Selective silencing of α-globin by the histone demethylase inhibitor IOX1: a potentially new pathway for treatment of β-thalassemia

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Methods

Human CD34+ erythroid differentiation culture

Human umbilical cord blood and peripheral blood component donation leucocyte cones were purchased from National Health Service Blood and Transplant (NHSBT), UK. Ethical approval for the study was granted by North West Research Ethics Committee of NHS National Research Ethics Services, UK (reference no. 03/08/097). Human CD34+ hematopoietic stem and progenitor cells were purified using CD34 MicroBead Kit (Miltenyl Biotech) and were cultured in a two-phase liquid culture system. In the expansion phase (phase 1) CD34+ cells were cultured in StemSpan serum free expansion medium (SFEM) II (Stem cell technologies), supplemented with 100U/ml penicillin/streptomycin (Gibco), 2mM glutamine (Gibco), 100ng/ml stem cell factor (SCF) (Peprotech), 10ng/ml interleukin-3 (Peprotech), 10ug/ml cholesterol rich lipids (Sigma) and 0.5IU/ml erythropoietin. After 7 days, cells were transferred into phase 2 differentiation medium, which is similar to phase 1 medium except for the addition of 0.5mg/ml iron saturated holotransferrin (Sigma) and higher concentration of erythropoietin (3U/ml). Throughout the culture, cells were maintained in a 5% CO_2 atmosphere at 37°C and the cell concentration was kept below 2 million/ml by adding fresh medium every 2-3 days.

Cellular morphology and flow cytometry

Cell viability was determined by trypan blue test. Cellular morphology was assessed using cytospins stained with modified Wright stain using an Hemateck slide stainer. For flow cytometry, washed cells were labelled for 20 minutes on ice, in 2% bovine serum albumin (Sigma), with the following monoclonal anti-human antibodies; allophycocyanin (APC) conjugated anti-CD34 (Miltenyl Biotech), fluorescein isothiocyanate (FITC) conjugated anti-CD71 (BD Pharmingen) and phycoerythrin (PE) conjugated anti-glycophorin A (BD Pharmingen). Analysis was performed on a CyanTM ADP analyzer using Summit v4.3 software after gating on viable cells identified with a Hoechst 33258 pentahydrate (Invitrogen) nucleic acid stain.

Small molecule library and treatment of cells

The epigenetic inhibitor library consisted of 37 epigenetically active small molecules and was a kind donation from the Structural Genome Consortium (SGC), Oxford, UK. Primary human erythroid cells at day 7 of the culture were incubated with compounds at the specified concentrations (supplemental table 1). Each compound was added to 10,000 cells in 200µL volume of phase 2 culture medium (cell concentration 50,000/ml) in 96-well flat bottom tissue culture plates. Cells were incubated for 72 hours without changing or adding medium and harvested on day 10.

RNA extraction, reverse transcription and gene expression analysis

Total RNA was purified using the RNeasy mini kit (Qiagen) and complementary DNA (cDNA) was prepared using the high capacity RNA to cDNA kit (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed in a 7500 fast real time

PCR system (Applied Biosystems) according to the manufacturer's protocol using validated, inventoried and exon-spanning TaqMan assays (Applied Biosystems) for human α-globin, β-globin, γ-globin and ribosomal protein L13A (*RPL13A*) (a complete list of TaqMan assays is available on request). Data were analyzed by 7500 software v2.0.6 using the delta delta CT method. *RPL13A* was used as the house keeping gene for normalizations as a previous study showed it to be constant throughout human erythropoiesis(1).

During the initial small molecule screen, high throughput qPCR was performed using a Fluidigm 48.48 gene expression chip with TaqMan assays (Applied Biosystems) and the Integrated fluidic circuit controller MX and BioMark HD system according to the manufacturer's protocol. Data were analysed using Fluidigm Real-Time PCR analysis 3.1.3 software.

For Nanostring experiments, we purchased a custom made capture probe set panel and consumables from Nanostring technologies. Hybridized samples were processed using the nCounter prep station and nCounter digital analyzer (Nanostring Technologies) according to the manufacturer's instructions. Raw data were normalized to an internal positive spike-in control to normalize to all of the platform's associated sources of variation and then to the geometric mean of eight housekeeping genes (*RPL13A, RPL18, GAPDH, PABPC1, CA2, FTH1, PAIP2* and *LAPTM4A*).

Microarray

Microarray whole genome gene expression analysis was performed using Illumina's Human HT12v4.0 Expression BeadChip and Illumina iScan Scanner. The experiments were performed in biological quadruplicate and the data were normalized in R using the lumi package, analyzed using Linear Models for Microarray (limma) and filtered using an adjusted P value of <0.05 to identify differentially expressed genes. Low expressing genes were filtered out when comparing the expression levels of genes in IOX1 treated and untreated cells (2,3)

Western blot

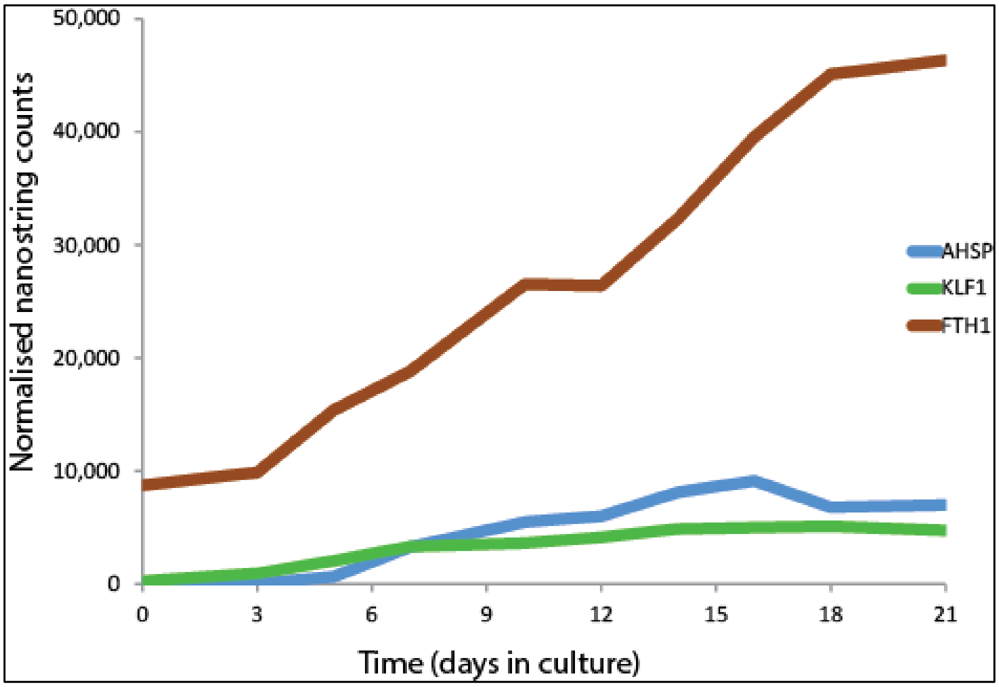
Cells were lysed and histone proteins were solubilized in 0.4M HCl and precipitated in acetone overnight at −20°C. Western blotting was performed using antibodies to H3K27me3 (Millipore, 07-449), H3K9me3 (Abcam ab8898) and histone H3 (Abcam ab1791).

