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Codanin-1 Mutations Engineered in Human Erythroid Cells Demonstrate Role of CDAN1 in Terminal Erythroid Maturation

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Abstract

The generation of a functional erythrocyte from a committed progenitor requires significant changes in gene expression during a time of hemoglobin accumulation, rapid cell division, and nuclear condensation. Congenital Dyserythropoietic Anemia type I (CDA-I), is an autosomal recessive disease that presents with erythroid hyperplasia in the bone marrow. Erythroblasts in patients with CDA-I are frequently binucleate, have chromatin bridging, and defective chromatin condensation. CDA-1 is most commonly caused by mutations in Codanin-1 (CDAN1). The function of CDAN1 is poorly understood but it is thought to regulate histone incorporation into nascent DNA during cellular replication. The study of CDA-1 has been limited by lack of in vitro models that recapitulate key features of the disease and most studies on CDAN1 function have been done in non-erythroid cells. To model CDA-I we generated HUDEP2 mutant lines with deletion or mutation of R1042 of CDAN1, mirroring mutations found in CDA-1 patients. CDAN1 mutant cell lines had decreased viability, increased intercellular bridges, and binucleate cells. Further, they had alterations in histone acetylation associated with prematurely elevated erythroid gene expression, including gamma-globin. Together, these data imply a specific functional role for CDAN1, specifically R1042 on exon 24, in the regulation of DNA replication and organization during erythroid maturation. Most importantly, generation of models with specific patient mutations, such as R1042, will provide further mechanistic insight into CDA-I pathology.

Authorship Statement

Experiments were performed by ZM, KB, EL, and MG. Data analyses was performed by ZM, JM, and LAS. HUDEP2 cells were supplied by RK and YN. The manuscript was written by ZM and LAS.

Introduction

Erythropoiesis is a process of enormous magnitude, with the average adult generating 2–3 million red blood cells per second to maintain steady state and avoid anemia[1]. This requires significant changes in gene expression during a time of hemoglobin accumulation and rapid cell division [2]. Terminal erythroid maturation (TEM) also requires the condensation of large regions of the erythroid genome, which is associated with downregulation of most non-erythroid genes[3]. Disruption of nuclear condensation is associated with myelodysplastic syndromes and congenital anemias, however the factors that regulate this process are poorly understood.

Congenital dyserythropoietic anemia type-I (CDA-I), is an autosomal recessive disease that often presents with macrocytic anemia in the neonatal period or early childhood [4]. Patients with CDA-I are frequently transfusion dependent and suffer from iron overload by early adulthood[5]. Bone marrow studies of CDA-1 patients demonstrate erythroblasts with chromatin bridging and defective chromatin condensation[6, 7]. CDA-1 is commonly caused by mutations in the ubiquitously expressed gene Codanin-1 (CDAN1)[8]. The function of CDAN1 is poorly understood but studies in HELA and other non-erythroid cells suggest it regulates histone incorporation into nascent DNA during cellular replication[9]. Homozygous null CDAN1 disruption is embryonic lethal pre-implantation, suggesting it has an essential function in multiple cell types [6]. However, the reason mutations in this ubiquitously expressed gene cause an erythroid-specific phenotype is unknown. Since knockdown of CDAN1 in Hela cells and patient-derived lymphocytes did not recapitulate key features of CDA-I[6,10], there is an urgent need for erythroid-derived models to analyze the CDAN1 mechanism.

In this report we establish an erythroid model system that recapitulates key phenotypic features of CDA-I and allows dissection of the molecular mechanisms that underlie CDA-I.

Results and Discussion

CDAN1 is robustly expressed during early erythroid maturation, with a progressive decline in both RNA and protein levels during maturation(SFigure 1a, b;[11, 12]). We confirmed this progressive decline in two erythroid cell systems, HUDEP2 and CD34+-derived erythroblasts (Figure 1a and SFigure 3). This expression pattern suggests that CDAN1 has a critical function in early and intermediate erythroblasts. Consistent with this observation, in bone marrow samples from CDA1 patients, chromatin bridging and binuclearity are most prominently observed in intermediate erythroblasts [13]. HUDEP2 cells, which are transcriptionally similar to intermediate erythroblasts (SFigure 2), are therefore good model system for studying CDAN1 dynamics in erythroid cells.

