annexin V/PI and TMRE, respectively (Figure 1C,1D and 1E). After 24 hour of Epo withdrawal, 63% (/+15%, n = 15) of the cells were Annexin V positive and a sharp decrease in mitochondrial potential was observed, indicative of apoptosis (Figure 1D and 1E). Loss of mitochondrial membrane potential was accompanied by cytochrome c release from the mitochondria (Figure 1F) and by translocation of cytosolic Bax to the mitochondria (Figure 1H). Epo removal further induced DNA condensation and nuclei fragmentation as well as reduced cortical actin and Glycophorin A staining (Figure 1G), indicating that these cells have lost the integrity of their plasma membrane. We also confirmed by Western blotting and flow cytometry that caspase 3, caspase 8 and caspase 9 were activated upon 24 hours of Epo removal (Figure 2) [26].
Table 1. Proteins with altered abundance in SDL with a change in average ratio of >2 and a t-test of p<0.05.

| Spot No. | t-test | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | p/ | No of peptides matched | Mascot Protein Score | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|--------|---------------|---------------------|---------------------------|---------------------|----|-----------------------|---------------------|---------------------|---------------------|----------|
| 1        | 0.016  | 3.58          | SET isoform 2       | gi|170763498 (SET, 6418)   | ~ 35               | 32 | ~4.2                   | 4                   | 9                   | 332                 | 1063.6047 |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 41       |
| 2        | 0.0074 | 3.55          | 14-3-3 gamma        | gi|5726310 (YWHAH, 7532) ~ 25 | 28.4               | ~4.9| 4.6                   | 9                   | 209                 | 1205.6559 | 4        |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 1208.6045 |
| 3        | 0.0043 | 3.54          | 14-3-3 beta/alpha   | gi|4507949 (YWHAH, 7529) ~ 25 | 28               | ~4.8| 4.7                   | 13                  | 308                 | 1205.6559 | 17       |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 1261.6106 |
| 4        | 0.025  | 3.27          | heterogeneous nuclear ribonucleoproteins A2/B1 isoform A2 | gi|4504447 (HNRNPA2B1, 3181) ~ 25 | 36               | ~9 | 8.7                   | 15                  | 367                 | 1013.4434 | 33       |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 1188.6471 |
| 5        | 0.037  | 3.03          | heat shock protein HSP 90-alpha isoform 2 | gi|154146691 (HSP90AA1, 3320) ~ 35 | 84.6           | ~4.3| 4.9                   | 12                  | 497                 | 1589.8759 | 110      |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 1778.9475 |
| 6        | 0.025  | 2.73          | 40S ribosomal protein SA | gi|8845502 (RPSA, 3921) ~ 29 | 32.8           | ~5.25| 4.7                   | 15                  | 500                 | 1203.6481 | 81       |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 1698.8599 |
| 7        | 0.023  | 2.71          | heat shock protein HSP 90-beta | gi|20149594 (HSP90AB1, 3326) ~ 30 | 83.3           | ~4.4| 4.9                   | 19                  | 593                 | 1194.6477 | 74       |
|          |        |               |                     |                           |                     |     |                       |                     |                     |                     | 1808.9581 |
| 8        | 0.00088 | 2.38        | 14-3-3 epsilon      | gi|5803225 (YWHAE, 7531) ~ 25 | 29.2           | ~4.7| 4.5                   | 13                  | 303                 | 1205.6559 | 30       |
| Spot No. | t-test | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kDa) | p/I | No of peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|---------|--------|---------------|---------------------|---------------------------|----------------------|-----|------------------------|-----------------------------|---------------------|----------------------|----------|
| 9       | 0.00045| 2.19          | Myosin 9            | gi|12667788 (MYH9, 4627) | 100                   | 226.5 | 5.5                    | 19                          | 185                 | YLAEFATGNDR      | 96       |
| 10      | 0.02   | 2.14          | stathmin isoform a  | gi|5031851 (STMN1, 3925) | 15                    | 17.3  | 5.9                    | 1                           | 81                  | ASGQAFELILSPR    | 75       |
| 11      | 0.019  | 2.05          | eukaryotic initiation factor 4A-I | gi|4503529 (EIF4A1, 1973) | 42                    | 46.1  | 5.3                    | 18                          | 419                 | GIDVQQVSLVINYDLPTNR | 80       |

Characterization of proteins fractionated by 2D-PAGE and identified by both the DeCyder software (all gels, independent t-test, change in average ratio of >2 with a t-test of p<0.05) and Mass Spectrometry, ranked in order of change in average ratio. From the 11 spots up-regulated in SDL, 11 different proteins were identified. Spot No. is number of the spot picked and shown on an SDL 2D gel (Figure 3A); t-test and change in average ratio calculated by the DeCyder 2D Differential Analysis software when comparing the four SDL with the four ESDL 2D gels; Identified Proteins: full name of the protein identified by mass spectrometry, including isoform, as given by NCBI; NCBI Accession number and Gene ID number together with the official symbol provided by HUGO Gene Nomenclature Committee (HGNC) in brackets; Experimental (E) & Theoretical (T) Molecular mass (in kDa) and p/I: the experimental (E) molecular mass and p/I were determined by eye for each spot picked; whereas the theoretical (T) molecular mass and p/I were determined using EditSeq (DNA lasergene 8) on the protein sequence of gi number identified; Number of peptide matched: total number of unique peptides matched to the protein identified; Mascot protein score: the protein score for that gi number is given. Protein score is defined as -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 66 (>66) are considered significant identifications (p<0.05). Where MSMS was performed, the calculated precursor ion mass and resulting peptide sequences are shown together with the corresponding ion score. The list of all the peptides identified for each spot is given in Table S1.

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Table 2. Proteins with altered abundance in SDL (all gels, change in average ratio between 1.3 and 2, with a t-test of \( p < 0.05 \)).

