# Codanin-1, the protein encoded by the gene mutated in congenital dyserythropoietic anemia type I (*CDAN1*), is cell cycle-regulated

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## ABSTRACT

### Background
Congenital dyserythropoietic anemia type I is an inherited autosomal recessive macrocytic anemia associated with ineffective erythropoiesis and the development of secondary hemochromatosis. Distinct erythroid precursors with internuclear chromatin bridges and spongy heterochromatin are pathognomonic for the disease. The mutated gene (*CDAN1*) encodes a ubiquitously expressed protein of unknown function, codanin-1. Based on the morphological features of congenital dyserythropoietic anemia type I erythroblasts and data on a role in cell cycle progression of codanin-1 homolog in *Drosophila* we investigated the cellular localization and possible involvement of codanin-1 during the cell cycle.

### Design and Methods
Codanin-1 localization was studied by immunofluorescence and immune electron microscopy. Cell cycle expression of codanin-1 was evaluated using synchronized HeLa cells. E2F proteins are the main regulator of $G_1$/S transition. An E2F1-inducible cell line (U20S-ER-E2F1) enabled us to study codanin-1 expression following ectopic E2F1 induction. Direct binding of E2F1 to codanin-1 promoter was assessed by chromatin immunoprecipitation. We used a luciferase-reporter plasmid to study activation of *CDAN1* transcription by E2F1.

### Results
We localized codanin-1 to heterochromatin in interphase cells. During the cell cycle, high levels of codanin-1 were observed in the S phase. At mitosis, codanin-1 underwent phosphorylation, which coincided with its exclusion from condensed chromosomes. The proximal *CDAN1* gene promoter region, containing five putative E2F binding sites, was found to be a direct target of E2F1.

### Conclusions
Taken together, these data suggest that codanin-1 is a cell cycle-regulated protein active in the S phase. The exact role of codanin-1 during the S phase remains to be determined. Nevertheless this represents the first step towards understanding the function of the proteins involved in congenital dyserythropoietic anemia.

Key words: codanin-1, cell cycle, heterochromatin, E2F.

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## Introduction

The congenital dyserythropoietic anemias (CDA) are rare inherited disorders characterized by ineffective erythropoiesis, pathognomonic cytopathology of nucleated red blood cells in bone marrow, and secondary hemochromatosis. In 1968 Heimpel[1] subdivided this group of diseases into three types. CDA type II, the most common type, is characterized by binucleated erythroblasts with marginal cisternae. The affected gene in this disease was localized in some, but not all, patients to chromosome 20q11.2.[2] CDA type III, the rarest of all types, is associated with gigantic erythroblasts in bone marrow.[3] There are both familial and sporadic forms of CDA type III and the affected gene in a Swedish family was localized to 15q21-25.[4]

CDA type I (CDA I) is an autosomal recessive disease associated with moderate-to-severe macrocytic anemia and bone abnormalities.[5-7] Bone marrow aspirates reveal binuclear intermediate and late erythroid precursors, as well as internuclear chromatin bridges. Ultrastructural erythroid features include spongy heterochromatin, enlargement of nuclear pores and invagination of the nuclear membrane. S-phase arrest in erythroid precursors has also been previously described.[7-9]

A cohort of 45 Israeli Bedouin patients with CDA I enabled us to map the disease gene to chromosome 15, between markers D15S779 and D15S778, and subsequently to clone the *CDAN1* gene.[10,11] The *CDAN1* gene, with its 28 exons, spans 15 kb of genomic DNA and encodes a 4738-nucleotide-long mRNA. Northern blot analysis revealed that the gene is ubiquitously expressed.[11] The gene encodes a protein of 1227 amino acids, which we designated codanin-1.[11] No intracellular localization domains have been identified.

The *CDAN1* gene is mutated in 88% of CDA I patients and more than 30 unique mutations have been identified so far[6,11,12] No patients have been found to be homozygous for a null-type mutation, suggesting that the complete absence of functional codanin-1, the product of the *CDAN1* gene, may be lethal.

Homology searches have revealed orthologs in a wide variety of metazoans, ranging from *Drosophila* to primates. No obvious human codanin-1 paralog has been found. Interestingly, the *Drosophila* homolog, *dlt*, which shares 23% homology with human *CDAN1*, was found to be required for cell survival and cell cycle progression, particularly progression through the S phase.[13]

Based on the morphological features of CDA I erythroblasts and the preliminary data on codanin-1 function in *Drosophila*, we investigated the location and the behavior of codanin-1 during cell cycling.

## Design and Methods

### Cell culture and synchronization

HeLa and U20S cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS). K562 cells were grown in RPMI medium (Biological Industries, Bet-Haemek, Israel) supplemented with 15% FCS. U2OS-ER-E2F1 cells (U20S cells stably expressing the ER–E2F1 fusion protein) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 500 μg/mL G418. To induce their activation, U2OS-ER-E2F1 cells were treated with 300 nM of 4-hydroxytamoxifen (OHT) for the time indicated. Upon exposure to OHT, ER-E2F1 translocates from the cytoplasm to the nucleus to activate target genes. Cycloheximide (Sigma, St. Louis, MO, USA) was used at a concentration of 10 μg/mL.

For HeLa cell synchronization, cells were arrested by double thymidine block. Briefly, cells were blocked for 16 hours (h) with 2 mM thymidine, washed, and released into normal growth medium for 8 h, and then blocked again with 2 mM thymidine for 16 h to arrest the cells at the beginning of the S phase. For *in vitro* dephosphorylation, cells were arrested at prometaphase using 50 ng/mL nocodazole (Sigma), and cells were collected from the suspended media following shaking of the plates.

