Codanin-1, mutated in the anaemic disease CDAI, regulates Asf1 function in S-phase histone supply

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Efficient supply of new histones during DNA replication is critical to restore chromatin organization and maintain genome function. The histone chaperone anti-silencing function 1 (Asf1) serves a key function in providing H3.1-H4 to CAF-1 for replication-coupled nucleosome assembly. We identify Codanin-1 as a novel interaction partner of Asf1 regulating S-phase histone supply. Mutations in Codanin-1 can cause congenital dyserythropoietic anaemia type I (CDAI), characterized by chromatin abnormalities in bone marrow erythroblasts. Codanin-1 is part of a cytosolic Asf1–H3.1-H4–Importin-4 complex and binds directly to Asf1 via a conserved B-domain, implying a mutually exclusive interaction with the chaperones CAF-1 and HIRA. Codanin-1 depletion accelerates the rate of DNA replication and increases the level of chromatin-bound Asf1, suggesting that Codanin-1 guards a limiting step in chromatin replication. Consistently, ectopic Codanin-1 expression arrests S-phase progression by sequestering Asf1 in the cytoplasm, blocking histone delivery. We propose that Codanin-1 acts as a negative regulator of Asf1 function in chromatin assembly. This function is compromised by two CDAI mutations that impair complex formation with Asf1, providing insight into the molecular basis for CDAI disease.

Introduction

During DNA replication, parental histones segregate onto leading and lagging strands in a random fashion (Groth *et al*, 2007b). In parallel, new histones are deposited to maintain nucleosomal density. Nucleosome assembly is the first step towards restoration of chromatin on new DNA. Given the role of histones and higher order chromatin

structures in epigenetic gene regulation and genome stability, histone supply pathways must be fine-tuned to meet the demands at replication forks (De Koning *et al*, 2007; Jasencakova and Groth, 2010; Annunziato, 2011). The histone H3-H4 chaperone anti-silencing function 1 (Asf1) is a key player in chromatin replication, donating new histones to CAF-1 (Tyler *et al*, 1999; Mello *et al*, 2002) and handling histones together with the MCM2-7 replicative helicase potentially for recycling (Groth *et al*, 2007a; Jasencakova *et al*, 2010). The two mammalian homologues, Asf1a and Asf1b, have largely redundant functions in S-phase histone dynamics, with Asf1b being more specialized to proliferating cells (Corpet *et al*, 2011). The current view is that Asf1 binds histones H3-H4 in the cytoplasm and in complex with Importin-4 accompanies histone dimers into the nucleus where they are transferred to downstream chromatin assembly factors (De Koning *et al*, 2007; Campos *et al*, 2010; Jasencakova *et al*, 2010; Alvarez *et al*, 2011). Asf1 binds canonical S-phase histones H3.1-H4 as well as replacement histones H3.3-H4, which are delivered to CAF-1 and HIRA, respectively (Tagami *et al*, 2004). It is not entirely clear how the specificity of Asf1 in these distinct assembly pathways is regulated, but CAF-1 p60 and HIRA bind in a mutually exclusive manner to the same binding pocket in Asf1 (Tang *et al*, 2006; Malay *et al*, 2008).

Purification of soluble histone H3 complexes recently revealed that the HSC70, HSP90 and NASP chaperones act early in the histone supply pathway upstream of Asf1 and Importin-4 (Campos *et al*, 2010; Alvarez *et al*, 2011). Whereas HSC70 and HSP90 probably are important for folding, NASP is required to maintain a soluble pool of histones available for deposition (Campos *et al*, 2010; Cook *et al*, 2011). However, it remains unclear how histones H3-H4 are transferred to Asf1 and whether additional factors regulate Asf1 histone shuttling. As an entry point to understand Asf1 function, we have characterized human Asf1 complexes and recently reported a comprehensive profiling of modifications on Asf1-bound histones (Groth *et al*, 2007a; Jasencakova *et al*, 2010). In addition to cytosolic binding partners with predicted roles in histone metabolism (such as sNASP, RbAp46-HAT1, and Importin-4), a protein of unknown function, Codanin-1, caught our attention due to its abundance and link to disease. Mutations in CDAN1, the gene encoding Codanin-1, cause congenital dyserythropoietic anaemia type I (CDAI), a rare recessive anaemic disorder (Dgany *et al*, 2002; Iolascon *et al*, 2011). Codanin-1 is a 134-kDa ubiquitously expressed protein conserved in flies, frogs and fish, but with no apparent homologue in worms and yeast (Dgany *et al*, 2002). The *Drosophila* homologue, Discs lost (Dlt), is required for cell survival and cell-cycle progression (Pielage *et al*, 2003) and mice homozygous for a gene-trap in the CDAN1 locus die during early embryogenesis (Renella *et al*, 2011). This argues that Codanin-1 is an essential protein and consistently the majority of CDAI cases show missense mutations leading to

