Reduced *DOCK4* Expression Leads to Erythroid Dysplasia in Myelodysplastic Syndromes

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Supplemental Materials and Methods

O-dianisidine staining

Embryos were treated with PTU at 24 hpf. Embryos were dechorionated at 48 hpf and stained for 20 minutes in the dark in 0.6 mg/ml o-dianisidine (Sigma), 0.01M sodium acetate (pH 4.5), 0.65% H_2O_2 and 40% (v/v) ethanol. Embryos were washed with dH_2O three times and post-fixed in 4% paraformaldehyde for 1hour at 4C and rinsed in PBST. Embryos were transferred from 1X PBST into 25%, 50%, and 75% glycerol/PBST for microscopy.

Flow cytometry

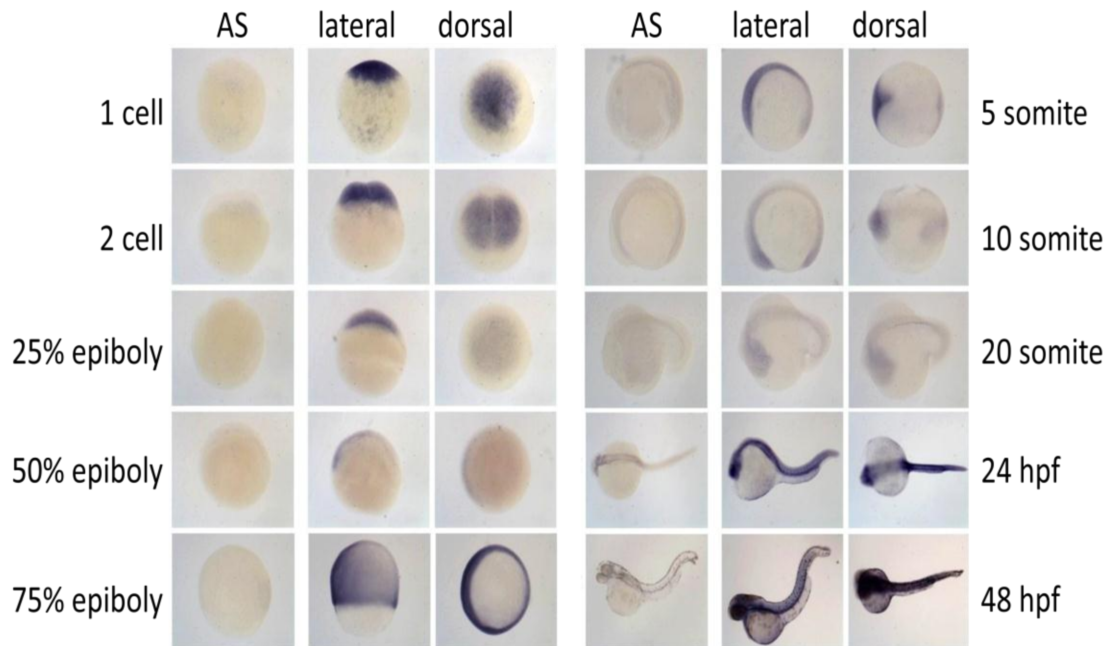
Cells were stained with antibodies for transferrin receptor, CD71 (BD biosciences) and Glycophorin A (eBioscience) at various time points to monitor the erythroid differentiation program. Unstained cells and Isotype specific antibody stained cells were used as controls to set gates. All the flow cytometric analyses were done on LSR-II flow cytometers (BD biosciences) in the University of Chicago Cytometry and Antibody Technology Facility. Data analyses were done using FlowJo software.