| Spot No. | t-test | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | \( p/ \) | No of peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|--------|---------------|---------------------|---------------------------|----------------------|--------|------------------------|---------------------------|------------------|---------------------|----------|
| 12       | 0.0083 | 1.74          | splicing factor 3A 677ptsubunit 1 isoform 1 | gi|5032087 | SF3A1, 10291 | −75 | 88.9 | −4.75 | 5.1 | 14 | 109 | 905.5203 | LTAQFVAR | 15 |
| 13       | 0.0055 | 1.71          | Actin, beta         | gi|4501885 | ACTB, 60 | −42 | 42 | −5.6 | 5.3 | 10 | 312 | 1198.7054 | AVFPSVGRPR | 41 |
| 13       | 0.0055 | 1.71          | Actin, gamma1       | gi|4501887 | ACTG1, 71 | −42 | 42 | −5.6 | 5.3 | 10 | 312 | 1198.7054 | AVFPSVGRPR | 41 |
| 14       | 0.013  | 1.71          | lamin-A/C isoform 2 | gi|5031875 | LMNA, 4000 | −25 | 65 | −5.75 | 6.7 | 28 | 561 | 849.4828 | LAVYIDR | 34 |
| 15       | 0.018  | 1.65          | peptidyl-prolyl cis-trans isomerase FKBP4 | gi|4503729 | FKBP4, 2288 | −50 | 51.8 | −5.25 | 5.3 | 20 | 313 | 1697.8717 | RGEAHLAVNDFLAR | 56 |
| 15       | 0.0016 | 1.62          | heterogeneous nuclear 677ptribonucleoproteins C1/C2 isoform b | gi|117190174 | HNRPC, 3183 | −36 | 32.3 | −5.2 | 4.8 | 9 | 218 | 943.5723 | VPPPPiAR | 56 |
| 16       | 0.0016 | 1.62          | transformer-2 protein 677ptribomolog beta | gi|4759098 | TRA2B, 6434 | −36 | 33.7 | −5.2 | 11.3 | 6 | 133 | 1329.6586 | GFAPVQYVNER | 85 |
| 17       | 0.0092 | 1.6           | protein CtnA isoform 2 | gi|7706244 | CUTA, 51596 | −14 | 16.8 | −4.5 | 5.1 | 2 | 85 | 1533.8279 | TQSSLVPALTFDR | 74 |
| 17       | 0.0092 | 1.6           | U6 snRNA-associated 677ptm-like protein LSm3 | gi|7657315 | LSM3, 27258 | −14 | 11.8 | −4.5 | 4.5 | 3 | 83 | 1005.5549 | NIPMLFVR | 13 |
| 18       | 0.021  | 1.43          | cofilin-1           | gi|5031635 | CFL1, 1072 | −15 | 18.5 | 5.8 | 8 | 12 | 296 | 1309.682 | AVLFCSEDKK | 15 |
| 19       | 0.015  | 1.42          | cofilin-1           | gi|5031635 | CFL1, 1072 | −15 | 18.5 | −5.65 | 8.1 | 10 | 294 | 1309.682 | AVLFCSEDKK | 45 |

Proteomic Analysis of Apoptotic Erythroblasts
Table 2. Cont.

| Spot No. | t-test  | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | p/No of peptides matched | Mascot Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|---------|---------------|---------------------|---------------------------|---------------------|-------------------------|-------------------|----------------------|-----------------------|----------|
| 20       | 0.014   | 1.36          | peptidyl-prolyl cis-trans isomerase FKBP4 | gi|4503729 (FKBP4, 2288) | −52 51.8 | −5.3 5.3 21 455 | 1059.4928 | LYNMFER | TQLAVCOOR 37 | 1 |
|          |         |               |                     |                           |                     |                         |                   | 1103.5626 |                     | 98 |
|          |         |               |                     |                           |                     |                         |                   | 1697.8717 |                     | 143 |
| 21       | 0.0063  | 1.33          | actin, beta         | gi|4501885 (ACTB, 60) | −40 42 | −5.4 5.3 13 187 | 1132.527 | GYSFTTAAE | AVFPSIVGRPR 10 | 2 |
|          |         |               |                     |                           |                     |                         |                   | 1198.7054 |                     | 29 |
|          |         |               |                     |                           |                     |                         |                   | 1516.7026 | SYELPDQGQTVGNR 51 |         |
| 22       | 0.0068  | 1.33          | carbonic anhydrase 1 | gi|4502517 (CA1, 759) | −75 28.9 | −6.5 6.9 8 258 | 1580.7915 | ESIVSSEQLAQR | EIINVGHSFHVNFEDDNIR 97 | 105 |
|          |         |               |                     |                           |                     |                         |                   | 2256.0427 |                     |         |
| 22       | 0.0068  | 1.33          | GMP synthase        | gi|4504035 (GMPS, 8833) | −75 76.7 | −6.5 6.7 17 160 | 1118.8686 | VYFDPVPAVR | HPFFGPGGLAIR 41 | 20 |
|          |         |               |                     |                           |                     |                         |                   | 1161.6527 |                     |         |
| 22       | 0.0068  | 1.33          | Phosphoenolpyruvate carboxykinase | gi|66364721 (PCK2, 5106) | −75 70.7 | −6.5 7.6 18 126 | 1118.5894 | IFHVNWFR | 17 |

Characterization of proteins fractionated by 2D-PAGE and identified by both the DeCyder software (all gels, independent t-test, change in average ratio between 1.3 and 2, with a t-test of p≤0.05) and Mass Spectrometry (MS), ranked in order of change in average ratio. From the 11 spots picked, 13 different proteins were identified by MS. A full list of all the peptides identified for each spot is given in Table S2.

doi:10.1371/journal.pone.0038356.t002
Figure 4. Western blot confirmation of protein proteolysis under SDL conditions. Western blotting of total cell lysates harvested from one independent culture after 6 hour, 12 hour and 24 hour in ESDL (+Epo) and SDL (-Epo) using antibodies against SET, 14-3-3 β, 14-3-3 γ, 14-3-3 ε, RPSA, Hsp90 isofoms alpha and beta. 20 μg of protein lysate was loaded per lane. Beta Actin and Hsc70 were used as loading controls. The arrows point to the smaller proteolytic fragments that occur in apoptotic erythroblasts.

doi:10.1371/journal.pone.0038356.g004
Table 3. Proteins with altered abundance in ESDL (all gels, change in average ratio below -1.3, with a t-test of \( p<0.05 \)).

| Spot No. | t-test | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | \( p/ \) | No of peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|--------|---------------|---------------------|---------------------------|---------------------|------|----------------------|----------------------|-------------------|---------------------|----------|
| 30       | 0.0052 | -1.42         | transcription factor BTF3 isoform A | gi|83641885 (BTF3, 689) | ~17 | 22.2 | ~7 | 9.4 | 10 | 434 | 863.5349 | LQFSLKK | 22 |
|          |        |               |                     |                           |                     |      |         |         |      |     |         |          |            |          |
| 31       | 0.013  | -1.37         | transcription factor BTF3 isoform A | gi|83641885 (BTF3, 689) | ~17 | 22.2 | ~6.4 | 9.4 | 7 | 233 | 863.5349 | LQFSLKK | 14 |
|          |        |               |                     |                           |                     |      |         |         |      |     |         |          |            |          |
| 32       | 0.0023 | -1.35         | SUMO-activating enzyme subunit 1 isoform a | gi|4885585 (SAE1, 10055) | ~37 | 38 | ~5.2 | 5.1 | 13 | 113 | 2214.1289 | NDVLSLGSPDLPEDPVR | 16 |
|          |        |               |                     |                           |                     |      |         |         |      |     |         |          |            |          |
| 33       | 0.017  | -1.35         | 40S ribosomal protein SA | gi|9845502 (RPSA, 3921) | ~40 | 33 | ~4.6 | 4.7 | 16 | 693 | 912.5313 | LLVTDIPR | 41 |
|          |        |               |                     |                           |                     |      |         |         |      |     |         |          |            |          |
| 34       | 0.046  | -1.34         | polypyrimidine tract-binding protein 1 isoform c | gi|14165466 (PTBP1, 5725) | ~50 | 57 | ~9 | 9.2 | 13 | 536 | 991.5432 | HQNQVLPR | 54 |
|          |        |               |                     |                           |                     |      |         |         |      |     |         |          |            |          |
| 35       | 0.042  | -1.31         | heat shock protein 105 kDa | gi|42544159 (HSHPH1, 10808) | ~100 | 97 | ~5.25 | 5.2 | 23 | 267 | 1418.6586 | NAVEYVEFPR | 60 |
|          |        |               |                     |                           |                     |      |         |         |      |     |         |          |            |          |