Quantitative Chromatin immunoprecipitation (ChIP)

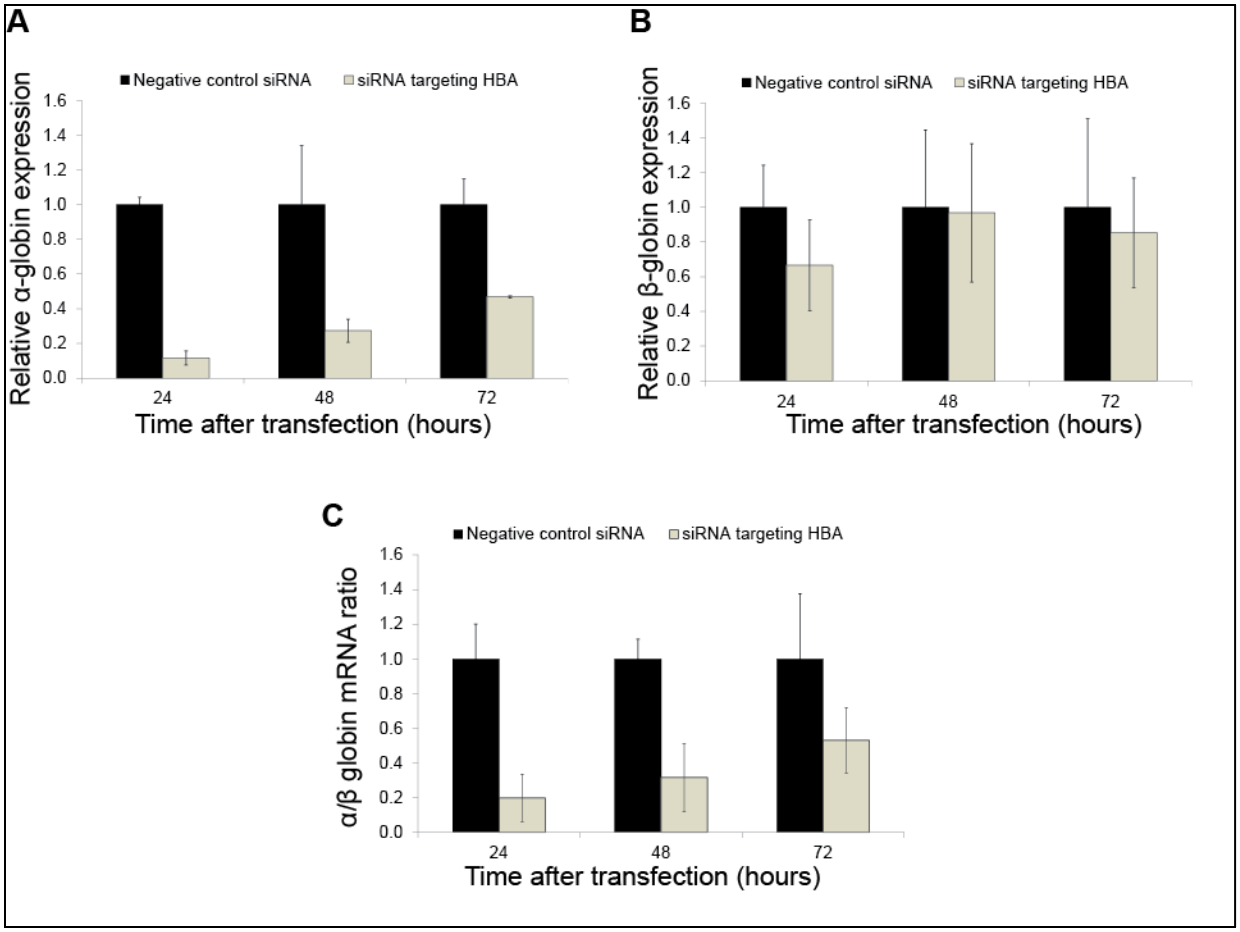
Quantitative ChIP assays were performed using Millipore ChIP assay kit (Merck Millipore, 17-295). Briefly, 10^7 cells per immunoprecipitation were fixed with 0.4% formaldehyde for 10 min at room temperature and the reaction quenched by adding 0.125M glycine. Cells were washed twice and lysed in SDS lysis buffer. The lysate was incubated on ice for 10 minutes and then sonicated to reduce chromatin fragments to an average size ~500 bp. ChIP assays were then carried out according to the manufacturer's instructions using antibodies to H3K27me3 (Millipore, 07-449) and

H3K9me3 (Abcam ab8898). Precipitated DNA was quantified by qPCR assay with previously described(4,5) primer pairs for human α-, β- and γ-globin promoter sequences and a primer pair for an intergenic region between the ε- and γ-globin genes as a negative control.