Previous studies have been conducted primarily in non-erythroid cells, with conflicting results regarding the cellular location of CDAN1, likely due to the use of different cell lines, as well as difficulty obtaining reliable antibodies[10, 14]. We therefore first sought to determine the cellular location of CDAN1 in erythroid cells. Immunocytochemistry using a validated commercial antibody (SFigure3) showed that CDAN1 is located both in the

cytoplasm and nucleus of erythroid cells (Figure 1b). We further interrogated the cellular location of CDAN1 using intracellular flow cytometry (IFC) and a clonal heterozygous tagged CDAN1 HUDEP2 cell line (SFigure 3). Intracellular IFC confirmed that CDAN1 is located in both the cytoplasm and nucleus of the cell and suggest that CDAN1 is perinuclear (Figure 1c). These findings are consistent with the proposed role for CDAN1 in the regulation of histone import and cell proliferation[9].

To gain insights into the function of CDAN1 in erythroid cells, we used genome editing to create clonal HUDEP2 mutant or control CDAN1 cell lines (SFigure 3). Mutants 1 and 2 (M.1 and M.2) contain a heterozygous deletion resulting in the loss of exon 24, which encodes the R1042 residue commonly mutated in CDAN1 patients. Mutant 3 (M.3) harbors a R1042W (cDNA, 3124C>T) mutation with an exon 23–27 deletion[8]. Consistent with mutations found in CDA-I patients[8] and an essential role for CDAN1 in erythropoiesis, we were unable to generate HUDEP2 lines with homozygous deletions or mutations of exon 24 [8]. Further supporting an essential role for CDAN1 in TEM, mutant CDAN1 cell lines had an observable increase in binuclearity, cytoplasmic invagination into the nucleus, and DNA bridging (Figure 1 f–g, SFigure 3)[15]. Quantification of binuclearity and intercellular bridging observed in TEM (Figure 1d and e) confirmed a CDA-I-like phenotype in mutant cell lines (Figure 1f and g). These data suggest that CDAN1, and more specifically the R1042 residue, is important for erythropoiesis and may have a critical function in intermediate erythroblasts.

All three mutant cell lines had decreased viability during expansion (Figure 2a). Using a fluorescent dye to track cell divisions, M.3 showed a decrease in cell doubling time opposed to increased doubling time in the mutants with the loss of the R1042 residue (Figure 2b). M.3 also had a unique cell cycle profile, with a significantly lower percent of cells in S-phase and a higher proportion of cells in M, based on both Ki-67 staining (Figure 2c) and the rate of DNA synthesis (Figure 2d). These data suggest that heterozygous loss of the R1042 residue has different functional consequences than compound heterozygosity for the exon 24 deletion and the point mutation, suggesting a functional role for this residue in erythroid cells. Previous studies, performed in non-erythroid cells, suggested that CDAN1 interacts with the histone chaperone and facilitator of histone acetylation, ASF1, to regulate histone availability during DNA replication[10]. Since ASF1 imports histone heterodimers into the nucleus, and can facilitate histone acetylation prior to DNA incorporation[16], we addressed histone acetylation and nuclear import[17, 18]. We assessed the level of histone acetylation with an antibody to H3K27Ac, which works robustly in intracellular IFC, demonstrating that there is an increase in H3K27Ac in all three mutant lines (Figure 2e and f). To assess histone import, we transduced M.3 with a plasmid encoding a histone H4-GFP fusion protein. Intriguingly, although the R1042 residue is not located in the ASF1 interacting domain,[10] we observed an increased proportion of H4 locating to the nucleus over 48 hours in M.3 cells compared to control (SFigure 4). Combined with the decrease in the proportion of M.3 cells in S-phase without a corresponding increase in BrdU median fluorescent intensity (Figures 2c and d), these results are consistent with increased histone incorporation, without a significant change in DNA replication. Together, these data suggest that mutations in CDAN1 result in alterations in histone availability and acetylation, which may be detrimental to TEM.

All three mutant lines had a decrease in cell viability during TEM (Figure 2g and h), with an increased rate of apoptosis observed in all mutant lines (SFigure 6). Mutant cells that survived TEM were phenotypically similar to controls. IFC analyses confirmed that the cell and nuclear size of the surviving mutant cells, as well as the cell surface markers, were similar to control, although there was increased variation in these measurements in M.3, consistent with an underlying disruption in the TEM process (Figure 2i–l). Collectively, these results suggest that the R1042 residue of CDAN1 is important for normal TEM, and that mutation and deletion of this residue may have distinct functional consequences.