Characterization of proteins fractionated by 2D-PAGE and identified by both the DeCyder software (all gels, independent t-test, change in average ratio below -1.3, with a t-test of \( p<0.05 \)) and Mass Spectrometry (MS), ranked in order of change in average ratio. From the 6 spots picked, 5 different proteins were identified by MS. A full list of all the peptides identified for each spot is given in Table S3.

doi:10.1371/journal.pone.0038356.t003
Table 4. Proteins with altered abundance in SDL (3 gels, independent t-test, change in average ratio above >1.3, with a t-test of $p<0.05$).

| Spot No. | t-test  | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | $p$/ | No of peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|---------|---------------|---------------------|----------------------------|---------------------|-----|------------------------|--------------------------|-------------------|-----------------------|----------|
| 23       | 0.019   | 1.88          | flavin reductase    | gi|4502419 (BLVRB, 645)      | ~15                 | 22.1 | ~7                    | 7.5                      | 6                 | 256                   | 1167.6117 | LQAVTDDHJR         | 62       |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |
| 24       | 0.029   | 1.76          | ubiquitin-conju-gating enzyme E2 | gi|40806164 (UBE2V1, 7335) | ~15                 | 19.3 | ~3.7                  | 9.6                      | 15               | 225                   | 2345.5625 | VVLoELR            | 21       |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |
| 25       | 0.00077 | 1.73          | serine/threonine-protein phosphatase PP1-alpha catalytic subunit | gi|4506003 (PPP1CA, 5499) | ~35                 | 37.5 | ~5.75                 | 6.1                      | 19               | 396                   | 1439.8046 | IKYPENFLLR          | 81       |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |
| 26       | 0.0068  | 1.41          | enoyl-CoA hydratase, mitochondrial precursor | gi|194097323 (ECHS1, 1892) | ~25                 | 31.4 | ~5.6                  | 8.1                      | 8                | 287                   | 1163.5957 | HWHDLTQVK          | 17       |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |
| 27       | 0.047   | 1.36          | cytochrome c oxidase subunit 4 | gi|45023981 (COX4I1, 1327) | <15                 | 19.6 | ~9                    | 9.5                      | 11               | 104                   | -        | -                   | -        |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |
| 28       | 0.00082 | 1.31          | haloadic dehalogenase-like hydrolase domain containing 3 | gi|13654296 (HDHD3, 81932) | ~25                 | 28   | ~6                    | 6.5                      | 11               | 372                   | 876.4937 | IFQEALR             | 40       |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |
| 29       | 0.046   | 1.31          | ubiquitin-conju-gating enzyme E2 N | gi|4507093 (UBE2N, 7334) | ~13                 | 17.1 | ~5.75                 | 6.4                      | 13               | 368                   | 9705.468 | WSPALQIR            | 25       |
|          |         |               |                     |                            |                     |      |                       |                          |                  |                       |          |

Characterization of proteins fractionated by 2D-PAGE and identified by both the DeCyder software (3 gels, independent t-test, change in average ratio above >1.3, with a t-test of $p<0.05$) and Mass Spectrometry (MS), ranked in order of change in average ratio. From the 7 spots picked, 7 different proteins were identified by MS. A full list of all the peptides identified for each spot is given in Table S4.

doi:10.1371/journal.pone.0038356.t004
Proteomic Analysis of Apoptotic Erythroblasts

Furthermore, no Fas Ligand was detectable on pro-erythroblasts by flow cytometry after 24 hours Epo removal (Figure S2).

2D DIGE Analysis of Primary Human Erythroid Cells 12 Hour After Epo Withdrawal

Proteins identified with a change in average intensity ratio of >2. To identify global proteome alterations in erythroblasts after Epo removal, a 2D DIGE approach was adopted. Late apoptotic events involve the proteolysis of many proteins and signalling events not directly involved in the initial induction of apoptosis. After 12 hours in SDL (no Epo), the first signs of apoptosis were observed by flow cytometry as the cells become Annexin V positive but are not yet TMRE low or PI+ (Figure S3). Therefore, to study the early events leading to apoptosis, rather than the later downstream events, we studied proteome changes after 12 hours of Epo removal by comparing the proteomes of erythroblasts kept in expansion medium (ESDL, 12 hours) with those switched to medium lacking Epo for 12 hours (SDL, 12 hours). By comparing 4 independent 2D DIGE experiments, 12 spots were consistently found to be up-regulated in SDL (apoptotic cells) with a change in average intensity ratio of >2 and a t-test of p<0.05. All 12 spots were picked from the gels (SDL, Figure 3A), and the identities of 11 of these spots were confirmed by mass spectrometry (Table 1 and Table S1). These include SET, 14-3-3 isoforms (beta, gamma and epsilon), Hsp90 alpha (Figure 3B) and beta, 40S ribosomal protein SA (RPSA) and non-muscle myosin heavy chain (myosin 9). Figure 3C illustrates the reproducibility of the observed alterations in abundance, showing the quantification of Hsp90 alpha isoform 2 (spot 5). For specific spots (e.g. spots 5 (Hsp90 alpha), 7 (Hsp90 beta) and 9 (Myosin 9)), the molecular weight of the spot picked for MS analysis was significantly smaller than the theoretical molecular weight of the full-length protein (Table 1), possibly as a result of caspase cleavage. Indeed, 9 out of the 11 proteins identified are known caspase substrates. Western blot analysis of total cell lysates from a different independent experiment validated the 2D DIGE proteomic results confirming the proteolysis of SET, 14-3-3 beta, 14-3-3 gamma, 14-3-3 epsilon, Hsp90 alpha, Hsp90 beta and RPSA upon Epo withdrawal (Figure 4).