### Western blotting and subcellular fractionation

Synchronized cells were lysed in NEB lysis buffer (10 mM HEPES, pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.2 mM EDTA, 0.2 mM EGTA, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (Sigma). Equal amounts of protein from each lysate, as determined by a Bradford assay, were subjected to 8% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto 0.2-μm cellulose nitrate membranes (Schleicher & Schuell, Dassel, Germany). Detection was performed with 1:1000 affinity-purified codanin-1-specific primary antibody and 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Subnuclear fractionation and chromatin extraction were performed as previously described.[14]

### mRNA levels in U2OS-ER-E2F1 cells

Total RNA was purified from U2OS-ER-E2F1 cells stimulated with 300 nM OHT using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript III First-Strand system (Invitrogen) according to the manufacturer's instructions. *GAPDH* cDNA was amplified using the primers: 5'-TCCCATCACCATCTTCCA and 5'-GTCATCATATTTGGCAGG. *CDAN1* cDNA was amplified using the primers: 5'-ATAAGCTTGCGCCTGTGGTGGACCAGCAG and 5'-ATGGATCCTCAGGCAGCAGGTTCAGGACCCTG. Products were separated on a 1% agarose gel and the intensity of the bands was measured using the public domain NIH Image J program (National Institutes of Health, Bethesda, MD, USA).

### Flow cytometry

Cells were trypsinized and washed once with phosphate-buffered saline (PBS). Next, the cells were fixed with 70% ethanol that had been pre-cooled to -20°C.

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The cells were stained with propidium iodide[15] and analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA, USA). The data were acquired and analyzed using CellQuest software.

### *Fluorescence microscopy*

HeLa cells were grown on coverslips. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and permeabilized in 0.25% Triton X-100 in PBS for 5 min, followed by two additional washes (in PBS) and then blocked in 10% FCS/PBS for 30 min. Next, cells were incubated with primary antibodies (diluted in 5% FCS/PBS) for 2 h, washed three times with PBS-T (PBS containing 0.1% Tween-20), and then incubated with secondary antibody for 30 min. Finally, the cells were washed once with PBS-T for 10 min at room temperature, once with 0.05 μg/mL 4',6'-diamidino-2-phenylindole (DAPI; Sigma) in PBS-T for 10 min at room temperature and once with PBS-T. The coverslips were mounted in fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA) and examined with an Olympus Fluoview-FV500 confocal laser scanning microscope. The objective was UPLAPO40x with N.A. 0.85. The antibodies used were affinity-purified rabbit polyclonal anti-codanin-1 and mouse monoclonal anti-α-tubulin (Sigma). Secondary antibodies were Cy3-donkey anti-rabbit antibody and Cy2-goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). All antibodies were used in a 1:150 dilution, except where otherwise mentioned.

### *Immunogold electron microscopy*

Post-embedded bone marrow cells from normal subjects, individuals with CDA I (Bedouin patients with the founder missense mutation R1042W) and subjects with non-dyserythropoietic hemolytic anemia were studied. Fixation, embedding, and post-embedding immunogold reactions were performed as previously described.[16] Ultra-thin sections of embedded materials were incubated overnight with affinity-purified polyclonal anti-codanin-1 antibodies, at a concentration of 0.06 mg/mL diluted 1:10. The secondary antibody applied was a conjugated 10-nm colloidal gold goat anti-rabbit IgG (BioCell, Cardiff, UK), diluted 1:50. Control experiments were run in parallel without primary antibody. The cells were examined with a Philips 201 electron microscope.

### In vitro *dephosphorylation*

For λ-phosphatase analysis, HeLa mitotic cell extracts were incubated at 30°C for 30 min in 50 μL of reaction buffer containing 200 units of λ-phosphatase (New England Biolabs, Beverly, MA, USA) in the presence or absence of phosphatase inhibitors. All reactions were stopped by addition of 4X loading buffer, and samples were subjected to SDS-PAGE and immunoblotting.

### *Chromatin immunoprecipitation*

Chromatin immunoprecipitation was performed with the Chromatin immunoprecipitation assay kit (Upstate, Lake Placid, NY, USA). Antibodies to HA (sc-805; Santa Cruz, CA, USA) and E2F1 (sc-193; Santa Cruz) (1 μg per immunoprecipitation) were used to precipitate chromosomal DNA in cross-linked chromatin prepared from exponentially growing U2OS cells. The immunoprecipitated DNA was analyzed by polymerase chain reaction (PCR) using the following primers: *-48CDAN1F*: 5'-TTGACTCCGTGGGTCTGG-3'; *+270CDAN1R*: 5'-CCTAGAGGAAGGGGACTGGA-3'; *GAPDH-F*: 5'-TACTAGCGGTTTTACGGGCG-3'; *GAPDH-R*: 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'