Immunoblot analysis

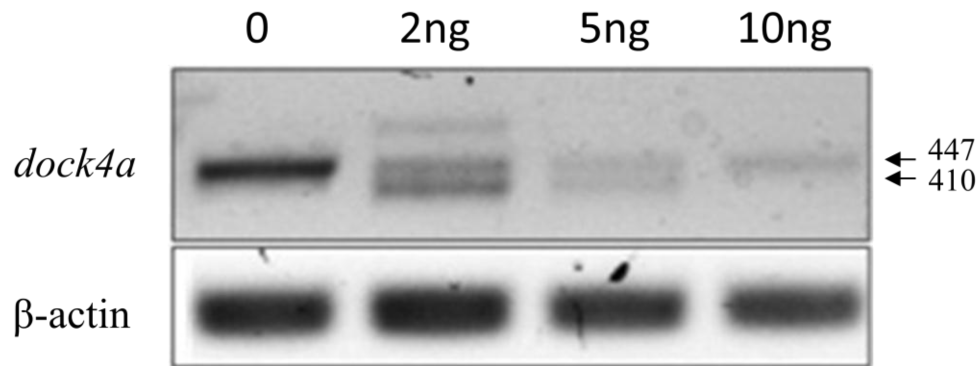
In experiments to determine phospho-ADDUCIN levels, cell lysates were prepared (M-PER, lysis reagent Thermoscientific) from day 10 (polychromatic erythroblasts) cells derived from purification and culture of CD34+ cells obtained from healthy donors or MDS patients. Protein concentrations were determined by the Lowry-Bradford method using a commercially available reagent (Bio-Rad). Electrophoresis was performed in a 4-16% gradient precast SDS gel (Bio-Rad) at 70-100 V using a gel electrophoresis kit (Bio-Rad). The gel was then electro-blotted onto a nitrocellulose membrane (Millipore) in a Semi-dry blot electrophoretic transfer unit (Bio-Rad) using transfer buffer (10mM CAPS + 10% Methanol, pH-11). The nitrocellulose membrane was

then incubated in blocking buffer (Li-Cor Biosciences) for 1 hour at room temperature. The membrane was incubated with mouse anti-human phospho-adducin (Ser 726) antibody (dilution 1:1000, Cat# 05-547, Upstate, NY) overnight on a shaker at 4°C. After three PBS-T washes, the membrane was incubated with IRDye 680RD goat anti-mouse IgG (dilution 1:3000, li-cor) for 2 hours on a shaker at room temperature. The membranes were washed three times (5 minutes each) with PBS scanned on the blot scanner (Odyssey CLx, Li-Cor). The blot was probed with an anti-tubulin antibody (dilution 1:2000, Neomarkers) as loading control.