Proteins identified with a change in average intensity ratio of >+/−1.3. When the cut-off value of change in average intensity ratio was lowered to >+1.3 (t-test of p<0.05) an additional 19 spots exhibited an increased abundance in SDL. Of these, 14 were picked from the gels (the 5 other spots were deemed too faint to pick) and 11 were identified by mass spectrometry (Table 2 and Table S2). These include splicing factors, actin and actin binding protein, colin-1, lamin A/C (cleaved), peptidyl-propyl cis trans isomerase FKBP4 and carbonic anhydrase. Western blot analysis confirmed proteolysis of lamin A/C during Epo withdrawal (Figure 4). 13 spots were found to be up-regulated in ESDL with a change in average ratio below <-1.3 and a t-test of p<0.05. Of these, 6 were picked and identified by mass spectrometry, including BTF3, a SUMO activating enzyme, RPSA (full length), polypyrimidine tract binding protein1 and Hsp105 (Table S3).

By monitoring the intensity of each spot across the 4 independent 2D DIGE experiments, we observed that spots from one sample pair (SDL and ESDL) were consistently outliers (see Figure 3C for an example of the Hsp90 alpha result). After exclusion of one sample pair, an additional 10 spots were found to be up-regulated in SDL with a change in average ratio above >1.3 and a t-test of p<0.05. Of these, 7 were picked and identified by mass spectrometry (Table 2 and Table S4) including several enzymes, ubiquitin-conjugating enzyme E2, and the serine/threonine protein phosphatase PP1-alpha catalytic subunit. Furthermore, after exclusion of this sample pair, an extra 20 spots had increased abundance in ESDL with a change in average ratio below <-1.3 and a t-test of p<0.05. Of these, 18 spots were picked from the gels, of which 14 were identified by mass spectrometry (Table 3 and Table S3) including secretory pathway proteins (clathrin and dynactin), Hsp70, the Hsp90 co-chaperone p23, lamin A/C (full length), splicing and ribonuclease proteins, and the serine/threonine protein kinase PAK2.

Investigating the changes in protein phosphorylation within the proteome after Epo withdrawal revealed 13 phospho-protein changes between ESDL and SDL (Figure 5). Of these 13 spots, 9 had increased phosphorylation in SDL compared to ESDL and these were identified by mass spectrometry (Table 6 and Table S6). The proteins detected as having potentially increased phosphorylation in SDL conditions included nascent polypeptide...
## Table 5. Proteins with altered abundance in ESDL (3 gels, independent t-test, change in average ratio below <-1.3, with a t-test of p<0.05).

| Spot No. | t-test | Average ratio | Identified Proteins | Accesion number (Gene ID) | Molecular mass (kD) | P/ | No of peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|--------|---------------|---------------------|--------------------------|---------------------|----|-----------------------|--------------------------|---------------------|----------------------|-----------|
| 36       | 0.024  | -1.71         | clathrin light chain A | gi|4502899 (CLTA, 1211) | ~30                | 23.7 | ~4.3               | 4.3                       | 9                  | 189                  | 1095.5105 | ELEEWYAR | 28         |
|          |        |               |                     |                          | 1407.7267          | AKKEEELYWAR        | 17            |
|          |        |               |                     |                          | 2352.0261          | AAEAFVDNIDESPGTEWER | 74            |
| 37       | 0.038  | -1.49         | dynactin subunit 2   | gi|5453429 (DCTN2, 105-40) | ~48                | 45               | ~5.2              | 5                        | 19                 | 210                  | 1598.8748 | VSAICLAVLQOEAVR | 43        |
|          |        |               |                     |                          | 1764.8398          | ENLAVTEGNASIDER    | 9             |
| 38       | 0.00305| -1.41         | heat shock 70 kDa protein 4 | gi|383327039 (HSPA4, 33018) | ~100               | 94               | ~5.1              | 5                        | 29                 | 269                  | 1321.7111 | VLATAFDTLTGGR | 33        |
|          |        |               |                     |                          | 1495.7023          | AGGIETIAEYSDR      | 5             |
|          |        |               |                     |                          | 1735.9265          | EFSITDWPVLPSLR     | 15            |
| 39       | 0.004  | -1.41         | polypyrimidine tract-binding protein 1 | gi|14165466 (PTBP1, 5725) | ~50                | 57               | ~9                | 9.2                      | 21                 | 337                  | 991.5482 | HENVQPLPR | 20         |
|          |        |               |                     |                          | 1431.7379          | GQPYYIQFSNHK       | 34            |
|          |        |               |                     |                          | 2039.0959          | VTPQSVFLFGVYGQDR   | 27            |
|          |        |               |                     |                          | 2243.1204          | NNQOALLQAYDPSAQHAK | 42            |
|          |        |               |                     |                          | 2275.2769          | IARQLAGAGSLLVSLNPER | 35        |
| 40       | 0.0001 | -1.39         | eukaryotic translation initiation factor 4H | gi|11559923 (EIF4H, 7458) | ~27                | 27.4             | ~5.8              | 7.1                      | 10                 | 153                  | 957.5264 | FRDGPPLR | 7          |
|          |        |               |                     |                          | 1393.6958          | EALTYDGALLGDR      | 55            |
|          |        |               |                     |                          | 2351.2578          | TVATPLNQVANPSAIQGPR | 17        |
| 41       | 0.024  | -1.38         | heterogeneous nuclear ribonucleoprotein K | gi|14165437 (HNRNPK, 3190) | ~60                | 51               | ~5.2              | 5.1                      | 17                 | 192                  | 1194.6993 | NLRPLPPPPLPR | 38        |
|          |        |               |                     |                          | 1780.7985          | TQYNAVSVPDSSGPER   | 32            |
| 42       | 0.02   | -1.37         | heterogeneous nuclear ribonucleoproteins C1/C2 | gi|117190174 (HNRPC, 3183) | ~37                | 32.3             | ~5.1              | 4.8                      | 13                 | 240                  | 943.5723 | VPPPPPIARS | 26         |
|          |        |               |                     |                          | 1316.7936          | VFQFLNLFTLVK       | 10            |
|          |        |               |                     |                          | 1329.6586          | GAFVOYVNER         | 66            |
|          |        |               |                     |                          | 2117.8757          | AAEMYGSFVDLGDFR    | 34            |
| 43       | 0.0019 | -1.36         | HSP90 co-chaperone p23 | gi|23308579 (PTGES3, 10728) | ~18                | 18.7             | ~4.3              | 4.1                      | 10                 | 113                  | 921.4644 | SILCCLR | 15         |
|          |        |               |                     |                          | 1305.6936          | VFGGLNLFLVVK       | 10            |
|          |        |               |                     |                          | 1329.6586          | GAFVOYVNER         | 66            |
|          |        |               |                     |                          | 2117.8757          | AAEMYGSFVDLGDFR    | 34            |
| Spot No. | t-test | Average ratio | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | p/ | No of peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|--------|--------------|---------------------|---------------------------|---------------------|----|------------------------|----------------------------|-------------------|----------------------|----------|
| 44       | 0.0086 | -1.35        | ATP-dependent RNA helicase DDX1 | gi|4826868 (DDX1, 1653) | ~80 82.4 ~6.5 7.1 | 23 | 150                    | 1131.5542 | KGE:SGOSWPR | 4        |
| 45       | 0.014  | -1.34        | heterogeneous nuclear ribonucleoproteins C1/C2 | gi|11790174 (HNRPC, 3183) | ~37 32.3 ~5.1 4.8 | 12 | 276                    | 943.5723 | VPPPPPIAR | 20       |
| 46       | 0.00096| -1.33        | lamin-A/C            | gi|5031875 (LMNA, 4000) | ~60 65 ~5.8 6.7 | 25 | 199                    | 1316.7936 | VPGNLNTLVVK | 11       |
| 47       | 0.011  | -1.32        | heterogeneous nuclear ribonucleoprotein K | gi|14159347 (HNRNPK, 3190) | ~60 51 ~5.3 5.1 | 18 | 235                    | 1053.6415 | VVLGKPD | 1        |
| 48       | 0.027  | -1.32        | heterogeneous nuclear ribonucleoproteins C1/C2 | gi|11790174 (HNRPC, 3183) | ~37 32.3 ~5.1 4.8 | 12 | 223                    | 943.5723 | VPPPPPIAR | 19       |
| 49       | 0.027  | -1.32        | alanyl-tRNA synthetase | gi|109148542 (AARS, -110 16) | 107 ~5.25 5.3 | 21 | 155                    | 1408.6743 | AVFDYTEPDR | 33       |
| 50       | 0.032  | -1.32        | Heterogeneous nuclear ribonucleoprotein K | gi|14159347 (HNRNPK, 3190) | ~60 51 ~5.3 5.1 | 16 | 209                    | 1194.6939 | NLPLLPPPPPR | 50       |

