

## **Functional impairment of erythropoiesis in Congenital Dyserythropoietic Anaemia type I arises at the progenitor level**

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## Introduction

The question of why certain cell types are differentially susceptible to mutations in broadly expressed genes remains largely unanswered. An important first step in determining vulnerability of a specific lineage is to identify at what stage its requirement for a given protein arises. Strikingly, many types of congenital anaemia result from mutations in widely, if not ubiquitously, expressed proteins involved in core cellular processes. Examples include: mutations in ribosomal proteins causing Diamond Blackfan Anaemia (Choesmel et al., 2007); mutations in the secretory vesicle protein SEC23B causing Congential Dyserythropoietic Anaemia (CDA) type II (Schwarz et al., 2009); in CDA type III, arising from mutations in the kinesin encoded by *KIF23* and *RACGAP1* (Liljeholm et al., 2013; Wontakal et al., 2021); and CDA type I (CDA-I) caused by mutations in *CDAN1* or *CDIN1* (Roy & Babbs, 2019).

CDA-I is an example of a rare disease which has the potential to inform about general cellular processes. *CDIN1* and *CDAN1* are widely expressed and loss of either protein is incompatible with life, however, only developing erythroblasts appear to be susceptible to biallelic hypomorphic mutations in either gene. We have recently shown that *ex-vivo* differentiated erythroid cells from patients with CDA-I are delayed during terminal erythroid differentiation and this is associated with increased proliferation and widespread changes in chromatin accessibility (Scott et al, 2021). However, these studies were performed on CD34<sup>+</sup> cells, which represent a mixture of haematopoietic stem and progenitor cells (HSPCs). The aim of this study was to investigate the precise cellular stage affected in CDA-I by assaying the functional consequences of *CDAN1/CDIN1* mutations (Table 1) on the frequency and erythroid output of myeloid progenitors in patients with CDA-I. We show CDA-I does not alter the myeloid compartment, however, the functional output of CDA-I megakaryocyte and erythroid progenitors (MEPs) is compromised. Both in clonogenic assays and in liquid culture, CDA-I MEPs produce a higher proportion of aberrant colonies and show reduced expression of the erythroid lineage markers CD235 and CD71.

## Results and Discussion

Whether the erythroid defect in CDA-I is confined to terminally maturing erythroblasts or also affects their progenitors remains an open question. To address this we first investigated the expression of both causative genes (*CDAN1* and *CDIN1*) in primary human HSPCs from healthy individuals and found that similar expression levels of both genes were maintained throughout the haematopoietic hierarchy from HSC through to committed myeloid and erythroid progenitors, decrease during terminal erythroid differentiation from the basophilic erythroblast stage onwards (Figure 1a,b; Olijnik & Roy et al., 2021). Next, to determine when defects first arise in CDA-I erythropoiesis, we compared the frequency of the progenitor cells upstream of erythroblasts in the haematopoietic hierarchy [Common Myeloid Progenitors (CMP), Megakaryocyte-Erythroid Progenitors (MEPs) and Granulocyte-Macrophage Progenitors (GMPs)] in peripheral blood (PB) of patients with CDA-I with controls (Figure 1c). This showed that the frequency of all three progenitor cell types was the same in CDA-I patients (n=5) compared to controls (n=17) (Figure 1c), indicating that biallelic mutations in *CDAN1* or *CDIN1* have no effect on the numbers of myeloid progenitors with erythroid potential, despite expression of both genes in progenitors (Figure 1b). This finding is in contrast to the situation in Diamond Blackfan Anaemia (DBA), where there are quantitative and qualitative abnormalities in erythroid progenitors (Iskander et al; 2015).