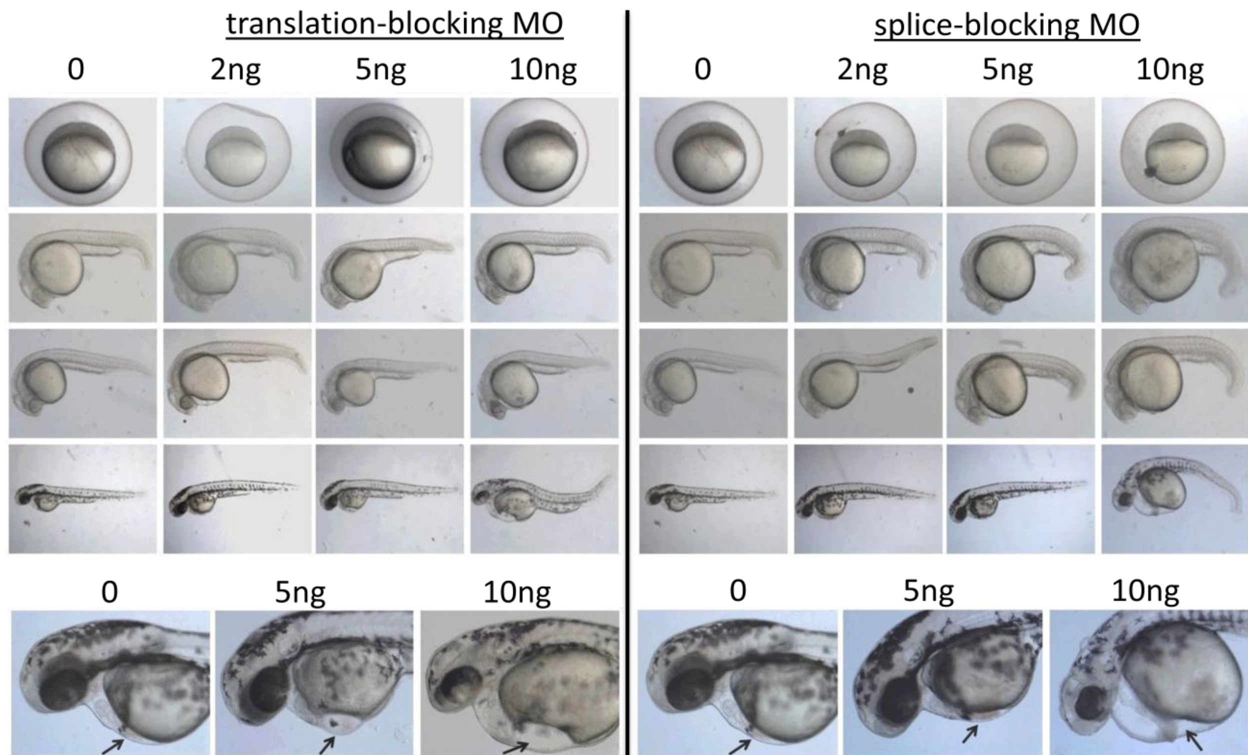
Supplemental Figures



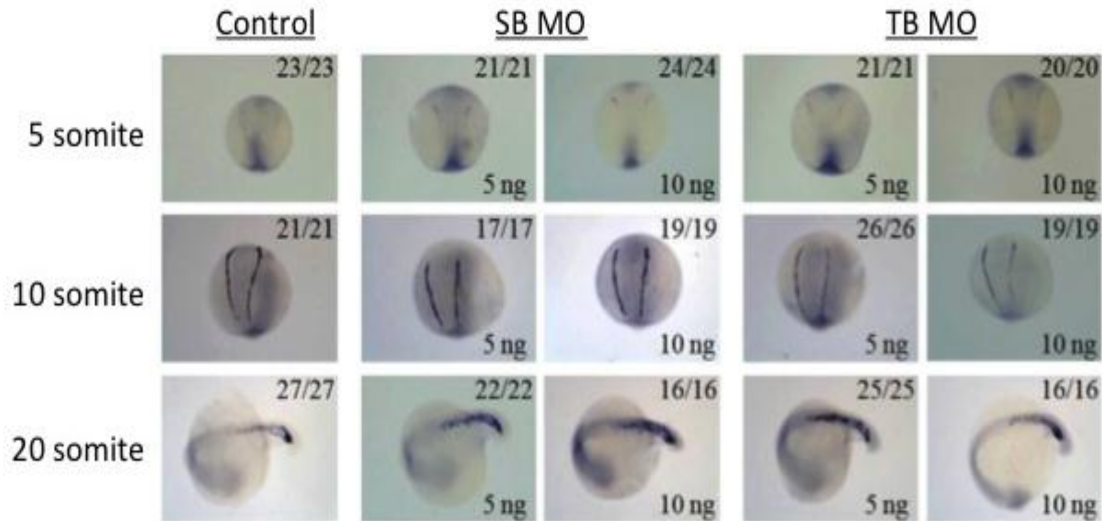
Supplemental Figure 1. ***Dock4a* is expressed during zebrafish embryogenesis.** Gene expression was determined using *in situ* hybridization. Wild-type zebrafish embryos were harvested and fixed at the stages indicated, from the 1-cell stage to 48 hpf. Embryos were hybridized to either a sense (control) probe or an antisense digoxygenin labeled RNA probe. Embryos were photographed with lateral or dorsal views as indicated (sense is a lateral view).