**Table 5.**
Table 5. Cont.

| Mascot | No of | Molecular | Average | MSMS Peptide | Ion | Ion score |
|--------|-------|-----------|---------|---------------|-----|-----------|
| No | peptides | mass (kD) | Identified | sequence | score | No |
| | | | protein | | | |
| | | | number | | | |
| | | | precursor | | | |
| | | | ion mass | | | |
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Characterization of proteins fractionated by 2D-PAGE and identified by both the DeCyder software (3 gels, independent t-test, change in average ratio below -1.3, with a test of p < 0.05) and Mass Spectrometry (MS), ranked in order of change in average ratio. From the 18 spots picked, 14 different proteins were identified by MS. A full list of all the peptides identified for each spot is given in Table S5.

Discussion

In this study we sought to determine the global proteome alterations that occur in erythroblasts during Epo withdrawal. We have shown that Fas-L independent cell death occurs in immature erythroblasts during Epo withdrawal and observed activation of both caspase 8 and caspase 9, alongside other classical features of the “intrinsic” apoptosis pathway. Caspase activation and apoptosis proceed through one of two major pathways, namely the ‘extrinsic’ pathway triggered by death receptor ligation and activation of the initiator caspase 8, or the ‘intrinsic’ pathway characterised by mitochondrial outer membrane permeabilisation (MOMP), cytochrome c release into the cytosol and activation of the initiator caspase 9 [27]. Both pathways then converge on activating executioner caspases, such as caspase 3 [27]. There is evidence that crosstalk can occur, as caspase 8 activation leads to cytochrome c release into the cytosol via tBid [28]. Conversely, caspase 8 activation downstream of caspase 9 has been reported [29]. Growth factor withdrawal from haematopoietic cells is generally thought to result in the activation of the mitochondrial pathway [30,31] and our studies are supportive of this. Further studies are required to determine the exact sequence of caspase activation, to specifically delineate which apoptotic pathway (extrinsic or intrinsic) is activated first and whether caspase 8 is activated downstream of caspase 9 or by a death receptor ligand (other than Fas-L) which is yet to be identified.

We used a 2D DIGE proteomic approach to provide a snapshot of the key differences in the proteomes of erythroblasts under continual expansion and erythroblasts undergoing apoptosis due to Epo deprivation. Overall we observed more alterations in protein abundance in apoptotic cells, probably because in this state cellular proteins are undergoing proteolytic cleavage by caspases or putative unknown proteases, and the resulting shift in size makes these proteins more evident in apoptotic cells. Indeed proteins known to be caspase targets were highly represented in our analysis (59% of the proteins identified (i.e. 34 proteins out of 57 in total, Tables 1–7) are known to be cleaved by caspases [32]). It is possible that proteolysis is not solely caspase-mediated but also due to cleavage by other types of proteases activated during apoptosis. However, caspases are the main proteolytic enzymes activated during apoptosis [33] and we confirmed activation of caspses in our culture system upon Epo withdrawal (Figure 3). Furthermore, the molecular weights of the proteolytic fragments detected in the lysates of apoptotic cells (Figure 4) match the associated complex alpha subunit (NACA), Hsp27, Hsp90 alpha and beta, and lamin A/C. The other 4 spots with altered phosphorylation profiles were increased in ESDL and from these 5 proteins were identified by mass spectrometry (Table 7 and Table S7) including matrix-3, nucleolin, splicing factor 1 and an initiation factor.

To confirm phosphorylation and identify possible phospho-sites on Hsp90 alpha and beta proteins, and nascent polypeptide associated complex alpha protein after Epo withdrawal, 2 additional independent 12 hour SDL samples were run on separate preparative 2D gels. Spots corresponding to NACA (spot 1), Hsp90 alpha (spot 4) and Hsp90 beta (spot 6) were picked, pooled, digested and analysed by Nano LC mass spectrometry. This technique confirmed the phosphorylated status of these proteins and we were able to identify several known and novel phospho-peptides (Table 8). It should be noted that for some peptides there was more than one possible phosphorylation site, so we have included all possibilities and the known phosphorylation site within the peptide has been indicated in Table 8.
Table 6. Phosphorylated spots up-regulated in SDL.