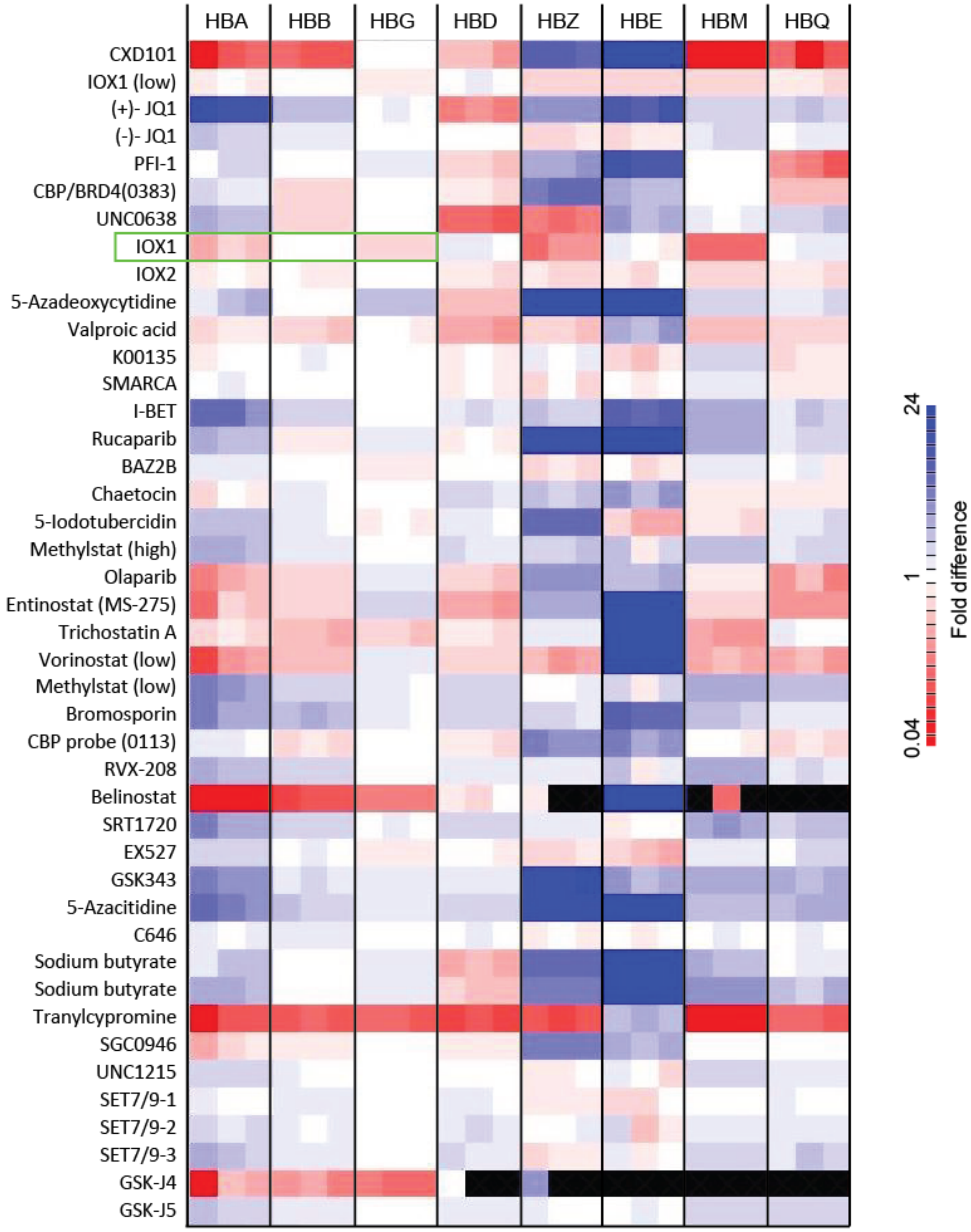
Supplemental Figures



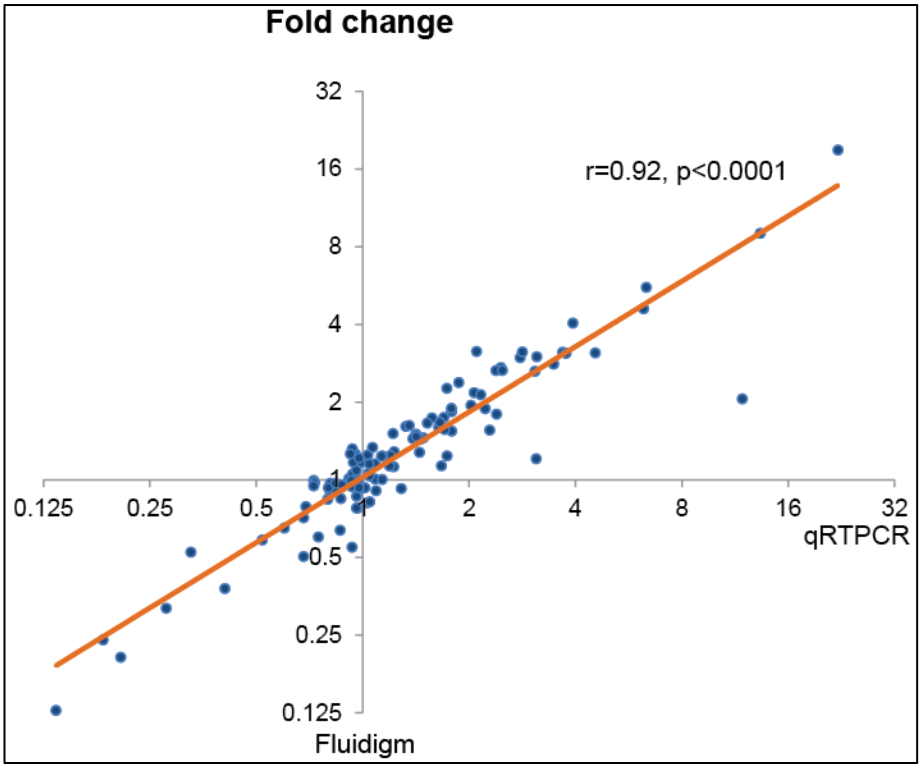
Supplemental figure 1: Relative expression levels of erythroid specific genes (*AHSP, KLF1* and *F*TH1) at different time points in culture.



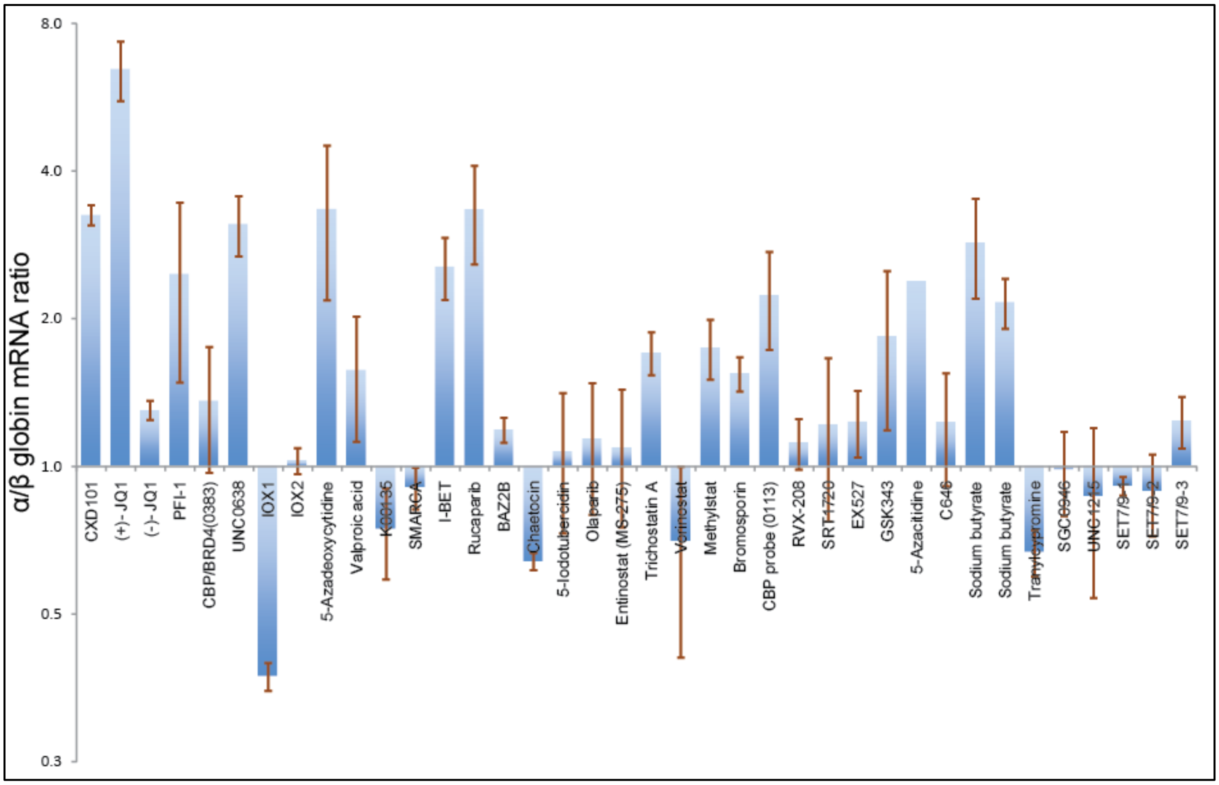
Supplemental figure 2: Validation of the erythroid differentiation system and the globin gene expression detection protocol. (A-B) α- and β-globin expression in erythroid cells transfected with a pair of siRNAs targeting human α-globin (*HBA*) and a negative control siRNA. siRNAs were added to the liquid culture medium on day 7 of erythroid cell differentiation (corresponding to the pro-erythroblast stage) and the cells were then incubated in a 5% CO2 atmosphere at 37°C. Mean level of globin gene expression relative to the expression of *RPL13A* is shown; error bars represent SD (n=2). (C) α/β globin mRNA ratio of erythroid cells transfected with a pair of siRNAs targeting human α-globin and a negative control siRNA; error bars represent SD (n=2).