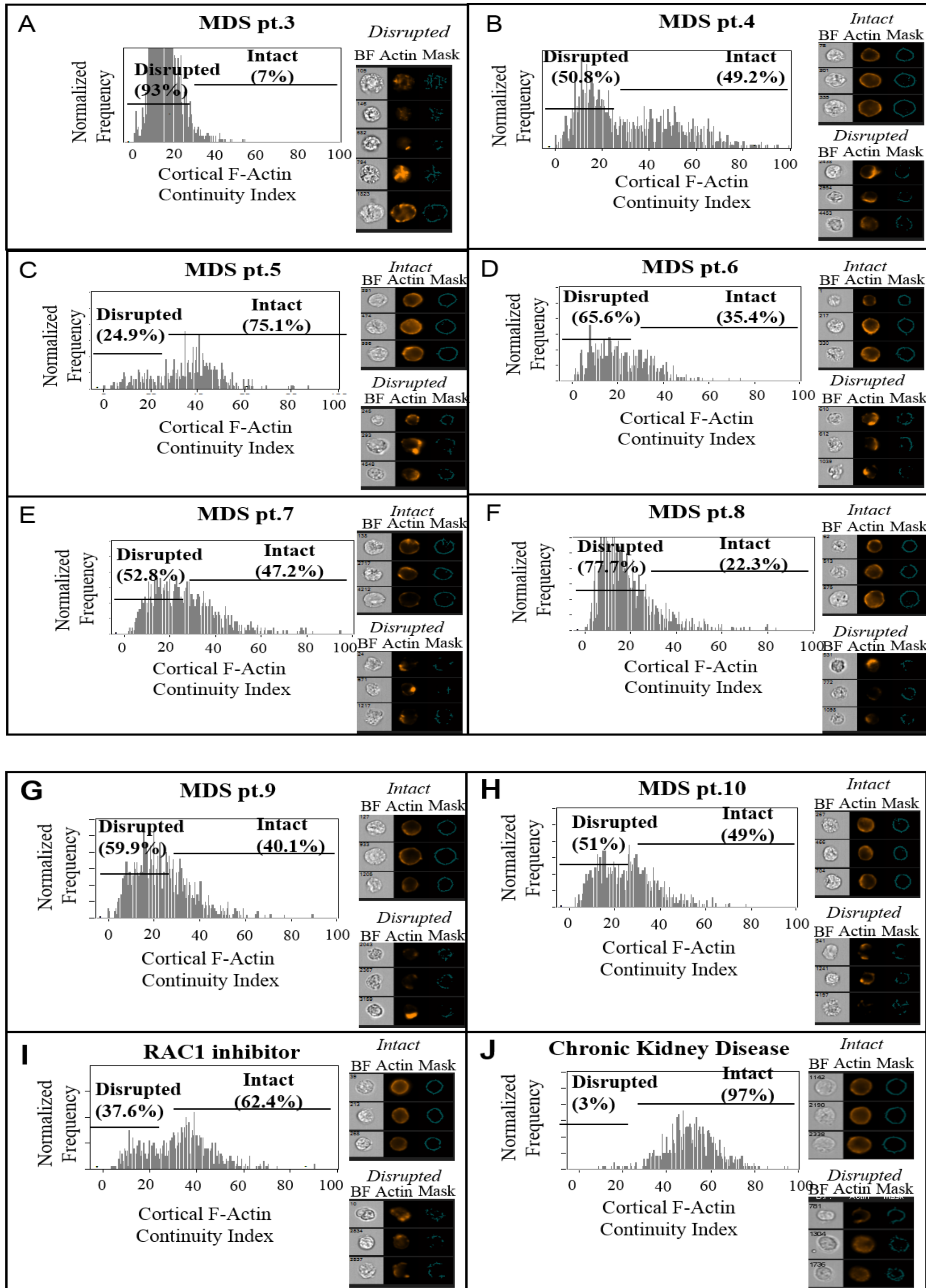
Supplemental Figure 2. Titration of the splice-blocking MO shows normal *dock4a* transcript depletion. Embryos were injected at the 1 cell stage with 0, 2.5, 5 or 10 ng of the *dock4a* TB MO. Embryos were harvested at 24 hpf and RNA extracted. The *dock4a* transcript levels were assessed using semi-quantitative RT-PCR. The expected normal transcript is 447 nt, and the SB MO causes some accumulation of a mis-spliced 410 nt transcript.



Supplemental Figure 3. Both the TB and SB MOs cause similar embryonic defects. Wild-type zebrafish embryos were injected at the one cell stage with 0, 2, 5, or 10 ng TB (left panels) or SB (right panels) morpholino. Shown are representative embryos (n>100). Panels below are higher magnification views demonstrating a pericardial edema and the location of blood pooling in the ventral part of the embryo (arrows)



Supplemental Figure 4. Primitive hematopoiesis initiates normally in the *dock4a* morphant embryos. Wild-type zebrafish embryos were injected with 0 (control), 5 ng, or 10 ng of the *dock4a* SB morpholino. The embryos were harvested and fixed in 4% PFA at the 5, 10, or 20 somite stage. *Gata1*transcript patterns were detected using an antisense digoxygenin labeled RNA probe, seen initially as two stripes in the lateral mesoderm, converging at the intermediate cell mass in the tail.