| Spot No. | Identified Proteins                                           | Accession number (Gene ID) | Molecular mass (kD) | pI  | No of Peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence       | Ion score |
|----------|---------------------------------------------------------------|----------------------------|---------------------|-----|------------------------|----------------------------|---------------------|--------------------------|-----------|
| 1        | Nascent poly-peptide-associated complex alpha subunit isoform b | gi|5031931 (NACA, 4666) | 23  | 25                     | 23.4                       | 4.4                 | 5  | 111                    | 1484.7267 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1549.8988               | NILFVIKPDVYK 58 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1614.8334               | IEDLSQAOAAAEK 4 |
| 2        | NDUFS3 NADH dehydrogenase (ubi-quinone) Fe-S protein 3        | gi|4758788 (NDUFS3, 4722) | 30  | 25                     | 30.2                       | 4.5                 | 5  | 127                    | 1295.663 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1366.7729               | FENVYNNLSLR 26 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1486.79                 | WAEPEVAQEFIR 21 |
| 2        | HSPB1 heat shock 27 kDa protein 1                             | gi|4504517 (HSPB1, 3315) | 22  | 25                     | 22.8                       | 3.3                 | 9  | 116                    | 1163.6207 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1905.9916               | LATOSNETIPVTFESR 37 |
|          |                                                               |                            |                     |     |                        |                            |                     | 74                       | 1295.663 |
| 2        | Lamin A/C                                                     | gi|5031931 (Lamina, 4008) | 74  | 25                     | 65                | 2.8                 | 16 | 93                     | 1022.5781 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1209.6344               | FVGDPMLR 14 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1664.8903               | DHINLPGFSQUNLR 30 |
|          |                                                               |                            |                     |     |                        |                            |                     | 2009.0127               | LTQAOIIFYGEPNFR 27 |
| 3        | purine nucleoside phosphorylase                               | gi|15718362 (PNP, 4860) | 32  | 25                     | 32                | 4.7                 | 16 | 253                    | 1589.8759 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1778.9475               | HSOFIGYRTLFVEK 5 |
|          |                                                               |                            |                     |     |                        |                            |                     | 2015.0443               | VILHaksiDQTEYLEER 4 |
| 4        | heat shock protein HSP 90-alpha                               | gi|13792990 (HSP90AA1, 3320) | 98  | 25                     | 98               | 4.3                 | 15 | 94                     | 1589.8759 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1778.9475               | HSOFIGYRTLFVEK 5 |
|          |                                                               |                            |                     |     |                        |                            |                     | 2015.0443               | VILHaksiDQTEYLEER 4 |
| 5        | heat shock protein HSP 90-alpha                               | gi|13792990 (HSP90AA1, 3320) | 98  | 25                     | 98               | 4.3                 | 12 | 103                    | 1589.8759 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1778.9475               | HSOFIGYRTLFVEK 5 |
|          |                                                               |                            |                     |     |                        |                            |                     | 2015.0443               | VILHaksiDQTEYLEER 4 |
| 6        | heat shock protein HSP 90-beta                                | gi|20149594 (HSP90AB1, 3326) | 83  | 25                     | 83               | 4.3                 | 16 | 111                    | 1194.6477 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1194.6477               | IDIPNPQER 18 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1194.6477               | IDIPNPQER 18 |
| 7        | heat shock protein HSP 90-beta                                | gi|20149594 (HSP90AB1, 3326) | 83  | 25                     | 83               | 4.3                 | 14 | 133                    | 1194.6477 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1194.6477               | IDIPNPQER 18 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1194.6477               | IDIPNPQER 18 |
|          |                                                               |                            |                     |     |                        |                            |                     | 1311.5699               | EDQTEYLEER 19 |

Proteomic Analysis of Apoptotic Erythroblasts
Characterization of proteins fractionated by 2D-PAGE, stained with Pro-Q Diamond phosphoprotein stain and identified by Image Quant v5.2 software analysis as being hyper-phosphorylated in SDL and then identified by Mass Spectrometry. From the 9 spots picked, 9 different proteins were identified by MS. A full list of all the peptides identified for each spot is given in Table S6.

| Spot No. | Identified Proteins | Precursor ion mass | Mascot Protein score (Ion score) | pI | No of Peptides | Molecular mass (kD) |
|----------|---------------------|-------------------|---------------------------------|----|----------------|--------------------|
| 8        | acdc-huence-ch nuclear phosphoprotein 31 family member B | 1998.9381 HSQFIGYPITLYLEK | 23 | 1808.9581 | 16 | 1566.8163 | 8 acidic leucine-rich nuclear phosphoprotein 32 family member B gi|5454088 (ANP32B, 15kD) |
| 9        | 60s acidic ribosomal protein P0 | 1805.0443 VILHLKEDQTEYLEER | 16 | 1972.9069 | 104 | 1313.71 | 9 60S acidic ribosomal protein P0 gi|4506667 (RPLP0, 35 kD) |

The Hsp90 proteins are chaperones to a multitude of client proteins, most of which are involved in signal transduction (i.e. Jak2, Pim-1, Akt/PKB) and inhibition of Hsp90 disrupts multiple pathways essential to cell survival [40]. Of interest, Hsp90 protects the pro-survival protein Pim-1 from proteasomal degradation [41] and we also found that Epo-withdrawal in our system leads to loss of the pro-survival protein Pim-1 from proteasomal degradation [41] and also noted that the Hsp90, SET, RPSA have all been implicated in signal transduction (i.e. Jak2, Pim-1, Akt/PKB) and inhibition of Hsp90 disrupts multiple pathways essential to cell survival [40].

Many of the changes in protein abundance on Epo withdrawal observed here in our 2D DIGE comparison with living cells, are consistent with the known characteristic alterations that occur during apoptosis, reflecting the universal nature of this fundamental process. Hence, the observed changes in cellular morphology that occur during apoptosis require alterations in actin and myosin cytoskeleton and nuclear lamins, whilst essential house keeping functions such as transcription, translation and the secretory pathway are targeted for destruction (reviewed by [36]). Therefore, the observed alterations in the abundance/cleavage of cytoskeletal proteins (myosin 9, actins, cofilin) and nuclear lamins (lamin A/C), β-NAC (protein translocation to the ER), transcription factors (ELF4A) and secretory pathway proteins were to be expected. However, it is highly significant that many of the novel changes in proteins reported here which were detected as more abundant upon Epo withdrawal are multifunctional proteins such as Hsp90, 14-3-3 isoforms, SET and RPSA that have undergone proteomic changes might not be detected at the 12 hour time point but could occur earlier or later.