To assess whether the function of these myeloid progenitors was affected by biallelic mutations in *CDAN1* or *CDIN1*, we sorted individual MEPs from healthy individuals (n=10) and CDA-I patients (n=5) into methylcellulose and cultured them for 14 days. The overall plating efficiency and proportion of MEPs that gave rise to erythroid colonies was similar in patients with CDA-I and controls ~70% (Figure 1d). However, whilst 100% of the control MEPs produced mature BFU-Es, 16.9%  $\pm$  3.7 of the erythroid colonies produced by CDA-I MEPs were small, poorly haemoglobinised erythroid clusters that were not seen in the MEP cultures from healthy donors (Figure 1d). These data suggest that in patients with CDA-I, mutations in *CDAN1* or *CDIN1* cause perturbation of erythropoiesis at an earlier stage of erythroid differentiation (MEP) than previously reported, with samples from patients with mutations in either gene being similarly affected. Consistent with this, in three CDA-I patients with *CDAN1* mutations, treatment with IFN- $\alpha$ 2a, the only current therapy for CDA-I (Roy and

Babbs, 2019), restored the normal frequency and pattern of MEP-derived erythroid colonies (Figure 1d) and also normalised Hb levels in patients (Figure 1e). Sorting of individual MEPs into liquid culture, to allow more terminal differentiation, confirmed a qualitative defect in the number of MEP derived colonies grown from CDA-I MEPs (n=36 colonies) compared to controls (n=62 colonies) (Supplementary Figure 1a) whilst, interestingly, MEPs (n=91) cultured from three CDA-I patients who were on IFN- $\alpha$ 2a treatment at the time of sampling, showed normal erythroid maturation *in vitro* with only 6% aberrant colonies compared to 36% from the untreated CDA-I patients (Supplementary Figure 1b).

In summary, we provide evidence that defects in erythroid development in the rare inherited anaemia CDA-I arise at the MEP stage of erythroid differentiation, much earlier than previously recognized. There is no reported effect on the megakaryocytic lineage and platelet counts in CDA-I patients appear to be normal (Table 1). In common with other cell types, the question of why megakaryocytes are unaffected in CDA-I remains unclear. One explanation may be functional redundancy, as is presumed to be the case in other cell types. Some insight into this may come from analysis of platelet RNA, which suggests that megakaryocytes express a higher level of *CDIN1* than *CDAN1*, the latter of which is below the level of detection (Simon et al., 2014). Our data also suggest that the erythroid defect can be corrected by IFN- $\alpha$ 2a. It remains to be shown whether abnormal MEPs in CDA-I give rise exclusively to abnormal erythroblasts. Current understanding suggests the proteins encoded by *CDIN1* and *CDAN1* play a role in DNA repair and/or chromatin assembly. Analysing erythroid cells harbouring mutations in both genes would be likely to elucidate this novel pathway, however, generating suitable models will be technically challenging. By refining the stages of erythroid differentiation affected in CDA-I, this study paves the way for further work to identify why erythroid progenitors at the MEP stage develop a specific requirement for the function of the proteins encoded by *CDAN1* and *CDIN1*. To this end, single-cell multiomic approaches to characterize the molecular defect in CDA-I progenitors are ongoing in our laboratory. Such studies would have the potential to inform novel processes in normal and disease erythropoiesis.

**Data Availability:** Raw RNAseq data from Gene Expression Omnibus (GEO) GSE74912 and GSE115684 was analysed in Figure 1b.

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**Contributions:** CS extracted CD34<sup>+</sup> cells from CDA-I patient blood and cones; CS and KB performed the flow cytometry analysis and sorting experiments with assistance from SC. CS and KB scored, imaged and flow cytometric analysis of colonies from the Methocult and liquid culture assays. IR, VJB, NR, CB, CS and KB conceived and designed experiments; NR provided conceptual advice and clinical oversight; CB created the figures; CS and CB wrote the paper and all authors reviewed and critically edited the manuscript. RB, QAH, SO, RR, KR, MC and NR are the clinicians responsible for the care of the CDA-I patients.