HBA
HBB
HBG
HBD
HBZ
HBE
HBM
HBQ
CXD101
IOX1 (low)
(+)- JQ1
(-)- JQ1
PFI-1
CBP/BRD4(0383)
UNC0638
IOX1
IOX2
5-Azadeoxycytidine
Valproic acid
K00135
SMARCA
I-BET
Rucaparib
BAZ2B
Chaetocin
5-Iodotubercidin
Methylstat (high)
Olaparib
Entinostat (MS-275)
Trichostatin A
Vorinostat (low)
Methylstat (low)
Bromosporin
CBP probe (0113)
RVX-208
Belinostat
SRT1720
EX527
GSK343
5-Azacitidine
C646
Sodium butyrate
Sodium butyrate
Tranylcypromine
SGC0946
UNC1215
SET7/9-1
SET7/9-2
SET7/9-3
GSK-J4
GSK-J5
24
1
0.04
Fold difference

Supplemental figure 3: Representative heat map (one of 3 biological repeats) demonstrating fold differences of globin mRNA levels in erythroid cells treated with small molecules. The expression levels were normalized to the mean of four housekeeping genes (*RPL13A, RPL18, GAPDH* and *FTH1*) and referenced to the vehicle (DMSO) control. Each row represents a single compound and each column represent one of three technical repeats performed for each globin gene with colours ranging from dark red (decreased expression) through white (no change) to blue (increased expression). HBA, α-globin; HBB, β-globin; HBG, γ-globin; HBD, δ-globin; HBZ, ζ-globin; HBE, ε-globin; HBM, μ-globin, HBQ- θ-globin.



Supplemental figure 4: Validation of Fluidigm BioMark data by standard qRTPCR. Fold changes of genes of the same cDNA samples used in the Fluidigm analysis were independently quantified by qRTPCR. The data indicates close correlation between the two data sets, and thus validates the Fluidigm analysis. Abbreviations: r, Pearson's correlation coefficient.



Supplemental figure 5: α/β globin mRNA ratios in cells treated with small molecule inhibitors. Error bars represent SD (n=3).

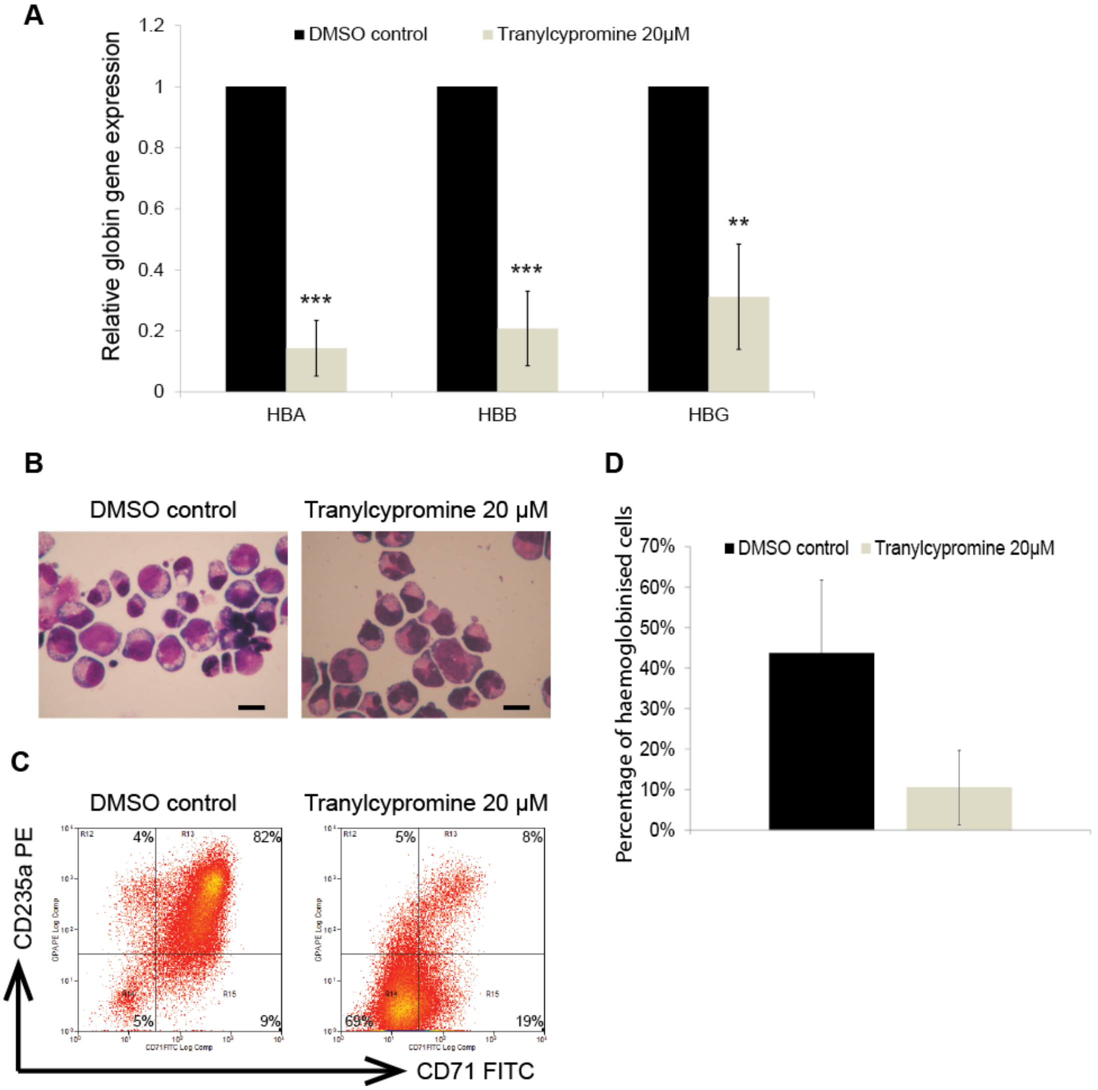


Figure 6: Effects of tranylcypromine on erythroid cells. Erythroid cells treated for 72 hours with tranylcypromine 20μM and DMSO control were analyzed on day 10 of erythroid differentiation. (A) Mean relative expression of α-, β- and γ-globin normalized to RPL13A and referenced to a DMSO control from 5 independent biological repeats; error bars represent SD; **p<0.01 and ***p<0.001 relative to the DMSO control. (B) Representative cytospins of cells stained by modified Wright stain; scale bar – 10μm. (C) Representative flow cytometry plots of cells stained with FITC-conjugated anti-CD71

and PE-conjugated anti-CD235a antibodies. (D) Mean haemoglobinised cell percentages from 2 independent biological repeats are shown; error bars represent SD. Abbreviations: HBA, α-globin; HBB, β-globin.