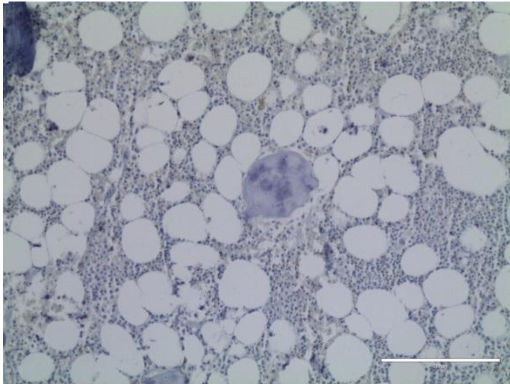

A
MDS pt.3
Disrupted
BF Actin Mask
Normalized Frequency
Disrupted (93%)
Intact (7%)
0 20 40 60 80 100
Cortical F-Actin Continuity Index
B
MDS pt.4
Intact
BF Actin Mask
Disrupted (50.8%)
Intact (49.2%)
Disrupted
BF Actin Mask
C
MDS pt.5
Disrupted (24.9%)
Intact (75.1%)
D
MDS pt.6
Disrupted (65.6%)
Intact (35.4%)
E
MDS pt.7
Disrupted (52.8%)
Intact (47.2%)
F
MDS pt.8
Disrupted (77.7%)
Intact (22.3%)
G
MDS pt.9
Disrupted (59.9%)
Intact (40.1%)
H
MDS pt.10
Disrupted (51%)
Intact (49%)
I
RAC1 inhibitor
Disrupted (37.6%)
Intact (62.4%)
J
Chronic Kidney Disease
Disrupted (3%)
Intact (97%)

Supplemental Figure 5. Quantitation of F-actin disruption by Multispectral flow cytometry (ImageStream^{TM}). A-J) Histograms displaying the extent of F-actin disruption in different subtypes of MDS. Cortical actin continuity index (CACI) developed to measure the staining pattern of actin was used to quantify actin filament lengths.

A

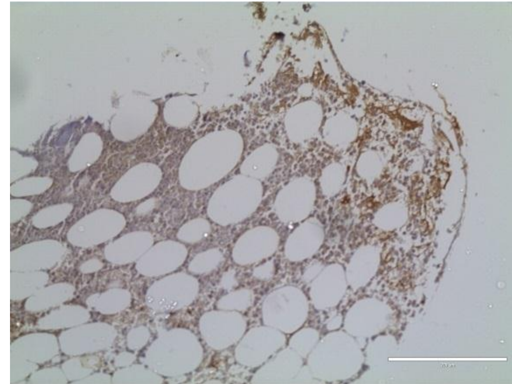
Pt.	Cytogenetics	Rx	% Actin disruption from Imagestream	DOCK4 IHC
13	Normal	MDS	75.4	0
14	CMML	CMML	73.1	0
15	Normal	UA	63.1	0
16	Normal	MDS	62.9	0
17	Trisomy 20	MDS	59.7	1
18	UA	UA	40.8	1
19	UA	UA	14.7	2
20	Normal	MDS	10.5	2