### *Luciferase assay*

For luciferase assays, the minimal *CDAN1* gene promoter region was inserted into the pGL4 luciferase reporter plasmid (Promega, Madison, WI, USA). The 1.3 kb of the *CDAN1* gene, including 1 kb upstream of the transcription start site (sequence includes the five E2F1 binding sites), was amplified from genomic DNA using the primers 5'-AAGAAATTACTCAGGCCG and 5'-CCCAGTTGGAGTGCACTC. The amplified fragment was ligated into the pDrive plasmid, from the Qiagen PCR cloning kit (Qiagen, Valencia, CA, USA). A *KpnI-XhoI* fragment containing all the PCR products was released from the plasmid and ligated into pGL4 (Promega). To determine which E2F binding site is used for transcription, smaller fragments of the *CDAN1* promoter were amplified by PCR from the 1.2 kb fragment and inserted into pGL4 (Figure 6). To add an *XhoI* site at the 3' end (corresponding to nucleotide +330 of *CDAN1*), all of the fragments were amplified using the primer 5'-ATACTCGAGCCCAGTTGGAGTGCACTC. The primers used to add a *KpnI* site at the 5' end of the fragments were as follows:

fragment -46 to +330: TATGGTACCTTGACTCCGTGGGTCTGG;

fragment 11 to +330: TATGGTACCTCCCCATCCCGCGACCCA;

fragment 57 to +330: TATGGTACCTCCCTGGCCGCCCCGCGG;

fragment 111 to +330: TATGGTACCGGGATGGCGGCCGTT.

The plasmids were transfected into U2OS cells using Lipofectamine (Invitrogen) according to the manufacturer's instructions, in the following proportions: 500 ng pGL4 (with or without *CDAN1* fragments), 300 ng Renilla plasmid (Promega), 100 ng E2F1 expression plasmid and, if necessary, the appropriate amount of pUC19 to have 900 ng total DNA in each transfection reaction. Cells were lysed 24 h after transfection using the passive lysis buffer from the Dual-Luciferase reporter assay kit (Promega). Luciferase and Renilla activity was measured using a single-injector luminometer, according to the manufacturer's instructions.

## Results

### *Codanin-1 localizes to heterochromatin*

In order to study codanin-1, affinity-purified rabbit polyclonal antibodies were raised against the peptide GPRDPDEGVSPEHLEQL, corresponding to amino acids 1040 to 1056 of the human codanin-1 sequence. Western blot analysis using this antibody and whole cell extracts

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from the HeLa, K562 (erythroid), and U2OS (osteosarcoma) cell lines identified the expected band of 130 kDa (Figure 1A). Immunofluorescence studies of growing HeLa cells stained with codanin-1 antibody, followed by Cy3 anti-rabbit secondary antibody, revealed a speckled pattern confined to the nucleus, but sparing the nucleoli (Figure 1B). Similar results were obtained in U2OS and K562 cells (*data not shown*). Cells stained with rabbit pre-immune serum showed no specific staining. Subcellular fractionation indicated that codanin-1 is present mostly in the nucleus, in the chromatin fraction (Figure 1C).

Electron microscopy sections of CDA I (Figure 1D, 1-2) bone marrow aspirates stained with gold-labeled codanin-1 antibodies revealed that the gold particles localize preferentially in the heterochromatin region of the nucleus. Virtually no gold particles were detected in the cytoplasm or in the nuclear euchromatin, nor were they found in the heterochromatin holes, the typical feature of CDA I. The pattern of the reaction in the non-dyserythropoietic hemolytic anemia bone marrow sample (Figure 1D, 3-4) was similar: the gold particles appeared dispersed or in small aggregates in the nuclear heterochromatin, but no particles were detected in the cytoplasm or in the euchromatin. No gold particles were detected in control sections.

Since the immunogold staining of codanin-1 in erythroid cells takes place preferentially in the nuclear heterochromatin, we were interested in seeing the pattern of the immunogold reaction in cells in which the nucleus consists mainly of euchromatin, such as in U2OS cells. In these cells, the heterochromatin is restricted to a very thin layer adjacent to the nuclear membrane (Figure 1D, 5). As Figure 1D, 6-7 clearly shows, the gold particles appear only in that small region, indicating that codanin-1 also localizes to the heterochromatin in these cells. A similar pattern was detected in HeLa and K562 cells (*data not shown*).

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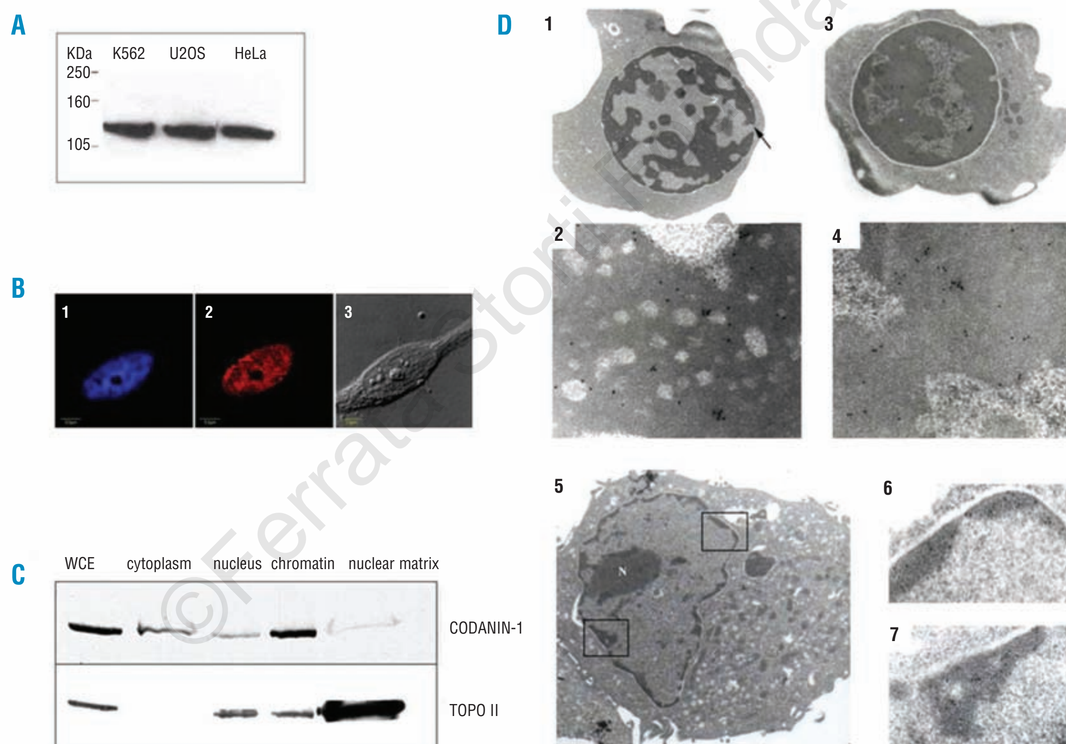