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single amino-acid substitutions in Codanin-1 (Dgany *et al*, 2002; Tamary *et al*, 2005; Heimpel *et al*, 2006; Ru *et al*, 2008). A principal cytological feature of bone marrow erythroblasts from CDAI patients is abnormal chromatin structure, known as 'spongy heterochromatin' having a Swiss cheese-like appearance (Wickramasinghe and Wood, 2005). Furthermore, cell-cycle analyses of patient samples show accumulation of erythroblasts in S phase (Wickramasinghe and Pippard, 1986; Tamary *et al*, 1996) suggesting replication defects. Given that Asf1 function is essential for DNA replication and chromatin assembly in human cells (Groth *et al*, 2005, 2007a), an appealing idea was that CDAI disease could be linked to defects in histone metabolism. Here, we characterize a molecular link between Codanin-1 and Asf1 function in histone supply and address the impact of CDAI mutations on this interplay.

Results

Codanin-1 is part of a cytosolic Asf1–H3-H4–Importin-4 complex

We identified Codanin-1 by mass spectrometry of e-Asf1a and e-Asf1b complexes isolated from asynchronous HeLa S3 cells (Figure 1A; Supplementary Figure S1A). This finding is consistent with a high-throughput proteomic screen identifying Codanin-1 as an Asf1b-associated protein (Ewing *et al*, 2007) and we further confirmed the interaction by reciprocal immunoprecipitation of endogenous Asf1 and Codanin-1 (Figure 1B and D). We previously found that Importin-4 is specific to cytosolic Asf1 complexes, while MCM4, 6, 7 are part of a nuclear Asf1 complex (Groth *et al*, 2007a; Jasencakova *et al*, 2010). Western blot analysis showed that Codanin-1 is a cytosolic protein, mainly found in cytosolic Asf1 complexes similarly to Importin-4 (Supplementary Figure S1A and B). Cytosolic Asf1 separates into two major forms on a sizing column; a histone-bound complex and a histone-free form eluting at lower molecular weight (Groth *et al*, 2005). To address whether Codanin-1 could be part of an Asf1 complex containing histones, we analysed purified cytosolic e-Asf1b complexes by gel filtration. Codanin-1 co-eluted with histone-bound Asf1, showing an elution profile highly similar to Importin-4 (Figure 1C). We confirmed that Codanin-1 co-purified with soluble non-nucleosomal histone H3.1 (Figure 1E), using a cell line expressing low levels of epitope-tagged histone H3.1 (Tagami *et al*, 2004). Moreover, Importin-4 co-immunoprecipitated with Codanin-1 (Figure 1D) supporting that these factors are present together in a complex with Asf1 and histone H3-H4. However, the chaperone sNASP and RbAp46 thought to act upstream of Asf1 and the downstream chaperones HIRA and CAF-1 did not co-purify with Codanin-1 (Supplementary Figure S1C and D). These biochemical data identify Codanin-1 as a new member of a cytosolic Asf1–H3.1-H4–Importin-4 complex. We asked whether the interaction between Codanin-1 and Asf1 is histone dependent, taking advantage of Asf1 carrying a mutation in the histone-binding site, V94R (Mousson *et al*, 2005; Groth *et al*, 2007a). While the interaction with Importin-4 is lost in the Asf1a V94R mutant (Figure 1F; Jasencakova *et al*, 2010), Codanin-1 bound wild-type Asf1 and the V94R mutant equally well (Figure 1F). Thus, demonstrating that the interaction between Codanin-1 and Asf1 is histone independent.

Codanin-1 binds Asf1 via a B-domain similar to HIRA and CAF-1 p60

To dissect the nature of the Codanin-1–Asf1 interaction, we performed a series of pull-down experiments using recombinant GST–Asf1 and *in-vitro* translated ³⁵S-labelled Codanin-1. Indeed, full-length Codanin-1 bound to both recombinant Asf1a and Asf1b (Figure 2A). Detailed mapping revealed that the N-terminal part of Codanin-1 interacts with the globular domain of Asf1a (Figure 2A; Supplementary Figure S2A). Additionally, Codanin-1 bound recombinant wild-type and the Asf1aV94R mutant equally well (Supplementary Figure S2A). Together, these data identify Codanin-1 as a direct Asf1 binding partner.