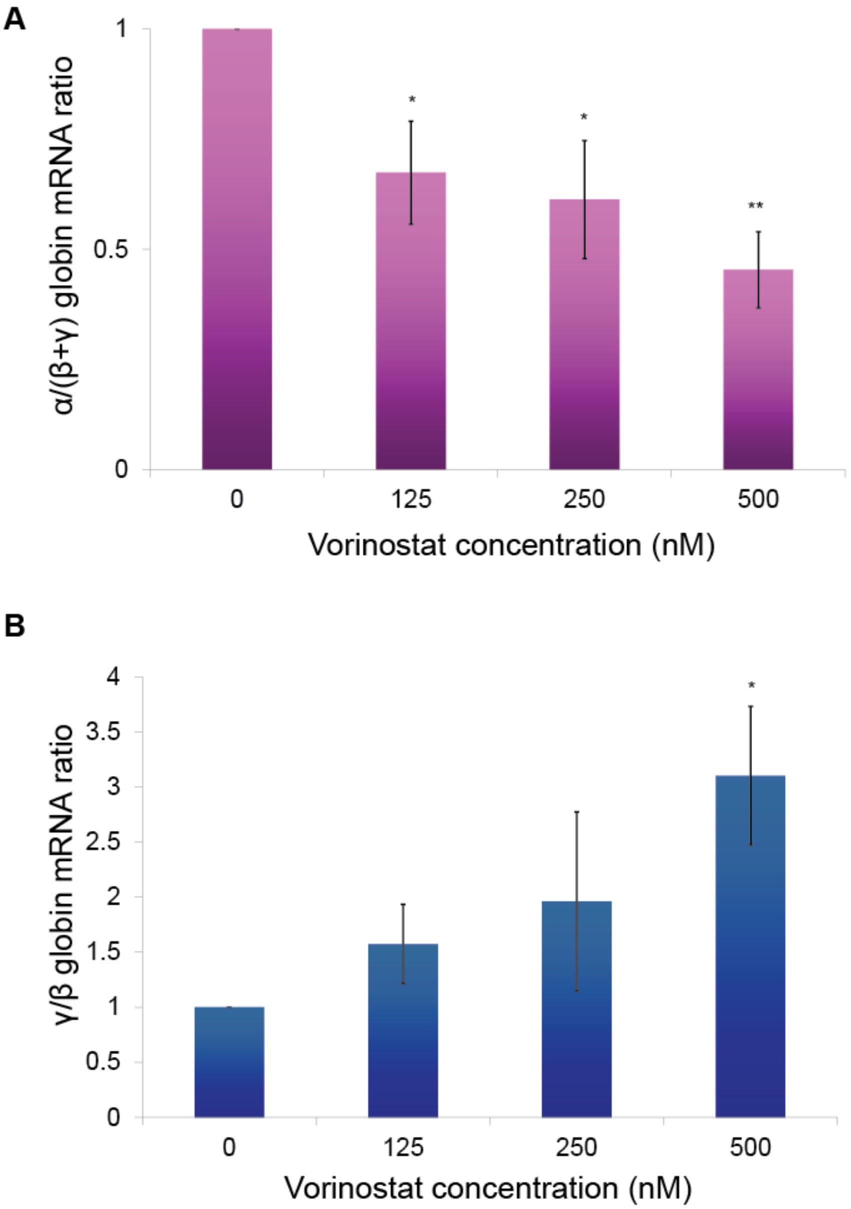
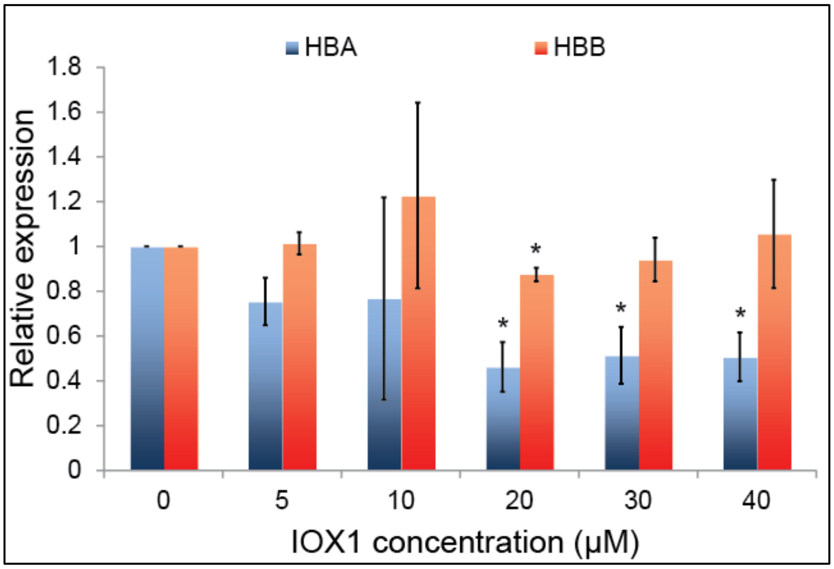
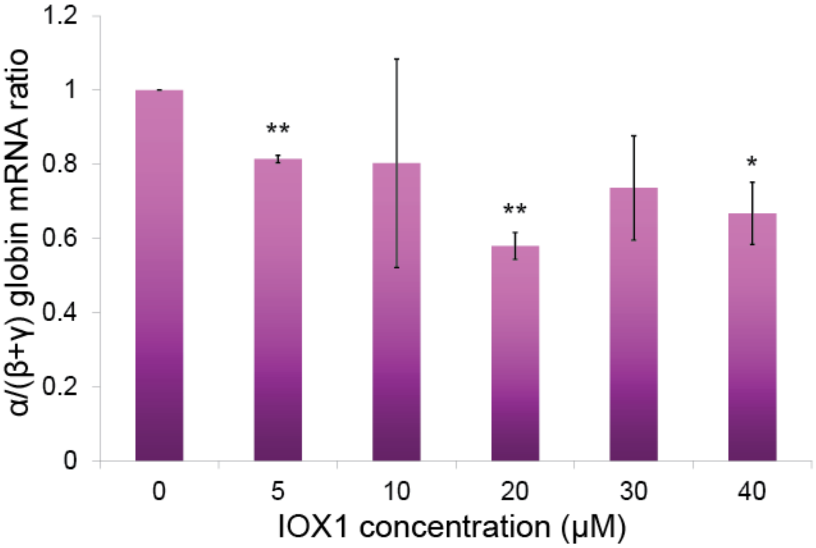


Figure 7: Effects of vorinostat on erythroid cells. Erythroid cells treated for 72 hours a dose range (0nM-500nM) of vorinostat were analysed on day 10 of erythroid differentiation. (A) α/(β+γ) globin mRNA ratio. (B) γ/β mRNA ratio. Error bars represent SD (n=3). *p<0.05, **p<0.01 relative to DMSO control.