Figure 1. Codanin-1 is a nuclear chromatin-bound protein. (A) Western blots using anti-codanin-1 against whole cell extracts of asynchronous K562, U2OS, and HeLa cells detected a 130-kDa band. (B) Nuclear localization of codanin-1 in HeLa cells; sparing of the nucleolus is evident. **(1)** 4'-6'-Diamidino-2-phenylindole (DAPI) DNA stain. **(2)** Anti-codanin-1 antibody (1:150), followed by secondary antibodies (Cy3-conjugated donkey anti-rabbit). **(3)** Differential interference contrast image of the cells. The pictures were taken using an Olympus Fluoview-FV500 confocal laser scanning microscope. The objective was UPLAPO60x with N.A. 0.85. (C) Biochemical sub-nuclear fractionation of HeLa cell extracts. WCE -whole cell extract. TOPO II-topoisomerase II. Fractions were immunoblotted with the antibodies indicated. Anti-TOPOII (an abundant nuclear matrix protein) was used as a control for the fractionation. Codanin-1 is present mainly in the chromatin fraction. (D) Immunogold ultrastructural localization of codanin-1 to heterochromatin. (D1) CDA I erythroblast showing the typical spongy appearance of the nuclear heterochromatin and a dilated membrane pore (arrow). (x7000). (D2) Enlarged portion of the nucleus of 1, showing localization of gold particles in the nuclear heterochromatin, but not in the euchromatin (x45000). (D3) An erythroblast from a case of autoimmune hemolytic anemia, without dyserythropoietic features, showing gold particles in the nuclear area (x10000). (D4) Enlarged portion of the nucleus in 3 showing preferential localization of gold particles in the nuclear heterochromatin (x45000). (D5) A typical U2OS cell showing a nucleus composed mainly of euchromatin. A very small amount of heterochromatin can be seen adjacent to the nuclear membrane (x4500). (D6,7) Two enlarged regions of the nucleus of 5 showing preferential localization of gold particles in two small areas of heterochromatin adjacent to the nuclear membrane (x45000). Cells were examined with a Philips 201 electron microscope.

### Codanin-1 localization and expression change through the cell cycle

As shown in Figure 1B, codanin-1 localizes mainly to the nucleus. Detailed confocal immunofluorescence microscopy was used to determine the subcellular localization of codanin-1 in different stages of the cell cycle (Figure 2). Cells were counterstained with α-tubulin and with 4', 6'-diamidino-2-phenylindole (DAPI). Figure 2 clearly shows that during mitosis (from prophase to telophase) codanin-1 is excluded from the condensed chromosomes. It becomes re-associated with DNA upon cytokinesis.

In order to examine the expression levels of codanin-1 protein during the cell cycle, we monitored the levels of codanin-1 in synchronized HeLa cells. Cells were arrested at the $G_1$/S boundary using a double-thymidine block. The cells were then released into fresh medium and harvested at different time points. Progression through the cell cycle was confirmed by FACS analysis. Immunoblotting revealed higher levels of codanin-1 during the S phase, with a decreased amount of codanin-1 in mitosis (Figure 3A). To examine whether these changes occur at the transcriptional level, we examined *CDAN1* mRNA levels, and found them to be up-regulated when quiescent cells re-enter the cell cycle (*data not shown*).

In experiments examining HeLa cells arrested at mitosis using nocodazole, we found that codanin-1 exhibits a higher mobility isoform (Figure 3B, a). We, therefore, investigated whether the slowed migration of mitotic codanin-1 was due to phosphorylation. To this end, proteins extracted from nocodazole-arrested HeLa cells were subjected to western blot analysis. Treatment with λ-phosphatase caused codanin-1 to migrate faster, compared with mock-treated samples. This effect was disrupted by adding the phosphatase inhibitors sodium fluoride and sodium orthovanadate (Figure 3B, b). These results indicate that codanin-1 is phosphorylated during mitosis.