B



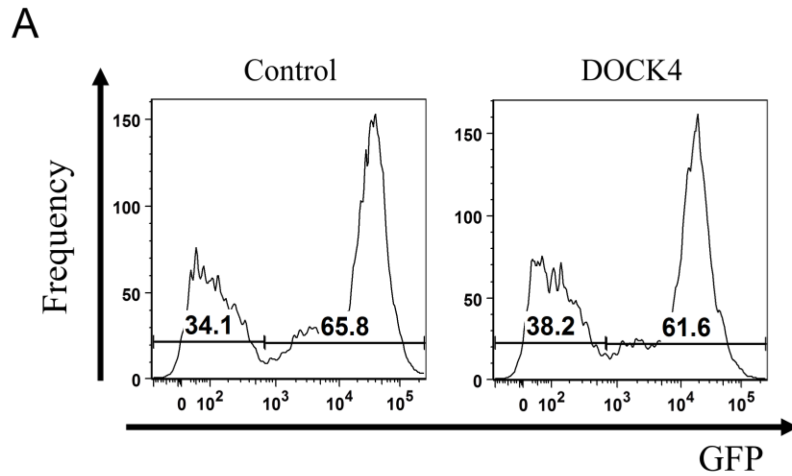
DOCK4 IHC 0

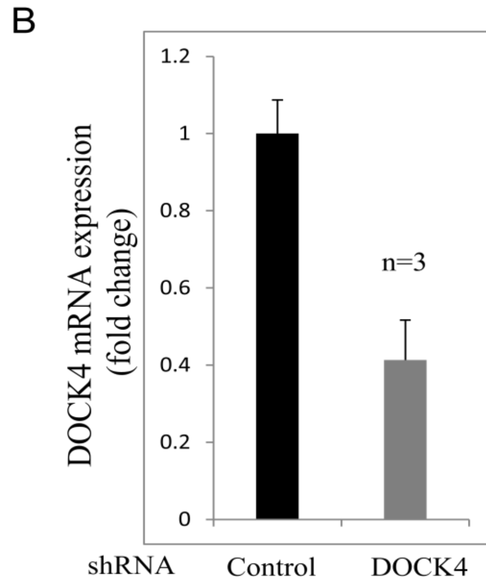
C



DOCK4 IHC 2+

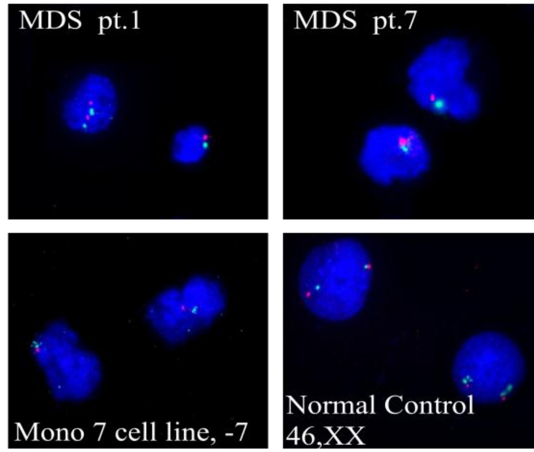
Supplemental Figure 6. Actin disruption in MDS erythroblasts without deletion of chr 7: Table showing actin disruption (by ImageStream analysis) and DOCK4 expression (by IHC) in MDS patients without deletion of chromosome 7 (A). Representative IHC plots showing low and high expression of DOCK4 in the marrow of MDS patients (B,C).