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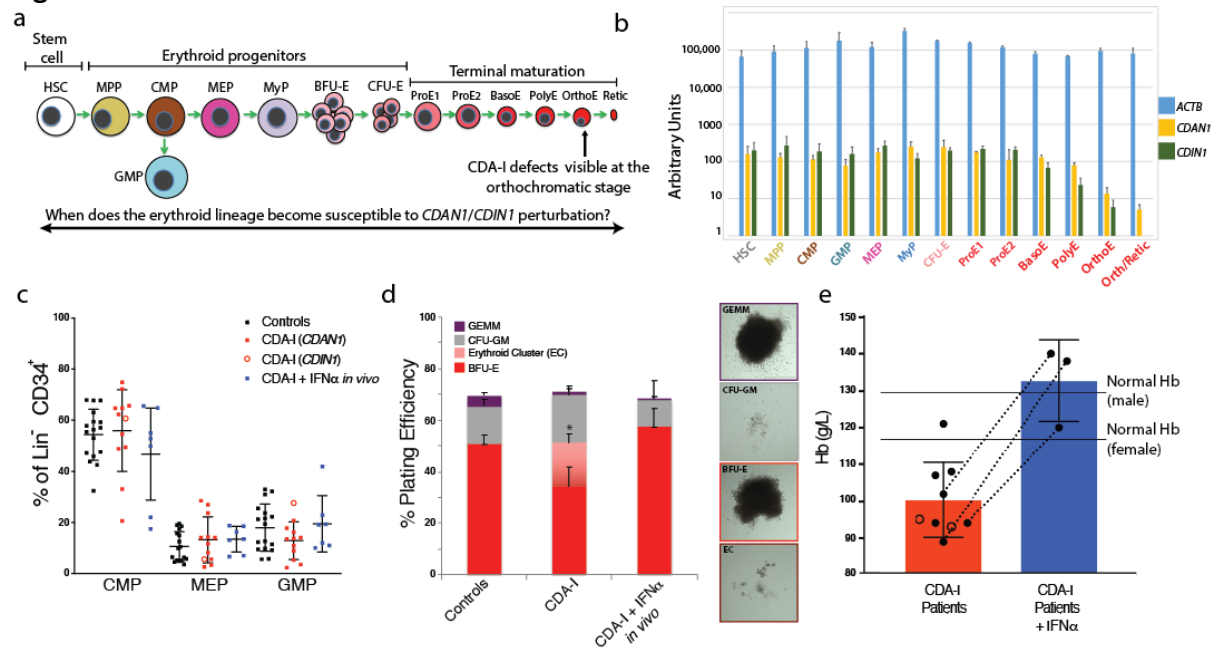
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**Table 1: Clinical Information**

Unique Identifier	Sex	Ethnicity	Hb (g/L)	MCH (pg)	MCHC (g/dL)	MCV (fl)	Plt (103/mm <sup>3</sup> )	RDW (%)	Gene	Alleles	Protein	Reference	Treatment
UPID01	F	Caucasian	94	37	346	107	364	20.4	CDAN1	c.152C>T	p.Pro51Leu	Roy et al., 2016	NA
UPID02	F	Caucasian	108	36.4	351	104	188	16.5	CDAN1	c.152C>T	p.Pro51Leu	Roy et al., 2016	NA
UPID10	M	NA	NA	NA	NA	NA	NA	NA	CDAN1	c.3124C>T	p.Arg1042Trp	Dgany et al., 2002	NA
UPID15	M	Caucasian	93	35.4	33.5	106	115	17.6	CDAN1	c.1104_1106delCTT c.3128A>T	p.Phe369del p.Asp1043Val	El-Sheikh et al., 2014, Dgany et al., 2002	NA
UPID17	F	Caucasian	140/96	NA	NA	118	596	NA	CDAN1	c.2015C>T c.2681_2682delAG	p.Pro672Leu p.Glu894ValfsTer109	Dgany et al; 2002 Olijnik & Roy et al 2020	Venesection IFN-α2a
UPID19	F	NA	94	NA	NA	96	289	NA	CDAN1	c.2015C>T c.2868+5G>C	p.Pro672Leu "Tier 3 variant, abnormal CDAN1 mRNA splicing" "ivs 22+5G to C"	Dgany et al; 2002 Olijnik & Roy et al 2020	IFN-α2a
UPID20	M	Caucasian	95	NA	NA	102	NA	NA	CDAN1	c.2015C>T c.2971_2977dupGCAGCAG	p.Pro672Leu p.Val993GlyfsTer13	Dgany <i>et al.</i> , 2002, Roy <i>et al.</i> , 2016	NA
UPID22	F	Caucasian	107	NA	NA	101.8	319	NA	CDAN1	c.2015C>T c.3338T>C	p.Pro672Leu p.Leu1113Pro	Dgany <i>et al</i> ; 2002 Olijnik & Roy et al 2020	NA
UPID23	F	Caucasian	138	NA	NA	104	NA	NA	CDAN1	c.2044C>T c.2744_2767del	p.Arg682Ter p.Leu915_Leu923del	Dgany et al; 2002 Olijnik & Roy et al 2020	IFN-α2a
UPID27	F	Caucasian	121	NA	NA	101	145	NA	CDIN1	c.467G>A	p.Cys156Tyr	Olijnik & Roy et al 2020	NA