Supplemental figure 8: Effects of a dose range (0μm-40μM) of IOX1 on α- and β-globin gene expression in human erythroid cells. α- and β-globin expression relative to *RPL13A* expression in cells differentiated from umbilical cord blood. Error bars represent SD (n=3). *p<0.05, **p<0.01 relative to DMSO control. HBA, α-globin; HBB, β-globin.



Supplemental figure 9: Effects of a dose range (0μm-40μM) of IOX1 on α/(β+γ) globin ratio in human erythroid cells (differentiated from cord blood CD34+ cells). Error bars represent SD (n=3); $^*p<0.05$, $^{**}p<0.01$ relative to DMSO control.

Supplemental Tables

Supplemental Table 1 – List of drugs/compounds in the epigenetic inhibitor small molecule library

Small molecule inhibitor	***Inhibitor target/class***	***Working concentration (µM)***
(+)- JQ1	Bromodomains	1
PFI-1	Bromodomains	5
CBP/ BRD4 (0383)	Bromodomains	5
SMARCA	Bromodomains - SMARCA	2.5
I-BET	Bromodomains - BRD2/3/4	1
BAZ2B	Bromodomains - BAZ2B	1
Bromosporin	Bromodomains - broad spectrum	1
CBP probe (0113)	Bromodomains - CBP Enantiomer	5
RVX-208	Bromodomains - BET clinic	5
5-Aza-deoxy-cytidine	DNA methyltransferase - DNMT1/3	5
5-Azacitidine	DNA methyltransferase	10
CXD101	HDAC	1
Valproic acid	HDAC -aliphatic acid compounds	1000
Entinostat (MS-275)	HDAC -ortho-amino anilides	0.5
SRT1720	HDAC -SIRT1	1
EX527	HDAC -SIRT1	1
Trichostatin A	HDAC -hydroxamic acid Class I & 2	0.5
Vorinostat	HDAC -hydroxamic acid	2.5
C646	Histone acetyltransferase p300/CBP	1
Methylstat	Histone demethylase	2.5
UNC0638	Histone methyltransferase - G9a/GLP	1
GSK343	Histone methyltransferase - EZH2	3
SGC0946 (DOT1L probe)	Histone methyltransferase - DOT1L	7.5
SET7/9-1	Histone methyltransferase - SETD7	2.5
SET7/9-2	Histone methyltransferase - SETD7	2.5
SET7/9-3	Histone methyltransferase - SETD7	2.5

Small molecule inhibitor	***Inhibitor target/class***	***Working concentration (µM)***
Chaetocin	Histone methyltransferase - SUV39H1	0.05
K00135	Kinase inhibitor - ATP competitive -PIM	1
5-Iodotubercidin "HASPIN"	Kinase inhibitor - ATP mimetic - Haspin	1
IOX1	Lysine demethylase - broad range	40
Tranylcypromine	Lysine demethylase - LSD1	20
GSK-J4	Lysine demethylase - KDM6A/B (JMJD3/UTX)	10
UNC1215	Malignant Brain Tumour Domains - L3MBTL3	5
(-)- JQ1	Bromodomains - Negative control	1
Rucaparib	Poly ADP ribose polymerase (PARP)	10
Olaparib	Poly ADP ribose polymerase (PARP)	1
IOX2	Prolyl hydroxylases EGLN1 (PHD2)	10

Supplemental Table 2 – List of genes significantly up-regulated in IOX1 treated cells in alphabetical order

Gene Symbol	***Gene Name***	***Fold change***
AAGAB	Alpha- and gamma-adaptin binding protein	1.28
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	1.28
ACTL6A	Actin-like 6A	1.28
AHSA1	Activator of heat shock 90kDa protein ATPase homolog 1	1.57
ANXA2	Annexin A2	1.49
BCCIP	BRCA2 and CDKN1A interacting protein	1.44
BRI3BP	BRI3 binding protein	1.50
C17ORF79	Chromosome 17 open reading frame 79	1.47
C18ORF55	Chromosome 18 open reading frame 55	1.39
C19ORF2	Chromosome 19 open reading frame 2	1.28
CACYBP	Calcyclin binding protein	1.77
CDC123	Cell division cycle 123 homolog (S. cerevisiae)	1.25
CEP78	Centrosomal protein 78kDa	1.35
CLP1	Cleavage and polyadenylation factor I subunit	1.20
CRKRS	Cdc2-related kinase, arginine/serine-rich	1.18
CSTF3	Cleavage stimulation factor, 3' pre-RNA, subunit 3	1.24
CYB5B	Cytochrome b5 type B	1.30
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	1.92
DCUN1D5	Defective in cullinneddylation 1, domain containing 5	1.34
DHX15	DEAH (Asp-Glu-Ala-His) box polypeptide 15	1.39
DYRK2	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	1.37
EBNA1BP2	EBNA1 binding protein 2	1.49
EI24	Etoposide induced 2.4 mRNA	1.37
EIF4G2	Eukaryotic translation initiation factor 4 gamma 2	1.43
EMG1	EMG1 nucleolar protein homolog (S. cerevisiae)	1.39
EMP3	Epithelial membrane protein 3	1.48
ENOPH1	Enolase-phosphatase 1	1.18
FABP5L2	Fatty acid binding protein 5-like 2	1.49
FAM92A1	Family with sequence similarity 92, member A1	1.26
FJX1	Four jointed box 1 (Drosophila)	1.34
FREQ	Frequenin homolog (Drosophila)	1.30
FTL	Ferritin, light polypeptide	1.17
G3BP1	GTPase activating protein (SH3 domain) binding protein 1	1.36