Of the many Asf1-associated proteins only a few are direct binding partners, including downstream histone chaperones HIRA and CAF-1 p60 that play key roles in chromatin assembly (Mello *et al*, 2002; Daganzo *et al*, 2003). These downstream histone chaperones interact with Asf1 in a mutually exclusive manner via a so-called B-domain motif (Kirov *et al*, 1998; Daganzo *et al*, 2003; Tang *et al*, 2006). Interestingly, closer examination of the N-terminal part of Codanin-1 revealed a putative B-domain with a high similarity to the domains present in CAF-1 and HIRA (Figure 2B, *left*). Importantly, the B-domain residues RRI, involved in direct contacts with Asf1 side chains (Tang *et al*, 2006), are also present in Codanin-1. Additionally, we noticed that the B-domain in Codanin-1 is evolutionary conserved (Figure 2B, *right*). Disruption of the B-domain in Codanin-1 reduced binding to Asf1 (a and b) *in vitro* (Figure 2C). Conversely, mutation of the Asf1b residues (D36A and D37A) critical for binding the B-domain in HIRA and CAF-1 p60 (Daganzo *et al*, 2003; Tang *et al*, 2006) also abolished Codanin-1 interaction (Figure 2C). Taken together, these results demonstrate that Asf1 binds Codanin-1 in a manner that compares with its interaction with HIRA and CAF-1, implying that these interactions are mutually exclusive.

Codanin-1 is a negative regulator of chromatin replication

Given the interaction with Asf1, it was important to analyse the effect of Codanin-1 downregulation on chromatin replication. We targeted Codanin-1 by an siRNA smart pool as well as an independent siRNA and both strategies efficiently downregulated Codanin-1 mRNA and protein levels (Figure 3A; Supplementary Figure S3A). Whereas depletion of Asf1 (a and b) leads to accumulation of cells in S phase due to inhibition of DNA replication (Groth *et al*, 2005, 2007a), cell cycle progression was not dramatically altered upon Codanin-1 knockdown (Supplementary Figure S3B). However, we consistently found higher cell counts in cultures depleted for Codanin-1 as compared with controls, suggesting a moderate proliferation advantage (Supplementary Figure S3C). To directly probe DNA synthesis, we labelled newly synthesized DNA with a short pulse of EdU (5-ethynyl-2-deoxyuridine), which can be visualized with Click-iT technology. Surprisingly, cells treated with Codanin-1 siRNAs for 56 h displayed stronger EdU signals than controls (Figure 3B) and quantitative analysis at several time points confirmed this observation (Figure 3C; Supplementary Figure S4A). The increase in EdU incorporation was not associated with DNA damage as indicated by the lack of γH2AX (Supplementary Figure S4B). This argues that the rate of DNA replication is

Figure 1 Codanin-1, a new partner of the cytosolic Asf1–H3-H4–Importin-4 complex. (**A**) Coomassie staining of Asf1a complexes isolated from asynchronous HeLa S3 cells stably expressing Onestrep-tagged (e−) Asf1a. Codanin-1 was identified in both cytosolic and nuclear e-Asf1a complexes by mass spectrometry analysis. Proteins annotated in black were previously reported (Groth *et al*, 2007a; Jasencakova *et al*, 2010). (**B**) Co-immunoprecipitation of Codanin-1 with endogenous Asf1 (a and b) from cytosolic HeLa S3 extracts. (**C**) Size-exclusion chromatography of cytosolic e-Asf1b complexes isolated as in (**A**). Codanin-1 co-elutes together with histone H3 and Importin-4. (**D**) Co-immunoprecipitation of Importin-4 and Asf1 (a and b) with endogenous Codanin-1 from cytosolic HeLa S3 extracts. (**E**) FLAG–HA-tagged (e−) H3.1 was immunoprecipitated from cytosolic extracts of HeLa S3 cells expressing e-H3.1 (Tagami *et al*, 2004) and analysed by western blotting. Pull down with sepharose beads was used as negative control. (**F**) Western blot analysis of cytosolic complexes containing wild-type e-Asf1a or the histone binding mutant, e-Asf1a V94R.

increased in the absence of Codanin-1. Consistent with this observation, we noted that Codanin-1-depleted cells also had more PCNA (proliferating cell nuclear antigen) loaded onto chromatin as compared with control cells (Figure 3B and D; Supplementary Figure S4C). PCNA acts as a processivity clamp for DNA polymerases and marks sites of ongoing replication (reviewed in Moldovan *et al*, 2007). The higher levels of PCNA on replicating chromatin thus probably reflect that more replication forks are active in Codanin-1-depleted cells.

It was remarkable that knockdown of Codanin-1 and Asf1, two factors forming a common complex, had entirely opposite effects on DNA synthesis (Supplementary Figure S4A; Groth *et al*, 2005, 2007a). Although Codanin-1 depletion did not affect Asf1 protein levels (Figure 3A), we speculated that it might influence Asf1 localization to chromatin. To test this

idea, we pre-extracted cells with Triton to remove soluble proteins and probed the levels of chromatin-bound Asf1 by immunostaining. Codanin-1-depleted cells showed a striking increase of chromatin-bound Asf1 and quantitative analysis of signal intensities substantiated this observation (Figure 3E and F; Supplementary Figure S4D). Together, our RNAi-based analysis suggests that Codanin-1 could act as a negative regulator of Asf1 function in histone supply, governing its association with chromatin.