We next addressed whether CDAN1 mutation altered the transcriptional profile of the erythroblasts using RNA-seq. Clustering of differential expressed genes (DEG) showed high correlation among the control or mutant lines (Figure 3a), and the DEG were enriched for pathways and disease associations correlating with pathways important for erythroid maturation (i.e. JAK cytokine signaling) (SFigure 5). Intriguingly, more genes were downregulated than upregulated in the mutant lines (189 downregulated, 104 upregulated, adjusted p-value <0.05) (Figure 3a). Since this is counterintuitive to the increased histone acetylation observed, we analyzed DEG for their associated transcriptional regulators. We found that upregulated genes were associated with canonical erythroid cell type specific transcription factors (Figure 3b) and downregulated DEG associated with other hematopoietic cell lineage-specific transcription factors (Figure 3c), suggesting that upregulated DEG were enriched for erythroid-specific transcription factor targets. Indeed, the upregulated genes included genes highly expressed in erythroid cells, including GYPA. Intriguingly, there was also upregulated expression of gamma globin in CDAN1 mutant cells (Figure 3d and e), mirroring the elevated fetal globin expression observed in some CDA-1 patients[19–21]. Together, these results indicate premature loss of CDAN1 function in erythroid cells alters the transcriptome.

In conclusion, introduction of CDAN1 mutations into HUDEP2 cells, which model the stage of erythropoiesis most dramatically impacted by CDAN1 mutations, can recapitulate key features of CDA1. HUDEP2 cells are amenable to the creation of stable lines that carry specific CDAN1 mutations, and are therefore useful for gaining insights into the function of CDAN1 in erythroid cells. Complementary studies in CD34+ derived erythroid cultures and other systems are likely to provide further insights. Our studies using suggest that CDAN1 is a required component of the transcriptional and epigenetic changes that occur during TEM. Together with recent proteomic studies demonstrating that histone number is stable during TEM[3], and studies suggesting that CDAN1 regulates ASF1 [10, 17, 18], our studies are consistent with a model where CDAN1 regulates histone import, acetylation, and incorporation into chromatin during TEM (Figure 3f). We speculate that CDAN1 deficiency leads to more rapid incorporation of acetylated histones during DNA replication, and that erythroblasts, which must rapidly proliferate to meet the demand of the body for red blood cells, are unable to adequately deacetylate these hastily incorporated histones, resulting in disruption of chromatin architecture in heterochromatic regions, augmentation of gene expression in euchromatic regions, and decreased viability.

Methods

Single guide RNA and associated CRISPR/Cas9 editing[22], cell culture[12, 22, 23], IFC[22, 24], flow cytometry[25], and transcriptomic analysis[26] were completed as previous described. For additional details please see supplementary methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Congenital dyserythropoietic anemia type 1 (CDA1) is caused by mutations in CDAN1.
- HUDEP2 lines with deletion or mutation of CDAN1 phenocopy CDA1 erythroblasts.
- CDAN1 mutant HUDEP2 cells have altered histone acetylation and gene expression.