### Codanin-1 is a direct target of E2F1

The highest levels of codanin-1 were recorded during the S phase of the cell cycle (Figure 3A), suggesting the involvement of codanin-1 in some aspects of this phase. Since the E2F family of transcription factors is necessary for initiating the S phase and for expression of several S-phase-specific genes, we explored the possible influence

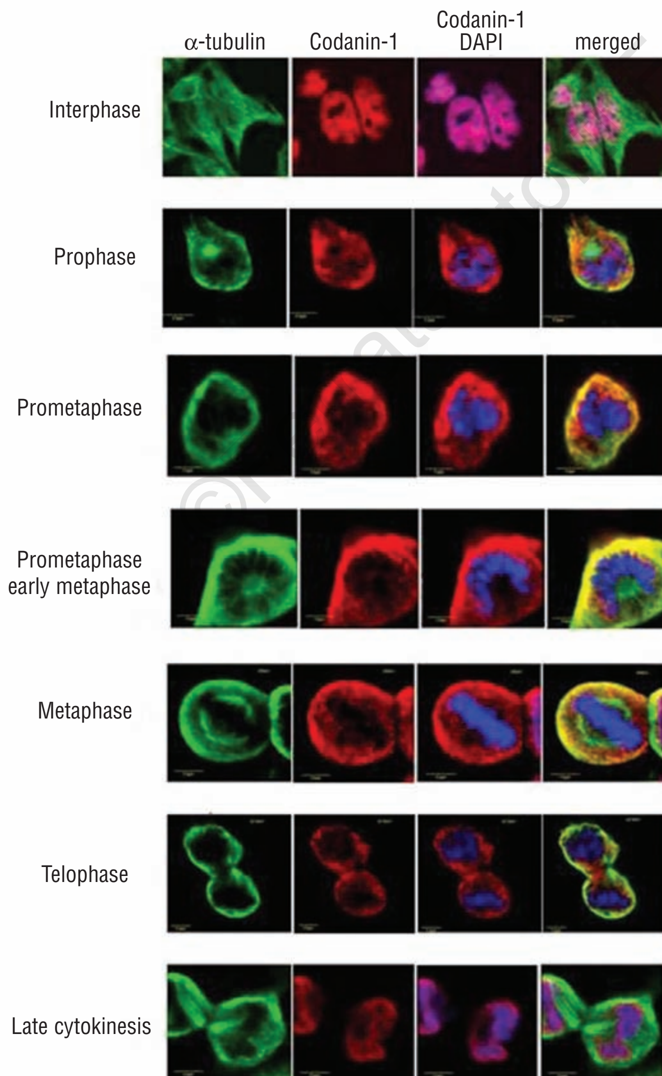


**Figure 2. Cell cycle-dependent localization of codanin-1. Asynchronized HeLa cells were fixed and co-stained with purified rabbit anti-codanin-1 and mouse anti-α-tubulin. DNA was stained with DAPI. While codanin-1 associates with DNA during interphase (top panel), it is clearly excluded from the mitotic condensing chromosomes (prophase to telophase). Images were photographed using a 60× objective on an Olympus Fluoview-FV500 confocal fluorescence microscope.**

of E2F1 on codanin-1 transcription. Sequence analysis of the region upstream of the codanin-1 transcription start site using the TESS program (*http://www.cbil.upenn.edu/cgi-bin/tess*) identified five putative E2F binding sites located at positions -832, -18, +34, +94, and +129 of the transcription start site. To determine the influence of E2F1 overexpression on codanin-1 protein level, we took advantage of an inducible U2OS cell line stably expressing E2F1 fused to a modified version of the estrogen receptor (ER) ligand binding domain.[17] To activate the E2F1 fusion protein, we treated U2OS-ER-E2F1 cells with 300 nM 4-hydroxytamoxifen (OHT). As shown in Figure 4A, this treatment resulted in a significant increase in the level of codanin-1 protein 8-12 h following the activation of E2F1. Activation was followed by a sharp decline in codanin-1 protein level after 16 h of incubation. The parental cell line, U2OS, was treated with OHT and showed no change in the levels of codanin-1 (Figure 4B). These results suggest that expression of the codanin-1 gene is regulated by E2F1.

To verify that the induction of codanin-1 expression by ectopic E2F1 occurred at the transcriptional level, we examined the levels of mRNA. RNA was extracted from U2OS-ER-E2F1 cells treated with 300 nM OHT and a fragment of *CDAN1* was amplified by PCR. Figure 4C suggests that the RNA levels of *CDAN1* rise after 4 h of ectopic E2F1 expression and remain stable for up to 24 h following OHT stimulation. Since protein levels of codanin-1 are reduced after 12 h of stimulation with OHT whereas RNA levels remain high, we investigated whether the reduced protein levels are due to proteasome-mediated degradation. Briefly, the MG-132 proteasome-inhibitor was introduced to U2OS-ER-E2F1 cells 8 h following OHT induction. Cells were harvested and lysates were prepared at the indicated times. The addition of the proteasome inhibitor abolished the previously seen decline in codanin-1, thus supporting the assumption that the rise in codanin-1 levels is followed by degradation via the proteasome (Figure 4D).

As shown in Figure 4C, E2F1 promotes *CDAN1* expression. This up-regulation could also be detected in the presence of cycloheximide, an inhibitor of protein synthesis (Figure 5A), indicating that *de novo* protein synthesis is not required for E2F1-induced up-regulation of the *CDANI* mRNA, thus suggesting that codanin-1 is a direct target of E2F1. To further validate the direct binding of endogenous E2F1 to *CDANI* promoter, we performed a chromatin immunoprecipitation analysis using chromatin from proliferating U2OS cells and an antibody directed against E2F1. Anti-HA antibody was used as a negative control. Endogenous E2F1 was found to be associated with the promoter (Figure 5B). No binding of endogenous E2F1 to an unrelated genomic fragment was detected (Figure 5B).