Gene Symbol	***Gene Name***	***Fold change***
G3BP2	GTPase activating protein (SH3 domain) binding protein 2	1.27
GART	Phosphoribosylglycinamide formyltransferase	1.36
GNL3	Guanine nucleotide binding protein-like 3	1.54
GPATCH4	G patch domain containing 4	1.27
GPN3	GPN-loop GTPase 3 (GPN3)	1.22
GTPBP4	GTP binding protein 4	1.51
HES5	Hairy and enhancer of split 5 (Drosophila)	1.45
HMGN2	High-mobility group nucleosomal binding domain 2	1.26
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	1.73
HNRNPD	Heterogeneous nuclear ribonucleoprotein D	1.40
HNRNPM	Heterogeneous nuclear ribonucleoprotein M	1.48
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1	1.47
HSPC111	Hypothetical protein HSPC111	1.62
LOC100130003	Misc_RNA	1.56
LOC389816	Cytokeratin associated protein	1.29
LOC389873	Misc_RNA	1.24
LOC642031	Hypothetical protein LOC642031	1.34
LOC647150	Misc_RNA	1.43
LOC654244	Similar to mitochondrial carrier protein MGC4399	1.29
LRRC26	Leucine rich repeat containing 26	1.25
LSM12	LSM12 homolog (S. cerevisiae)	1.27
MED27	Mediator complex subunit 27	1.30
MFAP1	Microfibrillar-associated protein 1	1.19
MIF	Macrophage migration inhibitory factor	1.38
MORF4L2	Mortality factor 4 like 2	1.35
MPP6	Membrane protein, palmitoylated 6	1.33
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	1.31
NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	1.32
NIP7	Nuclear import 7 homolog (S. cerevisiae)	1.38
NME1	Non-metastatic cells 1, protein	1.47
NOLC1	Nucleolar and coiled-body phosphoprotein 1	1.39
NOP16	NOP16 nucleolar protein homolog (yeast)	1.61
NSUN2	NOP2/Sun domain family, member 2	1.35
NXT1	NTF2-like export factor 1	1.44
PAK1IP1	PAK1 interacting protein 1	1.42

Gene Symbol	***Gene Name***	***Fold change***
PDCL3	Phosducin-like 3	1.43
PGRMC1	Progesterone receptor membrane component 1	1.43
PIAS2	Protein inhibitor of activated STAT, 2	1.24
PIGW	Phosphatidylinositol glycan anchor biosynthesis, class W	1.37
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	1.40
POLR2F	Polymerase (RNA) II (DNA directed) polypeptide F	1.42
POLR3K	Polymerase (RNA) III (DNA directed) polypeptide K	1.39
PPPDE1	PPPDE peptidase domain containing 1	1.30
PRDX1	Peroxiredoxin 1	1.44
PRPF38A	Pre-mRNA processing factor 38 (yeast) domain containing A	1.37
PSMA3	Proteasome (prosome, macropain) subunit, alpha type, 3	1.46
PSMA4	Proteasome (prosome, macropain) subunit, alpha type, 4	1.31
PTPLAD1	Protein tyrosine phosphatase-like A domain containing 1	1.32
PTRH2	Peptidyl-tRNA hydrolase 2	1.37
RABEPK	Rab9 effector protein with kelch motifs	1.37
RANBP1	RAN binding protein 1	1.52
RARS	Arginyl-tRNA synthetase	1.48
RBBP4	Retinoblastoma binding protein 4	1.20
RBM12	RNA binding motif protein 12	1.23
RCC1	Regulator of chromosome condensation 1	1.25
RPL36A	Rribosomal protein L36a	1.54
RPL6	Ribosomal protein L6	1.89
RRP15	Ribosomal RNA processing 15 homolog (S. cerevisiae)	1.36
SETMAR	SET domain and mariner transposase fusion gene	1.39
SNRPA1	Small nuclear ribonucleoprotein polypeptide A	1.26
SNRPF	Small nuclear ribonucleoprotein polypeptide F	1.33
TBRG4	Transforming growth factor beta regulator 4	1.34
TFB2M	Transcription factor B2, mitochondrial	1.32
THOC4	THO complex 4	1.41
TIMM23	Translocase of inner mitochondrial membrane 23 homolog (yeast)	1.39
TIPIN	TIMELESS interacting protein	1.35
TSPAN3	Tetraspanin 3	1.27
TTC4	Tetratricopeptide repeat domain 4	1.27
TXNRD1	Thioredoxin reductase 1	1.42
UCK2	Uridine-cytidine kinase 2	1.38

Gene Symbol	***Gene Name***	***Fold change***
UCRC	Ubiquinol-cytochrome c reductase complex	1.22
USP22	Ubiquitin specific peptidase 22	1.17
YWHAZ	Tyrosine 3-monooxygenase	1.33
ZBED1	Zinc finger, BED-type containing 1	1.30
ZNF275	Zinc finger protein 275	1.17
ZNF326	Zinc finger protein 326	1.27
ZNF410	Zinc finger protein 410	1.18

Supplementary table 3 – List of genes significantly down-regulated in IOX1 treated cells in alphabetical order

Gene Symbol	*Gene Name*	*Fold change*
AADACL1	Arylacetamide deacetylase-like 1	0.63
AARS	Alanyl-tRNA synthetase	0.64
ACSBG1	Acyl-CoA synthetase bubblegum family member 1	0.55
AMT	Aminomethyltransferase	0.74
AMY1A	Amylase, alpha 1A (salivary)	0.78
AMY1C	Amylase, alpha 1C (salivary)	0.76
ANKRA2	Ankyrin repeat, family A	0.67
ARHGEF2	Guanine nucleotide exchange factor	0.78
C10ORF33	Chromosome 10 open reading frame 33	0.69
C14ORF93	Chromosome 14 open reading frame 93	0.73
C17ORF90	Chromosome 17 open reading frame 90	0.75
C1ORF59	chromosome 1 open reading frame 59	0.67
C4ORF14	Chromosome 4 open reading frame 14	0.72
C6ORF192	Chromosome 6 open reading frame 192	0.76
CAPRIN2	Caprin family member 2	0.73
CARS	Cysteinyl-tRNA synthetase	0.62
CCDC26	Coiled-coil domain containing 26	0.80
CD37	CD37 antigen	0.72
CD53	CD53 molecule	0.81
CIDEB	Cell death-inducing DFFA-like effector b	0.87
CITED2	Cbp/p300-interacting transactivator	0.71
CTH	Cystathionase	0.77
CTSH	Cathepsin H	0.65
CXORF12	Chromosome X open reading frame 12	0.81
DECR1	2,4-dienoyl CoA reductase 1	0.71
DENND2D	DENN/MADD domain containing 2D	0.72
DLK1	Delta-like 1 homolog (Drosophila)	0.66
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	0.83
DPEP2	Dipeptidase 2	0.81
FAM113B	Family with sequence similarity 113, member B	0.56
FAM178B	Family with sequence similarity 178, member B	0.26
FAM83A	Family with sequence similarity 83, member A	0.55
FBXO11	F-box protein 11	0.77