Ectopic expression of Codanin-1 inhibits DNA replication by sequestering Asf1 in the cytoplasm

If Codanin-1 was a negative regulator of Asf1 function, then the prediction would be that ectopic Codanin-1 expression should inhibit S-phase progression similarly to Asf1 depletion

Figure 2 Codanin-1 binds directly to Asf1 via the same pocket as HIRA and CAF-1. (**A**) (*Top left*) Summary of the *in vitro* analysis of Codanin-1–Asf1 binding. '+', binding; '−', no binding; n.d., not done. (*Top right*) Coomassie staining of GST–Asf1 used for the pull-down assay. (*Lower panel*) *In vitro* binding analysis using GST–Asf1 (a and b) to pull down *in vitro* translated ^{35}S-labelled Codanin-1. Bound proteins were visualized by autoradiography. (**B**) (*Left*) Sequence alignment of the B-domains in Codanin-1, HIRA and CAF-1 p60. Note that CAF-1 p60 has two B-domain-like motifs designated (1) and (2). Conservation is indicated by red colour intensity. (*Right*) Sequence alignment showing conservation of the Codanin-1 B-domain in human (*Homo sapiens*, H.s.), mice (*Mus musculus*, M.m.), opossum (*Monodelphis domestica*, M.d.), frogs (*Xenopus tropicalis*, X.t.), fish (*Danio rerio*, D.r.) and flies (*Drosophila melanogaster*, D.m.). The degree of conservation is illustrated by red colour intensity. (**C**) (*Left*) Coomassie staining of GST–Asf1 used for the pull-down assay. (*Right*) *In vitro* binding analysis using GST fusions of Asf1a, Asf1b and Asf1b mutated in the B-domain binding pocket (Asf1b D36AD37A; Tang *et al*, 2006) and *in vitro* translated ^{35}S-labelled wild-type Codanin-1 or B-domain mutant (SRR/AAA). Bound proteins were visualized by autoradiography.

(Groth *et al*, 2005, 2007a; Supplementary Figures S3B and S4A). Indeed, Codanin-1 overexpression strongly impaired S-phase progression, as illustrated by the accumulation of cells in mid-S phase already 30 h post transfection (Figure 4A). Co-expression of Asf1a could rescue this arrest

(Figure 4A), providing additional support to the functional link between these factors.

Codanin-1 is mainly a cytoplasmic protein in primary erythroblasts and HeLa cells (Renella *et al*, 2011; Supplementary Figure S1A), and consistently ectopic

Figure 3 Codanin-1 depletion enhances DNA replication and Asf1 binding to chromatin. (**A**) U-2-OS cells were treated with an independent siRNA (siCdan1 #1) or an siRNA smart pool (siCdan1 #2) targeting Codanin-1 for 56 or 70 h. Knockdown efficiency was assessed by western blot analysis and qPCR (Supplementary Figure S3A). (**B**) Immunofluorescence analysis of U-2-OS cells treated with siRNA for 56 h followed by EdU pulse labelling. PCNA staining served as a marker for S-phase cells. Scale bar, 20 μm. (**C**) Quantification of EdU incorporation. (*Left*) Dot plot illustrating the distribution of EdU intensities within one experiment. Cells were treated as in (**B**) and EdU intensities were measured in PCNA-positive cells. $n>87$ and ***$P<0.0001$ calculated by Wilcoxon paired test. (*Right*) Bar diagram showing the average of three independent experiments with error bars indicating standard deviation. The values of siControl were set to 100%. In each experiment, we analysed between 87 and 187 cells per sample. (**D**) Quantification of chromatin-bound PCNA in cells treated as in (**B**). The bar diagram shows the average of three independent experiments with error bars indicating standard deviation. The values of siControl were set to 100%. In each experiment, we analysed between 87 and 187 cells per sample. The distribution of PCNA levels within one experiment is shown in Supplementary Figure S4C. (**E**) Immunofluorescence analysis of chromatin-bound Asf1 in U-2-OS cells stably expressing RFP-PCNA to mark S-phase cells. Codanin-1 was depleted by RNAi for 56 h prior to pre-extraction of soluble proteins and fixation. Scale bar, 20 μm. (**F**) Quantification of chromatin-bound Asf1 in cells treated as in (**E**). Dot plot illustrating the distribution of nuclear Asf1a intensities in pre-extracted cells treated for 56 h with the indicated siRNAs. $n>97$ and ***$P<0.0001$ calculated by Wilcoxon paired test. This result is representative of three independent experiments and similar results were observed with an independent siRNA (Supplementary Figure S4D).