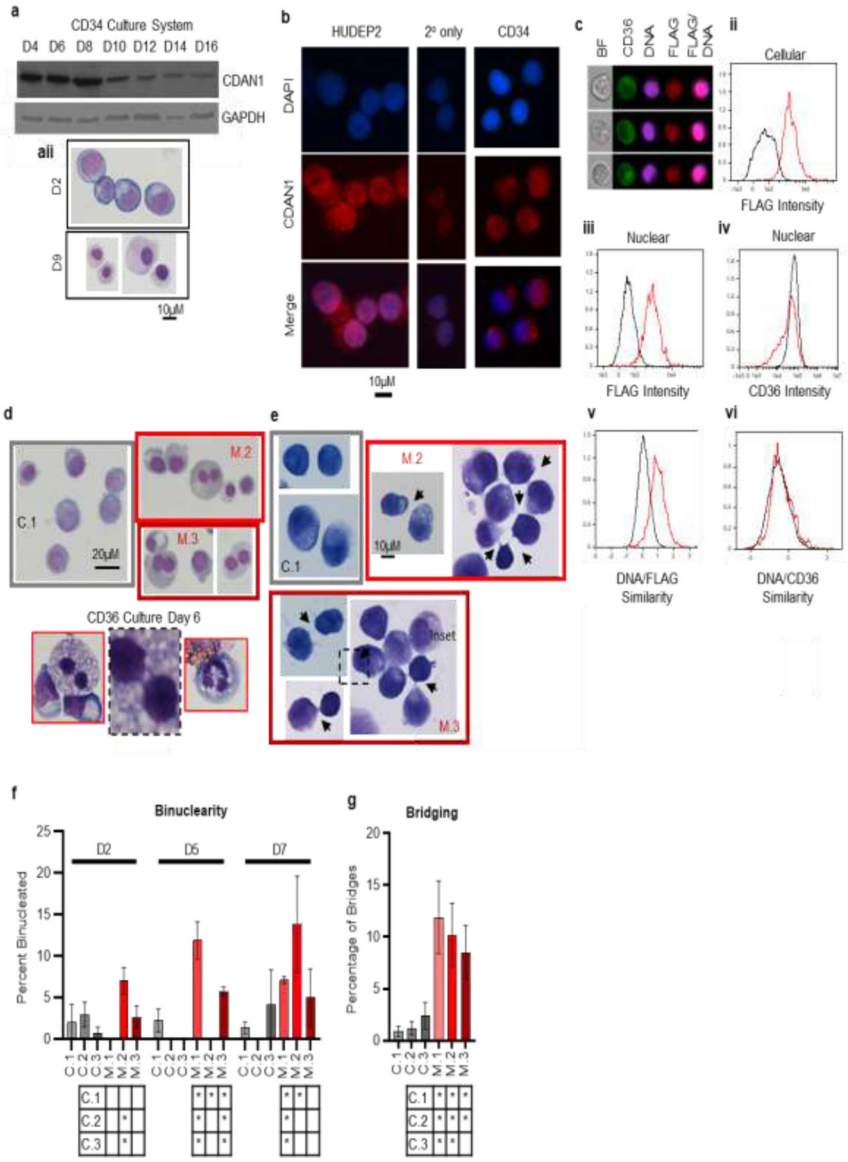


Figure 1. CDAN1 is perinuclear in erythroid cells and CDAN1 exon 24 mutations result in a CDA I phenotype.

(A) CDAN1 protein levels across erythroid maturation in CD34-derived erythroid culture (completed as in [11]). Representative cytospins showing expansion cells and intermediate erythroblasts (Day 2 and Day 9) (B) Immunocytochemistry showing perinuclear location of CDAN1 in HUDEP2 and CD34-dervied erythroid cells on culture Day 4. (C) Representative images from IFC showing location of tagged CDAN1 and total intensity of AF647 anti-FLAG antibody is quantified both in the cell (Ci) and in the nucleus as defined by the DNA stain (Cii, black line = non-immune control, red line = AF647 anti-FLAG stained cells). CD36 staining serves as a negative control for intensity in the nucleus (Eiv). (Cv) Analysis using a similarity score between the DNA stain and anti-FLAG antibody show an increased similarity between the DNA and CDAN1 location compared to control. (Cvi) Similar analysis between DNA and CD36 serves as a negative control. (D) Representative Cytospin cells showing increased frequency of binucleate cells in CDAN1 mutants and bulk CD36-

selected culture after exon 24 sgRNA targeting. (E) Sit-and-fix cells with intercellular bridging in mutant CDAN1 cells are shown with arrows indicating observed bridges. Increased binuclearity (F) and intercellular bridging (G) are confirmed by quantification. All replicates shown on graphs and statistics completed by two-way ANOVA followed by Fisher's LSD test for multiple comparisons ($p<0.05$ = *).