By cloning a fragment spanning from –901 to +330 bases of the transcription start site into a luciferase reporter plasmid (Figure 6A), we identified this fragment as a functional promoter. Co-transfection of an E2F1 expression plasmid increased luciferase activity, confirming that E2F1 activates the transcription of *CDAN1* (Figure 6B). In contrast, a DNA binding-deficient form of E2F1, E132[18] induced the expression of codanin-1 much less efficiently. Next, successive deletions of the promoter were performed to identify the E2F binding site(s) necessary for activation by E2F1. Our results indicate that the E2F binding site localized at 34 to 41 contributes to this activation because its deletion reduces activation by E2F1 overexpression. The E2F binding site localized at 94 to103 may also contribute to this activation, though it is possible that the region containing this site is needed for basal promoter activity, since its deletion causes complete loss of promoter activity.

## Discussion

This work represents the first attempt to elucidate the cellular role of codanin-1, the protein defective in CDA I. Using immunogold electron microscopy, we

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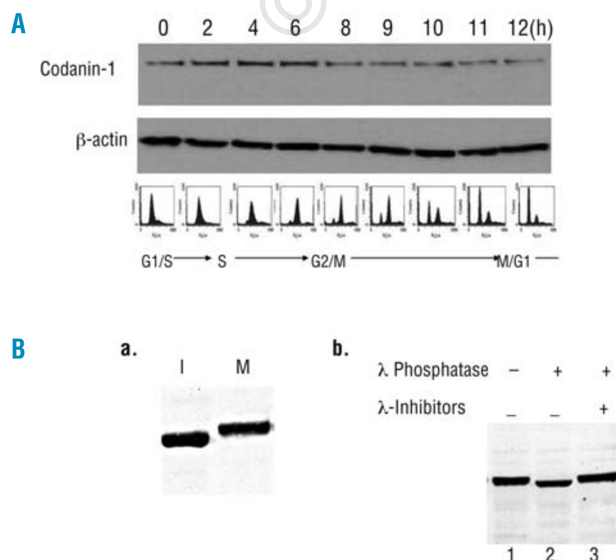


**Figure 3.** Codanin-1 expression and phosphorylation are cell cycle-regulated. (A) Lysates of HeLa cells released from double thymidine block were prepared at various times and immunoblotted with anti-codanin-1 antibody. β-actin was used as a loading control. Cell cycle distribution was analyzed using FACScan analysis of propidium iodide-stained cells. Codanin-1 concentration increases during the S phase and declines thereafter during $G_2$/M. (B) Codanin-1 is phosphorylated during mitosis. **(a)** Lysates prepared from interphase (I) or nocodazole-arrested (mitotic, M) HeLa cells were resolved on 8% gel and probed with anti-codanin-1. **(b)** Mitotic HeLa cell extracts were incubated (30 min, 30 °C) in the absence (lane 1) or presence (lane 2) of λ phosphatase as indicated. Lane 3 was loaded with mitotic extract treated with phosphatase in the presence of phosphatase inhibitors. Immunoblotting was performed with anti-codanin-1.

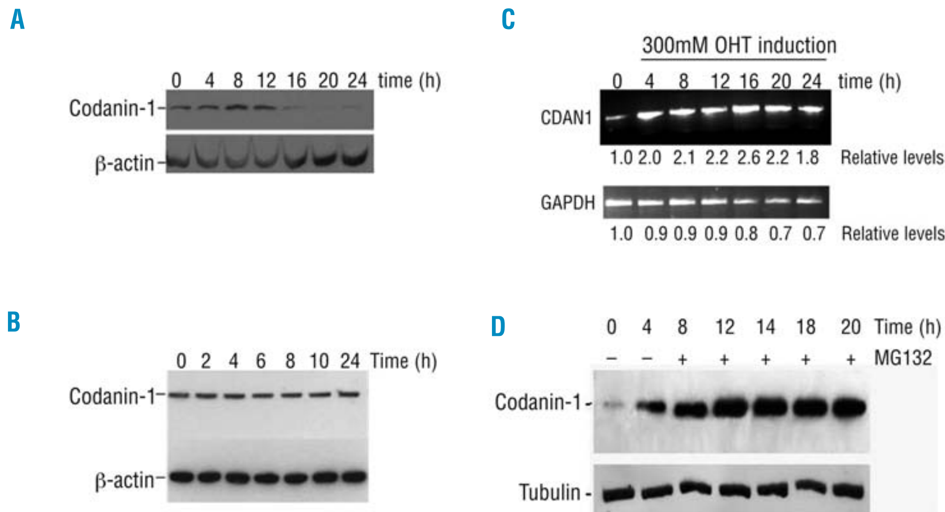


**Figure 4.** Overexpressed E2F1 is associated with high levels of codanin-1 in the S phase followed by a sharp decline. (A) U2OS cells expressing E2F1 fused to estrogen receptor (U2OS-ER-E2F1) were treated with 300 nM 4-hydroxytamoxifan (OHT) for the times indicated, to induce E2F1 activation. Lysates were prepared and immunoblotted with anti-codanin-1 antibody. β-actin was used as a loading control. Codanin-1 levels rise 8-12 h following E2F1 activation and sharply decline thereafter. (B) Parental U2OS cells were treated and analyzed as described in A. No change in codanin-1 levels was observed. (C) mRNA levels rise with E2F1 induction; U2OS-ER-E2F1 cells were treated with 300 nM OHT for the times indicated. RNA was isolated using Trizol and used for reverse transcriptase PCR. *CDAN1* and *GAPDH* were amplified by PCR and the products were separated on a 1% agarose gel. Relative levels of products indicated below the gel were calculated with the Image J program. (D) Codanin-1 is degraded by the proteasome. U2OS-ER-E2F1 cells were treated with 300 nM OHT for the times indicated. The proteasome inhibitor MG-132 was added to the cells at the time point indicated. Lysates were prepared and immunoblotted with anti-codanin-1 antibody. Tubulin was used as a loading control. MG-132 inhibited a reduction in codanin-1 levels observed after 12 h.