It should be noted that although we have identified many cleaved proteins more abundant in apoptotic cells, we only detected the equivalent full length protein in the living cells for RPSA (spot 6 in Table 1 and spot 33 Table 4), Lamin A/C (spot 14 Table 2 and spot 46 Table 5) and Ribonucleoprotein C1/C2. Moreover, we have identified a range of known caspase substrates but candidates such as the GATA-1 [18] were not detected by our analysis. One explanation for this may be that some key changes are obscured or masked by other proteins present on the 2D gel, and it is also likely that alterations in low abundant proteins might not be detected. In addition, approximately 30% of the spots detected as altered in our experiments were not confidently identified by mass spectrometry and this may explain some omissions. Also, the kinetics of proteolysis in response to Epo withdrawal might vary from protein to protein, such that certain proteomic changes might not be detected at the 12 hour time point but could occur earlier or later.
Table 7. Phosphorylated spots up-regulated in ESDL.

| Spot No. | Identified Proteins | Accession number (Gene ID) | Molecular mass (kD) | pI | No of Peptides matched | Mascot Protein Score (>66) | Precursor ion mass | MSMS Peptide sequence | Ion score |
|----------|---------------------|-----------------------------|---------------------|---|-----------------------|---------------------------|----------------------|----------------------|-----------|
| 1        | Matrin-3 isoform a  | gi|21626466 (MATR3, 9782)     | 125                 | -5.6 | 6.1 | 25 | 166 | 1324.6716 | GNLGAGGNLQGPR | 2 |
| 2        | Nucleolin          | gi|55956788 (NCL, 4691) ~ 100 | 77                  | -5.65 | 4.5 | 21 | 189 | 1160.5834 | SISLYYTGEK | 2 |
| 3        | Splicing Factor 1  | gi|2463198* (gi|42544130; gi|42544125; gi|42544123; gi|295842307) (SF1, 7536) | 70 | -9 | 9.1(9-9.5) | 9 | 71 | 1561.8584 | AYVQQIELDLTR | 11 |
| 4        | Eukaryotic translation initiation factor 4E type 2 | gi|4757702 (EIF4E2, 9470) | 27 | 28.4 | 8.3 | 9 | 68 | 1568.7855 | QIGTFASVEQFWR | 2 |
| 4        | Lysophospholipase-like 1 | gi|20270341 (LYPLAL1, 127018) | 27 | 26.3 | 8.3 | 7.8 | 9 | 68 | 1150.564 | GGISNWFDPR | 1 |

Characterization of proteins fractionated by 2D-PAGE, stained with Pro-Q Diamond phosphoprotein stain and identified by Image Quant v5.2 software analysis as being hyper-phosphorylated in ESDL and then identified by Mass Spectrometry. From the 4 spots picked, 5 different proteins were identified by MS. A full list of all the peptides identified for each spot is given in Table S7.

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been reported previously [34] but never for Epo withdrawal. To our knowledge Hsp90 beta isoform cleavage during apoptosis has never been reported. Furthermore, our observation that both Hsp90 isoforms are phosphorylated upon Epo withdrawal is significant because phosphorylation is reported to negatively regulate Hsp90 client protein interactions [42]. Additional studies are required to determine the role of Hsp90 alpha and beta phosphorylation sites during Epo withdrawal and to establish whether phosphorylation is important for inducing caspase-mediated cleavage of Hsp90 or occurs post-caspase cleavage.

The SET and RPSA proteins are less well studied but both proteins are involved in a broad range of cellular processes and were cleaved after Epo withdrawal. SET is also known as the inhibitor of protein phosphatase 2A (I2PP2A, [47]) or the myeloid leukaemia associated oncoprotein SET/TAF-1β [48]). It is a potent inhibitor of phosphatase 2A (PP2A) and interacts with several proteins involved in the regulation of cell cycle [49]. Interestingly, the abundance of the SET binding protein ribonucleoprotein A2 (spot 4, Table 1) increased in SDL but we did not confirm whether this protein was cleaved. Ribonucleoprotein A2 protein is also overexpressed in a variety of human tumours and is a potent inhibitor of phosphatase 2A [50]. RPSA is

#### Table 8. Confirmation of protein phosphorylation during 12 hour SDL.

| Protein name                                      | Accession Number | Peptide sequence                  | Potential site of phosphorylation | Ion score |
|---------------------------------------------------|------------------|-----------------------------------|----------------------------------|-----------|
| Heat shock protein HSP 90-alpha                   | P07900           | ELISNSSDALDKIR                   | Ser 50                           | 35        |
|                                                   |                  | ELISNSSDALDKIR                   | Ser 52                           | 51        |
|                                                   |                  | ELISNSSDALDKIR                   | Ser 53                           | 45        |
|                                                   |                  | ADJNNLGIIAK                      | Thr 104                          | 33        |
|                                                   |                  | EVIDDEAEEKD                      | Ser 231                          | 39        |
|                                                   |                  | DKEVvDDEAEEK                     | Ser 231                          | 53        |
|                                                   |                  | ESDEKPEIEIDVGSDIEEEEK            | Ser 252                          | 30        |
|                                                   |                  | ASDDEKPEIEIDVGSDEEEEEEK          | Ser 252                          | 30        |
|                                                   |                  | EESEDEKPEIEIDVGSDSEEKEE          | Ser 252                          | 36        |
|                                                   |                  | EDESEDEKPEIEIDVGSDSEEKEE         | Ser 263*                         | 49        |
| Heat shock protein HSP 90-beta                    | P08238           | ADJNNLGIIAK                      | Thr 104                          | 33        |
|                                                   |                  | EKEvDDEAEEK                      | Ser 226                          | 56        |
|                                                   |                  | EISvDDEAEEKEK                    | Ser 226*                         | 28        |
| Nascent polypeptide-associated complex subunit alpha | Q13765         | VQGEAVSNIQENIQTPTVQSSEEEEEVDETGVEVK | Thr 157                          | 24        |
|                                                   |                  | VQGEAVSNIQENIQTPTVQSSEEEEEVDETGVEVK | Thr 159                          | 32        |
|                                                   |                  | VQGEAVSNIQENIQTPTVQSeEEEEEVDETGVEVK | Thr 161*                        | 38        |
|                                                   |                  | VQGEAVSNIQENIQTPTVQSseEEEEEVDETGVEVK | Ser 166                          | 52        |
|                                                   |                  | VQGEAVSNIQENIQTPTVQSeEEEEEEVEtGVEVK | Thr 174                          | 23        |

12 hour SDL samples were fractionated by 2D PAGE and then hyper-phosphorylated spots previously identified by mass spectrometry as HSP90 alpha, Hsp90 beta and nascent polypeptide associated complex alpha subunit were subjected to Nano LC mass spectrometry to detect phosphopeptides as outlined in the materials and methods. It should be noted that for some peptides there may be more than one possible phosphorylation site, so we have included all possibilities and known phosphorylation sites within the peptide has been indicated with an *. For some peptides there are more than one possible phosphorylation site and so all matched options are presented. *Indicates a known reported phosphorylation site [43,61].

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a protein of the 40S ribosomal subunit as well as a cell surface protein that binds laminin, prion proteins and viruses [31].