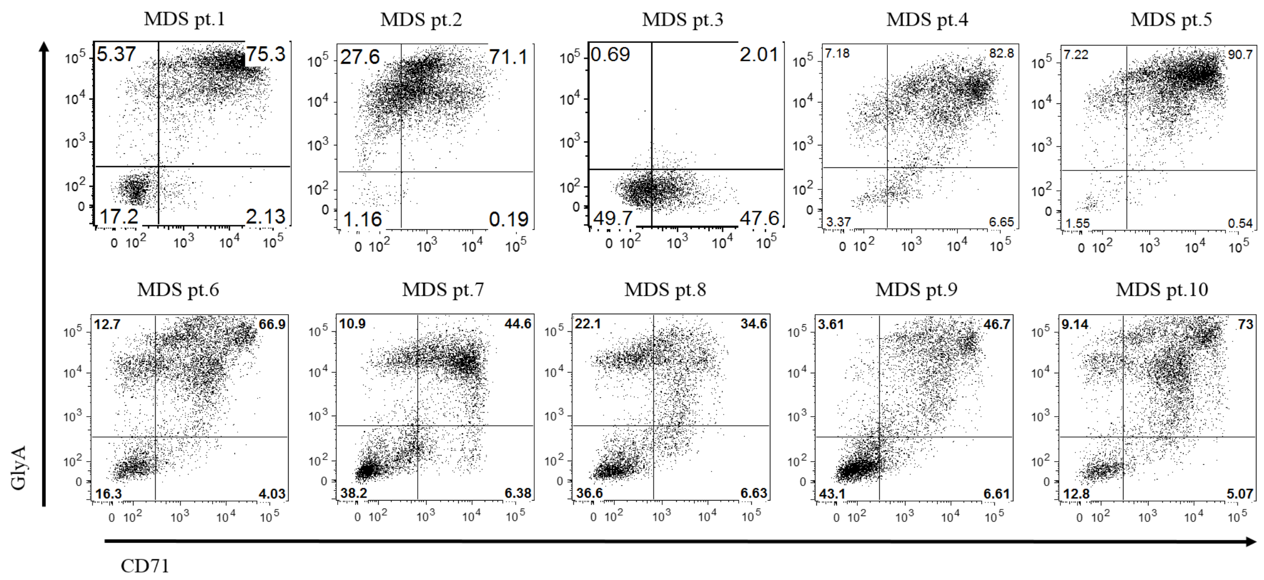


Supplemental Figure 7. *DOCK4* knockdown in primary human erythroblasts. Day 0 CD34+ cells were infected with lentiviral vector driven shRNAs for knockdown of DOCK4 and selected on puromycin. A) Flow cytometry analysis on day 6 erythroblasts to evaluate for lentiviral infection efficiency. B) Q-PCR showing fold reduction of DOCK4 mRNA expression compared to cells infected with control shRNA.