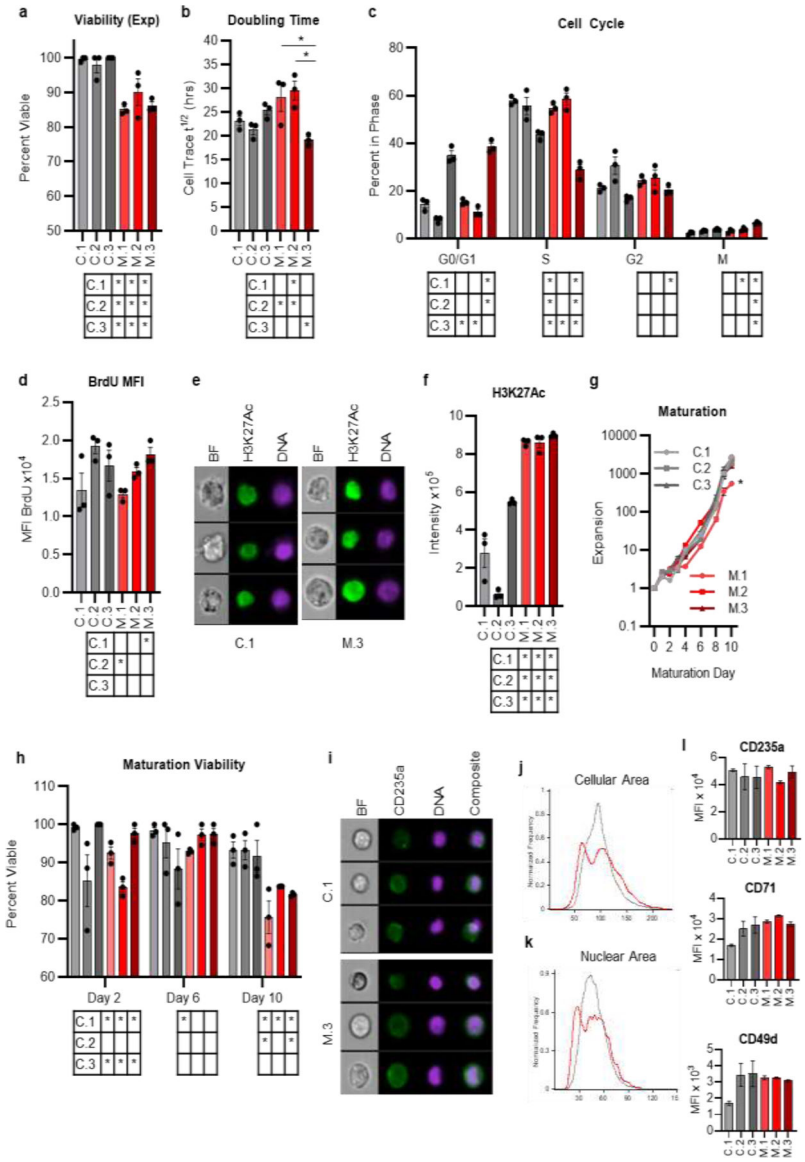
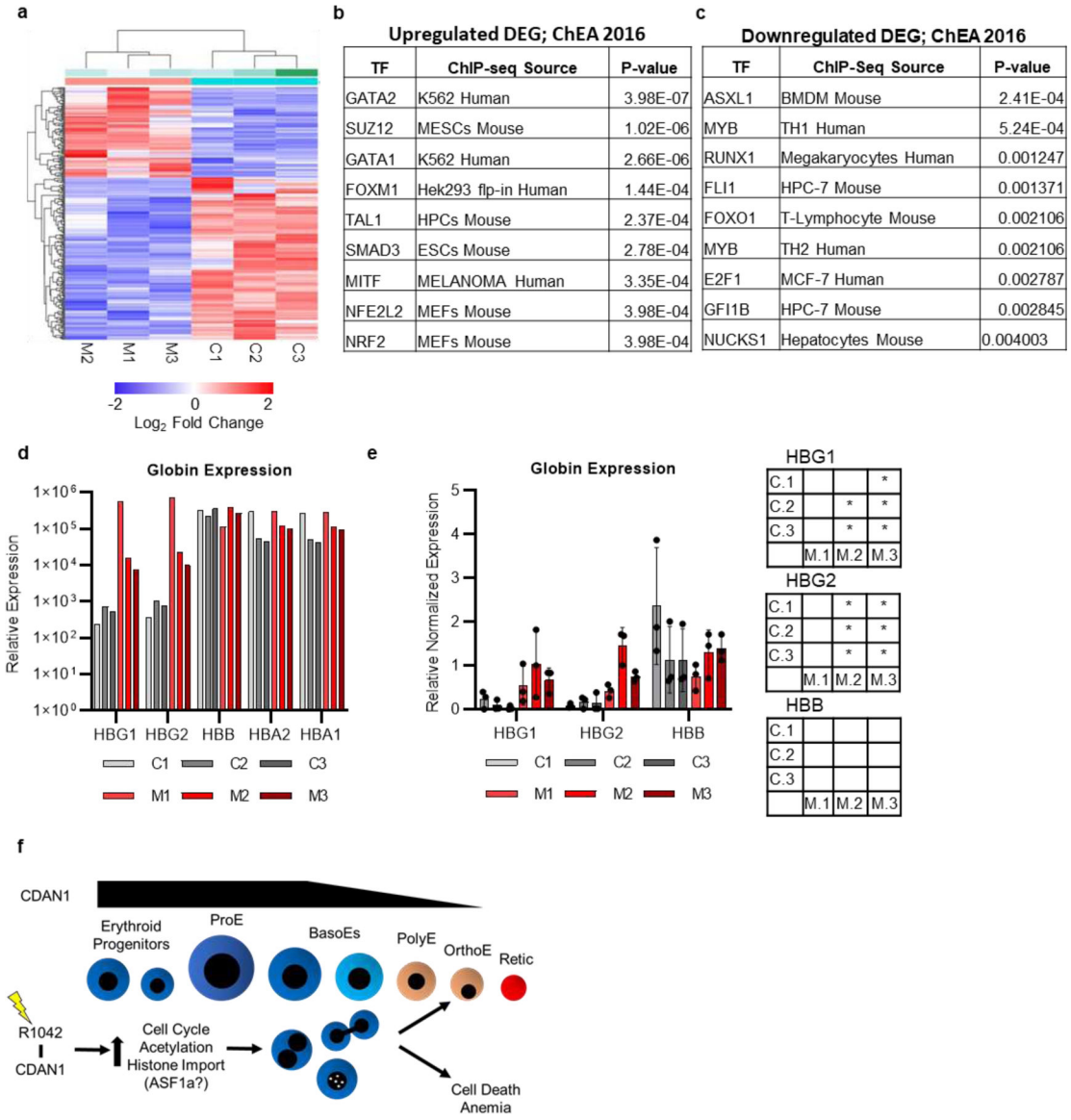