Codanin-1 localized primarily to the cytoplasm in U-2-OS cells (Figure 4B). Both Asf1a and Asf1b are mainly nuclear proteins (Jasencakova *et al*, 2010; Figure 4B), but given their role in histone import they are likely to shuttle between the cytoplasm and the nucleus. Remarkably, Asf1a shifted towards cytoplasmic localization in Codanin-1 expressing cells, becoming almost excluded from the nucleus (Figure 4B). The sequestration of Asf1 away from the sites of DNA replication could therefore explain the S-phase arrest in cells with ectopic Codanin-1 expression. Indeed, co-expression of Asf1a restored nuclear Asf1 levels (Figure 4B), consistent with the ability to rescue S-phase progression (Figure 4A). To further demonstrate that Asf1 binding is required for Codanin-1 to block S-phase progres-

sion, we generated cell lines conditional for expression of wild-type Codanin-1 and a B-domain mutant (Figure 4C). By immunoprecipitation of tagged proteins, we observed that Asf1 (a and b) binding was lost in the B-domain mutant (Figure 4D), consistent with our *in vitro* data (Figure 2C). Importantly, mutation of the B-domain clearly compromised the ability of Codanin-1 to induce S-phase arrest (Figure 4E). We thus conclude that Codanin-1 exerts a dominant-negative effect on S-phase progression chiefly by interfering with Asf1 function.

CDAI missense mutations disable the functional interaction with Asf1

Finally, we addressed whether the liaison between Codanin-1 and Asf1 is compromised by mutations found in CDAI

Figure 4 Ectopic Codanin-1 expression arrests S-phase progression by sequestering Asf1 in the cytoplasm. (**A**) Cell-cycle profiles of cells transfected with Myc–FLAG–Codanin-1 and/or e-Asf1a analysed 30 h post transfection. Cells were co-transfected with a GFP-Spectrin expression vector to gate for transfected cells. (**B**) Immunofluorescence analysis of cells treated as in (**A**). Cells were harvested 24 h after transfection and stained with antibodies against the Myc epitope and Asf1a. Scale bar, 20 µm. (**C**) Western blotting (*right*) and immuno-fluorescence analysis (*left*) of cells expressing ectopic wild-type FLAG–HA–Codanin-1 or a B-domain mutant. Conditional T-REx Flp-In U-2-OS cells were left untreated (−Tet) or induced with tetracycline for 24 h (+Tet). Scale bar, 20 µm. (**D**) Wild-type FLAG–HA-tagged Codanin-1 and B-mutant were immunoprecipitated from soluble protein extracts prepared from cells treated as in (**C**), and analysed by western blotting. (**E**) Cell-cycle profiles of cells from (**C**) analysed by FACS.

patients. We generated Flp-In cells conditionally expressing ectopic FLAG–HA–Codanin-1 carrying two common CDAI missense mutations, R714W and R1042W (Figure 5A; Dgany *et al*, 2002; Tamary *et al*, 2005; Heimpel *et al*, 2006; Ru *et al*, 2008). Western blotting and immunofluorescence analyses confirmed that wild-type and CDAI mutants were expressed at similar levels upon induction with tetracycline (Figure 5A). Like the majority of CDAI mutations, R714W and R1042W, fall in the C-terminal region, while the B-domain involved in the direct interaction with Asf1 is found in the N-terminal part of the protein. Consistently, the R714W and R1042W mutations did not affect Asf1 binding *in vitro* (Supplementary Figure S5A). However, immunoprecipitation of these CDAI disease mutants from cell extracts revealed that their ability to form complex with Asf1 (a and b) *in vivo* is significantly impaired as compared with wild-type Codanin-1 (Figure 5B). The cellular distribution of the mutants was similar to wild-type Codanin-1 (Figure 5A,

right), consistent with the finding that erythroblasts from CDAI patients show normal Codanin-1 localization (Renella *et al*, 2011). In cells expressing ectopic wild-type Codanin-1, Asf1 relocalized to the cytoplasm and only 13% of the cells maintained nuclear Asf1 staining (Figure 5C). This response was similar in cells expressing the R1042W mutant, although the nuclear exclusion of Asf1 generally appeared less pro-nounced. However, the R714W mutation significantly com-promised the ability of Codanin-1 to sequester Asf1 in the cytoplasm (Figure 5C). Importantly, this mutant had also lost the ability to arrest cells in S phase and inhibit DNA synthesis (Figure 5D and E), while the R1042W mutant showed a more modest defect.