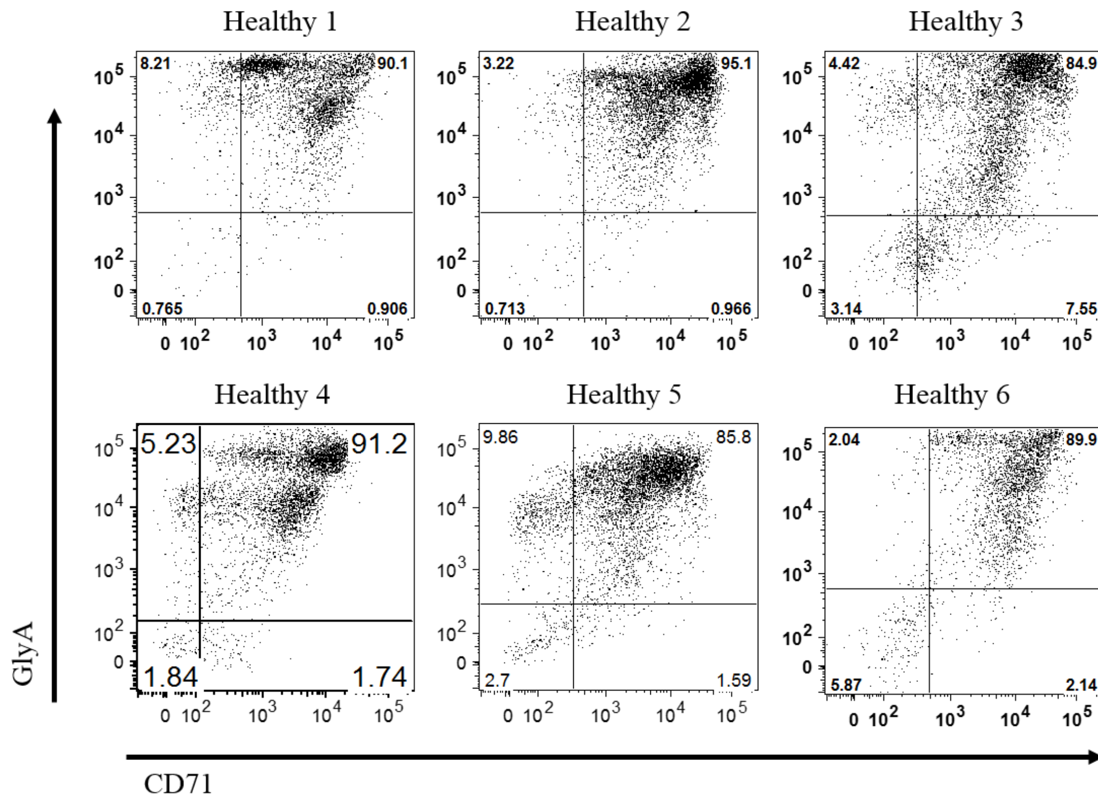
A



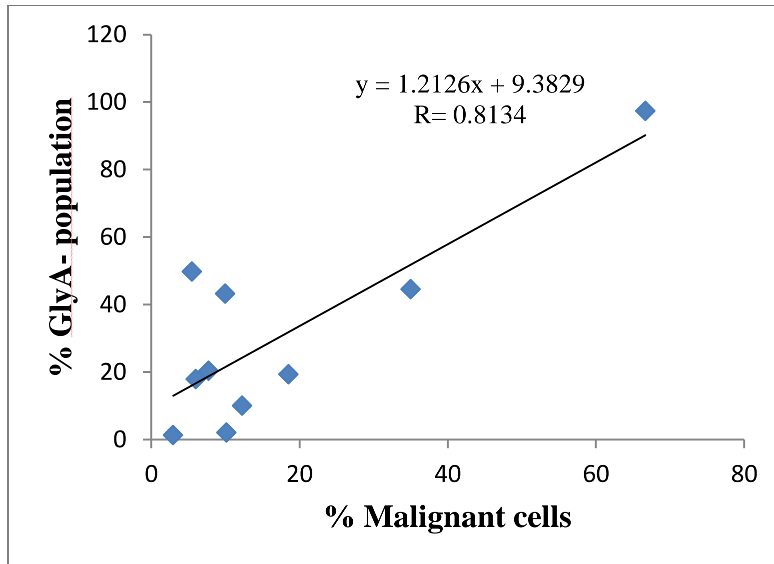
B



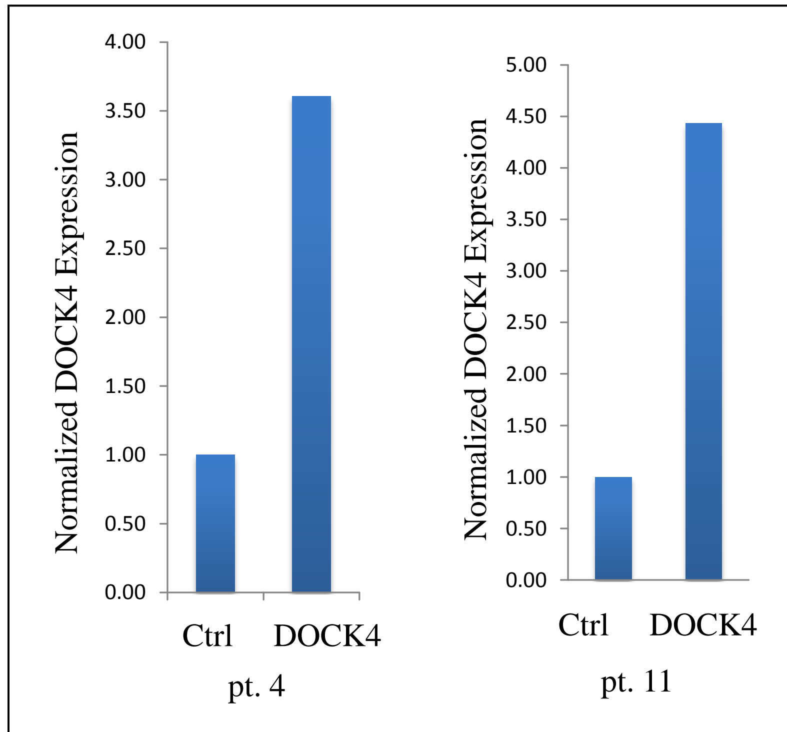
C



Supplemental Figure 8. Terminal differentiation of MDS malignant erythroblasts. A) Photomicrographs of day 10 cultured erythroblasts from MDS patients used for fluorescence in situ hybridization (FISH) that depict clonal malignant cells devoid of an arm of chromosome 7. Mono 7 cell line was used as a positive control and cells from a healthy donor used as a negative control. B) Flow cytometry analysis of erythroblasts from MDS patients derived from CD34+ cells on 14 of culture. C) Flow cytometry analysis of erythroblasts from healthy donors derived from CD34+ cells on 14 of culture.



Supplemental Figure 9. Correlation of the extent of erythroid terminal differentiation and the malignant cells in cultured primary MDS erythroblasts. Glycophorin A level were determined by flow cytometry as a measure of terminal differentiation in ten MDS samples along with FISH analysis to enumerate the numbers of malignant cells.



Supplemental Figure 10. Re-expression of DOCK4 in DOCK4 deficient MDS patient samples. Minicircle plasmid carrying *DOCK4* was transfected into CD34+ cell derived day 5 erythroid progenitors from two MDS patients lacking DOCK4 and qPCR was performed on day 10 of erythroblast culture. Normalized levels of *DOCK4* expression is shown compared to untransfected cells.