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demonstrated here that codanin-1 was exclusively found in heterochromatin, both in CDA I and autoimmune hemolytic anemia erythroblasts, as well as in the very small heterochromatin layer of non-erythroid proliferating cell lines. Heterochromatin, the tightly packed form of DNA, is thought to serve several functions, including chromosome segregation, control of genomic stability, and epigenetic regulation of gene expression.[19]

CDA I erythroid nuclear morphological features, as well as studies in *Drosophila*, which demonstrate involvement in cell cycle control,[13] prompted us to ascertain the possible cell cycle-dependent control of codanin-1. Support for a role in the S phase came from studying the expression pattern of endogenous codanin-1 during the cell cycle. Codanin-1 levels rose in the S phase, but later dropped at mitosis. The lower levels of codanin-1 present in mitosis may result from proteasome-mediated degradation, since the use of a proteasome-inhibitor, MG-132, both in U2OS-ER-E2F1 cells and in synchronized HeLa cells (*unpublished observation*), abolished its degradation. Detailed ubiquitination assays are required to confirm this mechanism.

Codanin-1 is expelled from the condensed chromosomes during mitosis, but becomes reassociated with chromatin in the newly formed nucleus at late telophase. A possible explanation for this phenomenon is that the expelled protein would otherwise interfere with chromatin-related events associated with mitosis, such as chromosome condensation. Many transcription factors, including Oct-1, Oct-2, c-Fos, E2F1, C/EBP and Sp1,[20,21] and chromatin remodeling factors are excluded from chromatin during mitosis.[21,22] In the human SWI/SNF chromatin remodeling complex, hBRM/BRG-

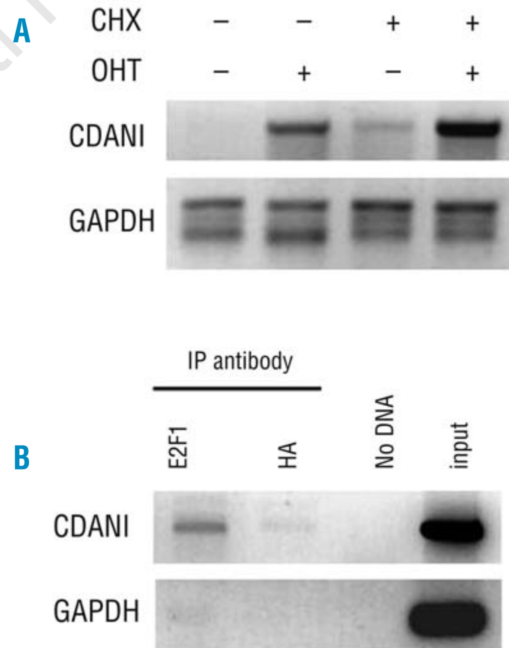


**Figure 5.** Codanin-1 is a direct transcriptional target of E2F1. (A) Reverse transcriptase-PCR analysis of *CDANI* and *GAPDH* mRNA levels: U2OS-ER-E2F1 cells treated with OHT for 8 h (+) or not treated (-) in the presence or absence of cycloheximide (CHX). (B) Chromatin immunoprecipitation analysis was performed using growing U2OS cells: cross-linked chromatin was immunoprecipitated with antibodies to E2F1 and HA, and then *CDANI* and *GAPDH* promoter fragments were amplified by PCR. Negative (no DNA) and positive (input DNA representing 0.2% of the total chromatin) control amplifications are shown.

1 interacts with nuclear elements during interphase, but this interaction is disrupted at the $G_2$/M transition.[22] The transitional inactivation and reactivation of hSWI/SNF is required for formation of a repressed chromatin structure during mitosis and reformation of an active chromatin structure as cells leave mitosis.[23] The temporal order of the displacement of transcription/chromatin-remodeling-factors, as well as their re-entry into the nuclei of daughter cells are incompletely understood, but the mechanism underlying phosphorylation has been well established by multiple investigators as a means of allowing for chromosomal condensation.[14, 22] The cell cycle-dependent phosphorylation of codanin-1 suggests a regulatory mechanism for its degradation and redistribution during mitosis.

The E2F family of transcription factors is a key regulator of genes required for $G_1$ to S transition, DNA replication, and cell cycle control. This family has also been implicated in development, apoptosis, signal transduction pathways, and differentiation, as well as in regulating genes involved in mitosis.[24-27] The codanin-1 promoter has not been previously cloned and characterized, but analysis of the sequence spanning -90 to +330 bases from the transcription start site using the TESS program (*http://www.cbil.upenn.edu/cgi-bin/tess*) identified putative transcriptional regulatory sites for a wide variety of transcription factors including E2F, Sp1, AP-1, and NF-1 as well as sites for erythroid-specific factors such as c-Myb and GATA-1, suggesting that this region is the *CDAN1* promoter. Cloning of this fragment into a luciferase reporter plasmid did indeed identify this region as a functional promoter, with E2F-induced transcription. However, codanin-1 transcription is probably regulated by other factors as well, since the elevated mRNA levels observed following OHT induction remain stable for up to 24 h, compared with protein levels, which dropped 12 h after stimulation. It is possible that other, yet unidentified, transcription factors are required to maintain stable *CDAN1* transcript levels, whereas protein levels are regulated through post-translational modifications. It is also possible that *CDAN1* mRNA is stable and is, therefore, present for a long time after *CDAN1* transcription.