Gene Symbol	***Gene Name***	***Fold change***
FCGR2A	Fc fragment of IgG	0.68
FHL2	Four and a half LIM domains 2	0.54
GALC	Galactosylceramidase	0.76
GALT	Galactose-1-phosphate uridylyltransferase	0.72
GBGT1	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1	0.66
IFNAR2	Interferon (alpha, beta and omega) receptor 2	0.79
IGSF3	Imunoglobulin superfamily, member 3	0.78
IRF9	Interferon regulatory factor 9	0.68
ITGA5	Integrin, alpha 5	0.75
ITGB1	Integrin, beta 1	0.68
KLF6	Kruppel-like factor 6	0.68
LMO2	LIM domain only 2	0.62
LOC130773	Similar to 60S ribosomal protein L23a	0.79
LOC284023	Hypothetical protein LOC284023	0.81
LOC339970	Misc_RNA	0.79
LOC387841	Similar to ribosomal protein L13a	0.70
LOC390940	Similar to R28379_1	0.72
LOC400464	Similar to FLJ43276 protein	0.68
LOC440348	Similar to nuclear pore complex interacting protein	0.73
LOC440353	Nuclear pore complex interacting protein pseudogene	0.72
LOC441013	Misc_RNA	0.75
LOC642299	Hypothetical protein	0.71
LOC653907	Similar to complement receptor related protein isoform 1	0.58
LOC729642	Hypothetical LOC729642	0.72
LOC91561	Similar to ribosomal protein S2	0.76
LRG1	Leucine-rich alpha-2-glycoprotein 1	0.80
MAP7	Microtubule-associated protein 7	0.65
MGC4677	Hypothetical protein MGC4677	0.77
MST1	Macrophage stimulating 1	0.57
MT2A	Metallothionein 2A	0.69
NPIP	Nuclear pore complex interacting protein	0.75
PARP3	Poly (ADP-ribose) polymerase family, member 3	0.76
PCK2	Phosphoenolpyruvate carboxykinase 2	0.53
PEAR1	Platelet endothelial aggregation receptor 1	0.81
PIP5K1B	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	0.80

Gene Symbol	***Gene Name***	***Fold change***
PLEKHM2	Pleckstrin homology domain containing, family M	0.80
PLSCR4	Phospholipid scramblase 4	0.76
PPM1M	Protein phosphatase 1M	0.67
PRG2	Proteoglycan 2, bone marrow	0.40
PTGS1	Prostaglandin-endoperoxide synthase 1	0.67
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	0.61
RENBP	Rrenin binding protein	0.81
RHCE	Rh blood group, CcEe antigens	0.43
RINL	Ras and Rab interactor-like	0.73
SKAP1	Src kinase associated phosphoprotein 1	0.59
SLC11A1	Solute carrier family 11 , member 1	0.56
SLC16A9	Solute carrier family 16, member 9	0.38
SLC1A5	Solute carrier family 1, member 5	0.68
SLC2A10	Solute carrier family 2, member 10	0.73
SLC7A1	Solute carrier family 7, member 1	0.69
TUBAL3	Tubulin, alpha-like 3	0.65
UCA1	Urothelial cancer associated 1	0.43
VEGFA	Vascular endothelial growth factor A	0.68
ZNF419	Zinc finger protein 419	0.76

Supplementary table 4 – Expression levels of genes essential for erythrocyte physiology in IOX1 cells compared to DMSO control cells (n=4)[1]

Gene symbol	*Gene name*	*Log_2 of mRNA abundance*		*Fold change*
		DMSO	*IOX1 40µM*	
RHCE	Rhesus blood group, CcEe antigens	10.16	8.94	0.43*
RHD	Rhesus blood group, D antigen	9.29	8.16	0.46
TMOD1	Tropomodulin 1	10.40	9.42	0.51
SLC4A1	Solute carrier family 4	11.45	10.65	0.58
EPB42	Erythrocyte membrane protein band 4.2	11.61	10.93	0.63
GYPB	Glycophorin B	12.36	11.69	0.63
HMBS	Hydroxymethylbilane synthase	12.20	11.71	0.71
GYPA	Glycophorin A	10.71	10.24	0.72
UROS	Uroporphyrinogen III synthase	12.52	12.05	0.72
PPOX	Protoporphyrinogen oxidase	11.31	10.85	0.73
ALAS2	Aminolevulinate, delta-, synthase 2	8.77	8.34	0.74
GPI	Glucose phosphate isomerase	11.26	10.85	0.75
CPOX	Coproporphyrinogen oxidase	12.61	12.21	0.76
GYPE	Glycophorin E	10.87	10.50	0.77
RHAG	Rhesus blood group-associated glycoprotein	12.51	12.17	0.79
STOM	Stomatin	11.85	11.53	0.80
PKLR	Pyruvate kinase, liver and RBC	10.65	10.36	0.82
TPM1	Tropomyosin 1	10.60	10.33	0.83
ALDOA	Aldolase A, fructose-bisphosphate	12.59	12.33	0.84
SPTA1	Spectrin, alpha	12.04	11.80	0.85
NT5C3	5'-nucleotidase, cytosolic III	10.63	10.41	0.86
FECH	Ferrochelatase	8.73	8.52	0.87
ANK1	Ankyrin 1	10.79	10.59	0.87
UROD	Uroporphyrinogen decarboxylase	13.10	12.91	0.88
GSTT1	Glutathione S-transferase theta 1	9.27	9.10	0.89
EPB49	Erythrocyte membrane protein band 4.9	9.34	9.17	0.89
ADD1	Adducin 1	8.47	8.31	0.89
CD47	CD47 antigen	9.68	9.53	0.90
ADD3	Adducin 3	9.38	9.24	0.91
GYPC	Glycophorin C	12.40	12.26	0.91

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[1] The mRNA abundance determined by microarray in cells treated with IOX1 (40µM) and DMSO (control) for 72 hours on day 7 of erythroid differentiation is shown here. The list of genes is adopted from Hembase (http://hembase.niddk.nih.gov/), a database of genes with specific and essential roles in erythrocyte physiology. Fold change represents the fold difference of mRNA abundance between IOX1 and DMSO treated cells. Only one (marked with *) out of 52 genes was differentially expressed in IOX1 treated cells compared to control.

Gene symbol	***Gene name***	***Log$_2$ of mRNA abundance***		***Fold change***
		DMSO	***IOX1 40µM***	
SPTB	Spectrin, beta	8.37	8.24	0.91
BPGM	2,3-bisphosphoglycerate mutase	8.84	8.73	0.92
GCLC	Glutamate-cysteine ligase	8.01	7.92	0.94
PGK1	Phosphoglycerate kinase 1	11.03	10.97	0.96
GSS	Glutathione synthetase	9.09	9.03	0.96
ALAD	Aminolevulinate, delta-, dehydratase	8.03	7.98	0.97
AQP3	Aquaporin 3	7.76	7.73	0.98
HK1	Hexokinase 1	13.17	13.14	0.98
TPI1	Triosephosphate isomerase 1	12.96	12.93	0.98
GSR	Glutathione reductase	7.82	7.80	0.99
AQP1	Aquaporin 1	7.73	7.71	0.99
HMOX1	Heme oxygenase (decycling) 1	7.93	7.92	0.99
ENO1	Enolase 1	13.13	13.14	1.00
GPX1	Glutathione peroxidase 1	10.93	10.93	1.00
PRDX2	Peroxiredoxin 2	7.82	7.83	1.01
PFKM	Phosphofructokinase, muscle	8.77	8.81	1.03
LDHB	Lactate dehydrogenase B	12.71	12.81	1.07
EPB41	Erythrocyte membrane protein band 4.1	8.07	8.18	1.08
G6PD	Glucose-6-phosphate dehydrogenase	8.30	8.47	1.12
PGD	Phosphogluconate dehydrogenase	10.71	10.90	1.14
ACTB	Actin, beta	13.49	13.73	1.18
ADA	Adenosine deaminase	8.59	8.84	1.18

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