These data argue that CDAI mutations compromise the ability of Codanin-1 to negatively regulate Asf1. The accumulation of Asf1 on chromatin is a palpable pheno-type of cells lacking Codanin-1 (Figure 3F; Supplementary Figure S4D). To directly test the ability of the R714W CDAI

Figure 5 CDAI missense mutations disable the functional interaction with Asf1. (**A**) Western blotting (*left*) and immunofluorescence analysis (*right*) of cells expressing ectopic wild-type FLAG–HA–Codanin-1 or CDAI mutants R714W and R1042W. U-2-OS cells conditional for expression of the indicated Codanin-1 proteins were induced with tetracycline for 24 h. The parental U-2-OS Flp-In cell line was used as a negative control. Scale bar, 20 μm. (**B**) Wild-type FLAG–HA-tagged Codanin-1 and CDAI mutants R714W and R1042W were immunoprecipitated from soluble protein extracts prepared from cells treated as in (**A**) and analysed by western blotting (*top*). Quantitative determination of Asf1 binding to CDAI mutants (*bottom*). Asf1 binding was analysed by western blotting and quantified in three independent experiments. The graph shows Asf1 (a or b) signals normalized to FLAG with error bars indicating standard deviation. (**C**) Immunofluorescence analysis of Asf1 localization in cells treated as in (**A**). Merged panel shows DAPI in blue, Asf1a in red and HA signals in green. Images representative of three independent experiments are shown with the percentage of cells showing nuclear Asf1 staining indicated below. Scale bar, 10 μm. (**D**) Cell-cycle profiles of conditional cells induced as in (**A**) and compared with the non-induced parental cell line. (**E**) EdU incorporation in cells expressing wild-type FLAG–HA–Codanin-1 and CDAI mutants R714W and R1042W. EdU intensities were quantified as in Figure 3C. The mean intensity of non-induced parental cells was set to 100%. The average of three independent experiments is presented with error bars indicating standard deviation. In each experiment, we analysed between 70 and 204 cells per sample.

mutant to substitute for endogenous Codanin-1 in regulation of Asf1, we carried out a complementation experiment using our cell lines inducible for siRNA-resistant Codanin-1 wild-type and R714W mutant (see Materials and methods). Codanin-1 depletion significantly increased the level of chromatin-bound Asf1 in non-induced cells (Figure 6A and B). Expression of wild-type Codanin-1 rescued this phenotype (Figure 6A; Supplementary Figure S6) and further reduced the level of Asf1 on chromatin. In contrast, the CDAI mutant R714W failed to rescue and Asf1 levels on chromatin remained high (Figure 6B; Supplementary Figure S6).

Discussion

Here, we characterize Codanin-1 as a novel interactor of the histone chaperone Asf1 and conclude that it acts as a negative regulator of Asf1 (Figure 7). The salient pieces of evidence supporting this view are (i) Codanin-1 is found in a cytosolic complex together with Asf1, histones H3.1-H4 and Importin-4; (ii) Asf1 binds via its HIRA/CAF-1 interaction pocket directly to a highly conserved B-domain motif in Codanin-1, making this interaction mutually exclusive with the two downstream chaperones; (iii) RNAi depletion of Codanin-1 elevates the level of chromatin-bound Asf1 and

Figure 6 The R714W CDAI disease mutant cannot substitute for endogenous Codanin-1 in regulation of Asf1. Quantitative analysis of Asf1 binding to chromatin (*left*) and western blotting (*right*) in cells depleted for endogenous Codanin-1 followed by expression of ectopic FLAG–HA–Codanin-1 wild-type (**A**) or R714W mutant (**B**). Conditional cells (described in Figure 5A) were treated with siRNA to deplete endogenous Codanin-1 (siCdan1 #2) or control siRNAs (siControl) and 32 h later either induced (+) or left uninduced (−). After additional 24 h, cells were harvested for immunofluorescence analysis (*left*) and western blotting (*right*). Soluble proteins were removed by pre-extraction prior to fixation and cells were stained with antibodies against Asf1 and PCNA. Asf1 levels on chromatin were measured as in Figure 3E. $n > 86$; ***$P < 0.0001$; NS, not significant $P = 0.66$ calculated by Wilcoxon paired test.

Figure 7 Model. We propose that Codanin-1 acts as a negative regulator of Asf1 function in provision of histones to replicating chromatin. Codanin-1 interacts directly with Asf1 and can sequester the chaperone in the cytoplasm away from replicating DNA. Moreover, Codanin-1 binding to Asf1 is mutually exclusive with CAF-1 and HIRA, making it a potential inhibitor of Asf1 histone donor function. In absence of Codanin-1, more Asf1 is present on chromatin, perhaps because Asf1–H3-H4 shuttling to the nucleus is accelerated. Concomitantly, cells show an increased rate of DNA synthesis, suggesting that Codanin-1 guards a limiting step in chromatin replication. We propose that Codanin-1 could regulate nuclear import of histones through its ability to bind the Asf1–H3.1-H4–Importin-4 complex. Only once Codanin-1 dissociates from the Asf1–H3.1-H4 complex in the nucleus, Asf1 would be able to bind CAF-1 and deliver histones for chromatin assembly.

enhances DNA synthesis in S-phase cells; (iv) ectopically expressed Codanin-1 binds and sequesters Asf1 in the cytoplasm, hereby interfering with Asf1 functions at replication forks including histone provision and inhibiting DNA synthesis. We also show that two CDAI disease mutations, R714W and R1042W, compromise Asf1 interaction *in vivo* and disable the ability of Codanin-1 to block S-phase progression when overexpressed. Moreover, the R714W CDAI mutant cannot substitute for endogenous Codanin-1 in regulation of Asf1 binding to chromatin. This illustrates that Codanin-1 mutants found in CDAI patients are defective in Asf1 regulation and suggests that this defect might underlie at least some of the phenotypes associated with CDAI.