In summary, we report here that codanin-1, the protein encoded by the gene mutated in CDA I, is a heterochromatin protein that is transcriptionally regulated by E2F1. Codanin-1 levels rise during the S phase and the protein is phosphorylated and expelled from condensed chromosomes during mitosis. These findings are in accordance with the morphological features of CDA I erythroblasts and with early studies by Queisser *et al.*,[9] who described suppressed $^3$H-thymidine uptake during the S phase in those cells.

The function of codanin-1 during the S phase is presently unknown. However, the spongy heterochromatin in CDA I may point to it having a role in heterochromatin organization during DNA replication. Interestingly, knock-out of several cell cycle-regulating proteins in mice resulted in narrow tissue-specific abnormalities. Double knock-out of E2F1 and E2F2, as well as E2F4 and cyclin D2 or D3 knock-out resulted mainly in erythroid-specific pathological features including S phase arrest and defective erythroid cell maturation with dyserythropoiesis.[28-32] Cell cycle

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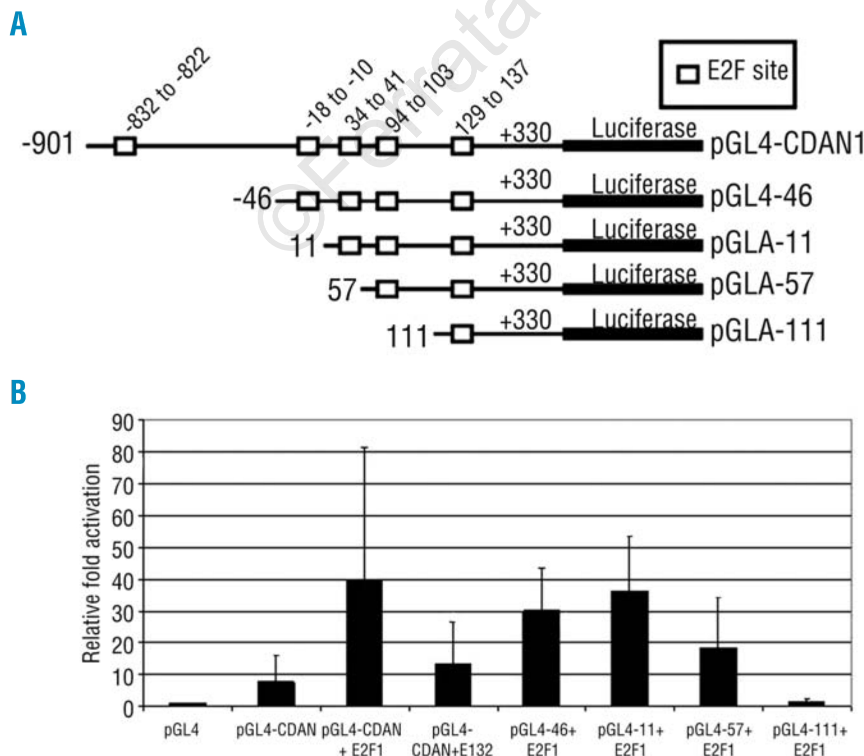


Figure 6. **Codanin-1 promoter is sensitive to E2F1 (A) Schematic representation of the codanin-1 promoter-containing luciferase reporter plasmids. (B) U2OS cells were co-transfected with the empty pGL4 or the codanin-1 promoter constructs described in A and either wild-type E2F1 or a mutated form of E2F1 (E132) that cannot bind to DNA. Cells were also transfected with the pRenilla reporter plasmid to evaluate transfection efficiency. Values obtained for luciferase activity by luminometer were divided by the values obtained for Renilla activity. Values obtained for the pGL4 transfected cells were set at 0 and other samples were calculated as fractions of that value to determine the relative activity. Results shown represent the average of three independent experiments.**

defects are also associated with a shorter $G_1$ phase, loss of size control, and macrocytosis.[33] It has recently been suggested that in the erythroid lineage, terminal differentiation is coupled to proliferation with few cell cycle divisions.[33] Theoretically, a cell cycle defect caused by mutated codanin-1 could account for the macrocytosis and dyserythropoiesis observed in CDA I. The exact mechanism by which the mutated codanin-1 interferes with normal erythropoiesis and its role in bringing about the typical morphological and functional features of CDA I are subjects for other investigations. Nevertheless, this is the first study that indicates the area of cell biology in which codanin-1 operates.

## Authorship and Disclosures

SNL: performed cell cycle experiments, analyzed and interpreted data, drafted the paper; OD: analyzed and interpreted data, drafted the paper; RL: performed E2F experiments; NM: laboratory analysis of the data and interpretation; TK: laboratory analysis and interpretation; NY: laboratory analysis; DG, BM, HT: conception and design of the study, revised the paper; PR: performed the EM studies; IY: revised the paper; GMK: performed the phosphorylation experiments.

The authors reported no potential conflicts of interest.

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