Apart from our novel observation here that both Hsp90 alpha and Hsp90 beta are cleaved in pro-erythroblasts deprived of Epo, we also report that several Hsp90 co-chaperones have altered abundance in living and apoptotic pro-erythroblasts, p23/PTGEG23, a member of the Hsp90 chaperone complex suggested to stabilise the Hsp90-ATP form [52] is more abundant in cells maintained in the presence of Epo (spot 43, Table 5). In contrast, cells deprived of Epo exhibit an increase in full length or cleaved peptidyl-prolyl cis-trans isomerase (PPIase) immunophilin FKBP4/FKBP52 (spots 15 and 20, Table 2), which is known to bind Hsp90 and Hsp70 and is important for the intracellular trafficking of the steroid hormone receptors [53]. Cells cultured in ESDL have a higher abundance of Hsp105 (HspH1) and Hsp70 (HspA4) (spot 35, Table 4 and spot 38 Table 5, respectively). Hsp70 prevents Gata-1 cleavage by caspase 3 [18] and AIF translocation to the nucleus [19]. On the other hand, phosphorylation of Hsp27 is induced in cells deprived of Epo (spot 2, Table 6). Although we did not confirm the phosphorylation or identity of the specific phosphorylation site(s) on Hsp27, it is notable that Hsp27 phosphorylation is required for its association with GATA-1 and for inducing GATA-1 degradation [54]. Taken together these results provide further evidence that chaperone proteins play an essential role in the regulation of Epo-induced survival in erythroblasts.

Commitment to apoptosis is post-translationally regulated by reversible phosphorylation of apoptotic signalling proteins. The abundance of several signalling proteins are altered between the two conditions some of which have already been mentioned above. In ESDL, the serine/threonine kinase p21 protein (Cdc42/Rac)-activated kinase 2 (PAK2, spot 52, Table 5) was up-regulated. PAK2 is cleaved by caspases during apoptosis [55] and its up-regulation in living cells in this study might result from loss of full length PAK2 by proteolysis in dying cells (although a smaller fragment was not identified in SDL). In apoptotic erythroblasts, the serine/threonine phosphatase PPIalpha (PPP1CA, spot 25, Table 4) increased in abundance. PPIalpha is known to be pro-apoptotic because it dephosphorylates the pro-apoptotic protein Bad [56].

Finally, several proteins involved in mRNA processing, translation and post-translational modifications were altered in presence or absence of Epo (see Tables 1–5). Interestingly, the heterogeneous nuclear ribonucleoprotein K (hnRNPK) which is detected as less abundant upon Epo withdrawal (spot 41, Table 5) has been shown to prevent the production of the pro-apoptotic BclXs splice isoform [57] and BcIX is well documented to be an Epo-responsive protein important for the survival of erythroblasts at the later stages of erythropoiesis [58]. Ribonucleoprotein C1/C2 detected both in ESDL (Spot 42, Table 5) and SDL (smaller Spot 16, Table 2) is reported to be induced by stimulators of apoptosis and p53, and has been proposed to regulate p53 mRNA during apoptosis [59]. Furthermore, Polypyrimidine tract binding protein 1 was increased in ESDL (spot 34 Table 4, and spot 39 Table 5) and this protein is reported to regulate apoptotic genes and susceptibility to caspase dependent apoptosis in differentiating cardiac myocytes [60]. Further work will need to be carried out to determine the roles of these proteins in survival and death of erythroblasts.

In summary, we have conducted the first ever comparison of the proteomes of expanding primary human erythroblasts and primary human erythroblasts undergoing the early phase of apoptotic death due to Epo withdrawal. This study has dramatically increased the repertoire of proteins that alter abundance during Epo withdrawal. In particular we report for the first time that several key multifunctional proteins are cleaved in response to Epo withdrawal from erythroblasts. Two of these proteins, Hsp90 alpha and Hsp90 beta, were also shown to be phosphorylated in apoptotic cells and we have identified these phosphorylation sites. This study validates the use of 2D DIGE to gain a comprehensive insight into cellular events leading to apoptosis in erythroblasts and as a means of identifying proteins whose aberrant regulation may contribute to human blood diseases. Furthermore, we provide an exciting new resource of candidate proteins, which will form the foundation for further studies on the mechanism of apoptosis caused by Epo withdrawal and also for studies on human diseases where there is ineffective erythropoiesis.

Supporting Information

Figure S1 A) Flow cytometry analysis of cell surface markers expressed by erythroblasts between day 6 and day 10 in culture in ESDL medium. FL2 fluorescence (x axis) versus cell number (y axis) of cells labelled with the isotype control antibody (grey line) and antibodies against CD117/c-kit, CD71, GPA (BRIC256) and band3 (BRIC6). By day 9 the majority of cells are are c-kit⁺ positive, CD71high, GPAlow/med and band 32neg/low B) Graph showing the average percentage of live erythroblasts after 24 h in ESDL or SDL, normalised to the percentage of live cells in ESDL between day 7 and day 10. This shows that similar level of cell death is achieved irrespective of the number of days expanding in culture. (TIF)

Figure S2 The expression of Fas and Fasl was analyzed by flow cytometry for Fas and FasL expression on day 9 erythroblasts after 24 hours in the presence (ESDL) or absence (SDL) of Epo. The figure shows that erythroblasts from both treatments express Fas. In contrast, the expression of Fasl in these cells was absent and this did not change upon withdrawal of Epo. (TIF)

Figure S3 Erythroblasts kept in expansion medium (ESDL, +Epo, green histograms) and erythroblasts switched to SDL (no Epo, pink histograms) were analysed by flow cytometry using Annexin V, TMRE and propidium iodide at 6 hours, 12 hour and 24 hour. After 12 hour, the cells switched to SDL start showing signs of apoptosis and this time point was chosen for proteomic analyses by 2D DIGE. (TIF)

Table S1. Table S1 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 1. (DOCX)

Table S2 Table S2 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 2. (DOCX)

Table S3 Table S3 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 3. (DOCX)

Table S4 Table S4 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 4. (DOCX)

Table S5 Table S5 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 5. (DOCX)
Table S6  Table S6 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 6.

(DOCX)

Table S7  Table S7 lists all peptides identified by mass spectrometry from each individual spot detailed in Table 7.

(DOCX)

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Author Contributions

Conceived and designed the experiments: SP KJH TJS GD EvdA AMT. Performed the experiments: SP KJH. Analyzed the data: SP KJH TJS GD EvdA AMT. Wrote the paper: SP KJH TJS GD EvdA AMT. Principal investigator: AMT. Conducted additional experiments for Figure 2 and Figure 4: TJS BRH. Conducted the duplicate experiments to provide protein samples for the Orbitrap mass spectrometry phosphorylation data: EvdA.
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