Codanin-1 is ubiquitously expressed in adult tissues and essential for early embryonic development (Dgany *et al*, 2002; Renella *et al*, 2011), arguing for a basic biological function. Indeed, no patients with a null genotype have been identified. We propose that Codanin-1 could guard a limiting step in chromatin replication. While ectopic Codanin-1 inhibits DNA replication by sequestering Asf1 in the cytoplasm, lack of the protein increases both DNA synthesis and the level of Asf1 on chromatin. Thus, in the absence of Codanin-1, cells may lack an essential control of

Asf1–histone dynamics. Codanin-1 is found in complex with Asf1–H3.1-H4 and Importin-4, which facilitates nuclear import of histones and Asf1 (Campos *et al*, 2010; Jasencakova *et al*, 2010; Alvarez *et al*, 2011). Moreover, Codanin-1 interacts directly with Asf1 via a B-domain that is conserved in all organisms possessing a Codanin-1 homologue. Similarly, the globular domain of Asf1 that contains the B-domain binding pocket is also conserved through evolution (De Koning *et al*, 2007). This argues that the interaction with Asf1 and the mutually exclusive relationship with CAF-1 and HIRA chaperones are integral to Codanin-1 function. While CAF-1 and HIRA are chromatin assembly factors, Codanin-1 acts upstream probably at the level of nuclear import. We envision that Codanin-1 acts in the cytoplasm and regulates Asf1–H3-H4 delivery to chromatin. Consistently, Codanin-1 is more abundant in soluble Asf1 cytosolic complexes, in agreement with its mainly cytosolic localization in HeLa cells (this study) and erythroblasts (Renella *et al*, 2011). It remains unclear whether the less abundant nuclear Asf1–Codanin-1 complex has a link to a reported heterochromatin localization of Codanin-1 (Noy-Lotan *et al*, 2009). Given that Codanin-1 binds to Asf1 with high affinity, translocation of Codanin-1 together with Asf1–H3-H4 into the nucleus will most likely block histone transfer to downstream chaperones. Thus, Codanin-1 could have a dual inhibitory function:

(i) sequestration of Asf1 in the cytoplasm and (ii) via its B-domain hindering the binding of Asf1 to CAF-1 and HIRA.

Congenital dyserythropoietic anaemias (CDAs) are a heterogeneous group of rare disorders resulting from various abnormalities in erythropoiesis (Iolascon *et al*, 2011). The two most common forms, CDAI and CDAII, have been defined by morphological features of bone marrow erythroblasts and are caused by mutations in Codanin-1 and Sec23B, respectively. In addition, CDAII patients have distinct glycosylation abnormalities, not manifested in CDAI (Iolascon *et al*, 2011, Schwarz *et al*, 2009). Several features are specific to CDAI erythroblasts, including abnormal heterochromatin organization, internuclear chromatin bridges and S-phase defects (Wickramasinghe and Pippard, 1986; Tamary *et al*, 1996). CDAI erythroblasts show aberrant accumulation of the heterochromatin HP1α in the Golgi, which led to the idea that Codanin-1 would be involved in protein processing and/or trafficking (Renella *et al*, 2011). However, it remains unclear why erythropoiesis is particularly sensitive to Codanin-1 mutations, since Codanin-1 is a ubiquitously expressed protein. The cellular features of CDAI erythroblasts such as spongy heterochromatin organization and internuclear chromatin bridges were not recapitulated in our systems for RNAi depletion or overexpression of Codanin-1, preventing dissection of these phenotypes. We speculate that high proliferation rates in early erythroid progenitors, along with gradual condensation of the nucleus in later differentiation stages, sensitizes these cells to chromatin assembly defects that in turn can jeopardize chromatin organization and chromosome segregation during mitosis (Kaufman *et al*, 1997; Taddei *et al*, 2001; Myung *et al*, 2003). Asf1 was first identified as an S-phase factor that upon overexpression interferes with heterochromatin silencing in yeast (Le *et al*, 1997), and it is thus possible to envision that CDAI mutations by disabling Asf1 regulation may unleash an antisilencing activity. The vast majority of mutations identified in CDAI patients, including R714W and R1042W examined here, fall in clusters within the C-terminal region away from the B-domain involved in Asf1 binding. However, the R714W and R1042W mutations partially disrupt Asf1 binding *in vivo* and the R714W mutant fails to functionally complement for endogenous Codanin-1 in regulation of Asf1 chromatin binding. We therefore speculate that these mutations may abrogate an interphase that indirectly aids efficient complex formation with Asf1. Structural analysis will be required to reveal whether CDAI mutations cluster on a potential interaction surface and how they are situated three dimensionally with respect to Asf1 binding. In addition, profiling of Codanin-1 complexes may identify partners that contribute to Asf1 regulation or participate in other, yet unknown, functions that also could be targeted by CDAI mutations.