Figure 2. CDAN1 mutations increase histone acetylation, disrupt cell cycle, and decrease cell viability during erythroid maturation.

(A) Cell viability counted over 10 days of HUDEP2 expansion culture quantified by trypan blue exclusion. (B) Doubling time, as determined by flow cytometric analysis of CFSE intensity. (C–D) Representative intracellular IFC images of AF488 anti-H3K27ac and DNA staining in C.1 and M.3 clones followed by quantification of median fluorescent intensity. (E) Cell cycle determined by DNA staining and Ki67 intensity[25]. (F) BrdU incorporation quantified by anti-BrdU antibody to determine relative length of time for DNA replication. (G) Growth curve of clonal lines in maturation conditions, determined by daily counts. (H) Viability of the maturing culture, determined by trypan blue exclusion and shown at three days in culture. (I) Representative images of matured, Day 10, C.1 and M.3 clones show similar cell and nuclear size. (J–K) Size distribution or the cell and nucleus between C.1 and M.3 show increased variation in CDAN1 mutant regardless of similar median value. (L) MFI analysis of cell surface markers associated with TEM are shown at maturation day 6. All

replicates shown on graphs and statistics completed by two-way ANOVA followed by Fisher's LSD test for multiple comparisons ($p<0.05$ = *).



TF	ChIP-seq Source	P-value
GATA2	K562 Human	3.98E-07
SUZ12	MESCs Mouse	1.02E-06
GATA1	K562 Human	2.66E-06
FOXM1	Hek293 flp-in Human	1.44E-04
TAL1	HPCs Mouse	2.37E-04
SMAD3	ESCs Mouse	2.78E-04
MITF	MELANOMA Human	3.35E-04
NFE2L2	MEFs Mouse	3.98E-04
NRF2	MEFs Mouse	3.98E-04

TF	ChIP-Seq Source	P-value
ASXL1	BMDM Mouse	2.41E-04
MYB	TH1 Human	5.24E-04
RUNX1	Megakaryocytes Human	0.001247
FLI1	HPC-7 Mouse	0.001371
FOXO1	T-Lymphocyte Mouse	0.002106
MYB	TH2 Human	0.002106
E2F1	MCF-7 Human	0.002787
GFI1B	HPC-7 Mouse	0.002845
NUCKS1	Hepatocytes Mouse	0.004003

Figure 3. CDAN1 mutations result in upregulation of fetal globin and genes associated with erythroid maturation.

(A) Heat map shown of hierarchically clustered log transformed differentially expressed genes generated from DeSeq2 analysis. Enrichr web platform[27] used to analyze transcription factors predicted to be relevant based on upregulated (B) or downregulated (C) DEG. (D) Globin expression in each clonal population shown using RNA-seq data (D) and qPCR in triplicate (E). (F) CDAN1 expression is highest in early erythroblasts before progressive downregulation. We propose a model where CDAN1 mutation leads to altered cell cycle progression, and disrupted histone homeostasis, including increased histone acetylation, and histone import, in early and intermediate erythroblasts. Although some CDAN1 mutant erythroblasts are able to complete maturation, these changes result in an increased frequency of dysmorphic erythroid precursors with decreased viability, and

ultimately anemia. All replicates shown on graphs and statistics completed by two-way ANOVA followed by Fisher's LSD test for multiple comparisons ($p<0.05$ = *).