Each patient has been assigned a unique personal identifier (UPID). The table includes full blood counts where available on the day of the CD34<sup>+</sup> HSPC extraction (shaded boxes), mutations in *CDIN1* and *CDAN1* and treatment the patients are receiving. NA, data not available.

**Figure 1**



Assessment of the myeloid compartment and clonogenicity of MEPs from untreated and IFN- $\alpha$ 2a treated CDA-I patients. a. Overview of erythroid differentiation. HSC, Haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, Granulocyte-monocyte progenitor; MEP, Megakaryocyte-erythroid progenitor; MyP, Myeloid progenitor; BFU-E, burst forming unit erythroid; CFU-E, Colony forming unit erythroid; ProE1/2, Pro-erythroblast1/2; BasoE, Basophilic erythroblast; PolyE, Polychromatic erythroblast; OrthoE, orthochromatic erythroblast; Retic, reticulocyte. b. Expression of *CDAN1* and *CDIN1* during terminal erythroid differentiation. Expression of beta-actin (*ACTB*) is shown for comparison. Data were generated using RNAseq data obtained from healthy individuals published by Corces et al., 2016 and Ludwig et al., 2019. d. Unchanged frequency of CMP (Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>+</sup> CD123<sup>+</sup> CD45RA<sup>-</sup>), MEP (Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>+</sup> CD123<sup>-</sup> CD45RA<sup>-</sup>) and GMP (Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>+</sup> CD123<sup>+</sup> CD45RA<sup>+</sup>) between healthy donors (n=17), untreated CDA-I patients (n=5) or those receiving IFN- $\alpha$ 2a treatment (n=3). Data from the single patient with a *CDIN1* mutation are shown with open circles. d. Colony output of MEPs 14 days after single-cell sorting into methylcellulose from donors (n=10), untreated CDA-I patients (n=5) or patients receiving IFN- $\alpha$ 2a treatment (n=3). BFUE numbers were not significantly different, however, erythroid clusters were significantly increased from cells from CDA-I patients (\*p=0.0114). Insets show representative images of colony types obtained. GEMM, colony-forming unit granulocyte, erythrocyte, macrophage, megakaryocyte; GM, colony-forming unit-granulocyte, macrophage; EC, erythroid cluster (10x magnification). e. *in vivo* IFN- $\alpha$ 2a treatment improves haemoglobin (Hb) levels in CDA-I patients. Dotted lines represent Hb values of individual patients before and after starting IFN- $\alpha$ 2a treatment. Thresholds represent the Oxford University Hospital NHS Foundation Trust normal Hb for adult males (open circles) and females (closed circles).