Interestingly, DNA synthesis is deregulated in Codanin-1-depleted cells, illustrating that replication control is coordinated with histone provision. We also show that ectopic Codanin-1 sequesters Asf1 in the cytoplasm, thereby blocking Asf1-dependent histone transfer to the nucleus and inhibiting DNA replication. We thus anticipate that the interaction between Asf1 and endogenous Codanin-1 must be regulated, perhaps via post-translational modifications, in order to allow Asf1 to shuttle into the nucleus and deliver its cargo to CAF-1 or HIRA for chromatin assembly. Interestingly, high-throughput studies have identified an interaction between

Codanin-1 and PDPK1, a cytoplasmic kinase acting upstream of AKT, as well as several putative Cdk phosphorylation sites in the vicinity of the B-domain (Stelzl *et al*, 2005; Choudhary *et al*, 2009; Huttlin *et al*, 2010). It is thus appealing to imagine that the binding of Asf1 to Codanin-1 provides a means to fine-tune histone supply in response to external stimuli and cell-cycle cues.

Materials and methods

Cell culture and siRNA treatment

HeLa S3 cells stably expressing OneStrep-tagged (e−) Asf1 (a and b) were described (Groth *et al*, 2007a). U-2-OS cells conditional for Codanin-1 were generated in the T-REx Flp-In system (Invitrogen) and expression was induced with tetracycline (80–100 ng/ml). Of note, Codanin-1 was expressed from a synthetic human codon-optimized cDNA (synthesized by GeneArt) resistant to siRNAs (siCdan1#2). The CDAN1-targeting siRNAs were siCdan1 #1 (Sigma): 5′-CGUAGAGUUCGUGGCAGAAAGAAUU-3′ (sense strand), siCdan1 #2: ON-TARGET plus SMART pool (Dharmacon). Cell-cycle profiles were determined by FACS analysis of DNA content and analysed by FlowJo 8.8.4 software (Tree Star).

Biochemistry and immunocytochemistry

Purification of e-Asf1 complexes using stringent washing conditions (500 mM NaCl, 20 mM Tris pH 7.8, 0.2 mM EDTA, 0.2% NP-40, 1 mM DTT, 5% glycerol, and protease and phosphatase inhibitors) and gel filtration analysis were described previously (Jasencakova *et al*, 2010). Co-immunoprecipitation of cytosolic proteins was carried out in a buffer containing 200 mM NaCl, 0.2% NP-40, 20 mM Tris pH 7.6, 0.2 mM EDTA pH 8, 5% glycerol and inhibitors (see Supplementary data). For GST pull-down experiments, ^{35}S-labelled Codanin-1 was produced using the TnT T7 Quick Coupled Transcription/Translation System (Promega) and incubated with recombinant GST–Asf1 proteins in binding buffer (150 mM NaCl, 0.2% NP-40, 50 mM Tris, pH 7.6, 2 mM EDTA, 5% glycerol and inhibitors) for 2 h at 4°C. The reactions were washed five times and analysed by digital autoradiography.

Mass spectrometry

Excised protein bands were analysed by Liquid chromatography-MS/MS on a Qstar elite machine at the LSMP Platform, Curie Institute, as described (Groth *et al*, 2007a).

Immunocytochemistry

Cells were either pre-extracted with 0.5% Triton in CSK buffer (10 mM PIPES pH 7, 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl$_2$) 5 min at 4°C to remove soluble proteins or fixed directly with 4% formaldehyde and processed as described (Jasencakova *et al*, 2010). Images were collected using Axiovert 200M confocal microscope equipped with LSM510 Laser module (Zeiss), or a DeltaVision system and analysed with SoftWoRx 5.0.0 software (Applied Precision). All images in the individual panels were acquired with the same settings and adjusted for brightness and contrast identically. Quantification was carried out using the polygon tool in SoftWoRX to measure fluorescence intensity in individual channels of nuclei defined by DAPI staining. Statistics and dot plot presentation of data were made in Prism 4.0 (GraphPad Software). See Supplementary data for the complete list of antibodies used in this study.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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