Fanconi anemia protein FANCI functions in ribosome biogenesis

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Fanconi anemia (FA) is a disease of DNA repair characterized by bone marrow failure and a reduced ability to remove DNA interstrand cross-links. Here, we provide evidence that the FA protein FANCI also functions in ribosome biogenesis, the process of making ribosomes that initiates in the nucleolus. We show that FANCI localizes to the nucleolus and is functionally and physically tied to the transcription of pre-ribosomal RNA (pre-rRNA) and to large ribosomal subunit (LSU) pre-rRNA processing independent of FANCD2. While FANCI is known to be monoubiquitinated when activated for DNA repair, we find that it is predominantly in the deubiquitinated state in the nucleolus, requiring the nucleoplasmic deubiquitinase (DUB) USP1 and the nucleolar DUB USP36. Our model suggests a possible dual pathophysiology for FA that includes defects in DNA repair and in ribosome biogenesis.

Fanconi anemia | FANCI | ribosome | pre-ribosomal RNA | nucleolus

Fanconi anemia (FA) is an inherited bone marrow failure syndrome (1–7). Individuals with FA often present early in childhood with aplastic anemia and congenital abnormalities, including skeletal, cardiac, and renal defects (8, 9). At the cellular level, cells from individuals with FA are hypersensitive to DNA-damaging agents such as diepoxybutane (DEB) or mitomycin C (MMC) (8). Hence, FA has thus far been known as a disease of DNA repair.

The 22 proteins that are mutated in individuals with FA are involved in maintaining genomic stability through repair of DNA interstrand cross-links (5, 6, 10–13). One such protein in the FA pathway is FANCI (14–16), mutated in 1% of FA patients (5). FANCI and its binding partner, FANCD2, together are called the ID complex and are activated for DNA repair by phosphorylation and monoubiquitination (6, 17–19). Deubiquitination of the ID complex is also important for DNA repair because impairing the removal of ubiquitin from FANCI or FANCD2 causes sensitivity to DNA cross-linking agents (20). The deubiquitinase (DUB) responsible for deubiquitination of FANCI and FANCD2 is USP1 (14, 21). While FANCI and FANCD2 act in a 1:1 stoichiometry in DNA repair, FANCI is more abundant than FANCD2 (22), raising the intriguing question, What is the function of the excess FANCI?

In addition to FA, bone marrow dysfunction is a sign of multiple diseases of ribosome biogenesis, collectively called ribosomopathies (23–25). Diamond–Blackfan anemia (DBA), an inherited bone marrow failure syndrome, is associated with mutations in large and small subunit ribosomal proteins (26, 27). Shwachman–Diamond syndrome, which results in neutropenia and exocrine pancreatic deficiency, is caused by mutations in the SBDS protein, required for late-stage maturation of the large subunit of the ribosome by catalyzing the removal of eukaryotic initiation factor 6 (28, 29). Furthermore, whereas defects in telomere maintenance are central to the pathogenesis of dyskeratosis congenita (DC), some forms of DC are caused by mutations in genes that also function in ribosome biogenesis, such as DKC1. DC patients present with aplastic anemia as well as skin and nail defects (30, 31). Moreover, BRCA1 [also known as FANCS (32)] has recently been shown to interact with and regulate RNA polymerase I (RNAPI), the enzyme responsible for the transcription of pre-ribosomal RNA (pre-rRNA) (33). The clear phenotypic overlap between FA and bone marrow failure ribosomopathies and the connection between BRCA1 (FANCS) and RNAPI suggest a possible connection between FA and defects in ribosome biogenesis.

The process of making ribosomes in eukaryotes, termed ribosome biogenesis, initiates in the large nonmembrane-bound nuclear organelle, the nucleolus (NO) (34, 35). In the NO, tandem repeats of ribosomal DNA (rDNA) (36) are transcribed by RNAPI, which generates a long pre-rRNA (37). A carefully coordinated series of endonucleolytic and exonucleolytic reactions results in the formation of the small ribosomal subunit (SSU) rRNA (18S) and two of the three large ribosomal subunit (LSU) rRNAs (5.8S and 28S) (38–40). Ribosomal subunits, containing rRNA and ribosomal proteins, are exported from the nucleus to the cytoplasm where the LSU and SSU together perform the essential function of translation of mRNA into protein (41, 42). Recently, the sensitivity of the bone marrow to defects in ribosome biogenesis was linked to decreased ribosome recycling factors in developing hematologic cell lineages (43, 44), highlighting a strong mechanistic link between defects in making ribosomes and bone marrow failure.

Here, we provide evidence for a nucleolar function for FANCI in ribosome biogenesis. FANCI is localized to the NO, the site of the initial stages of ribosome biogenesis, and is required for pre-rRNA transcription and processing. Additionally, FANCI, like other proteins that function in ribosome biogenesis, is required

Significance

FANCI is a Fanconi anemia (FA) protein and functions in DNA interstrand cross-link repair. Surprisingly, in addition to DNA repair proteins, we find that FANCI interacts with proteins that function in ribosome biogenesis, the synthesis of ribosomes in cells. Furthermore, FANCI localizes to the nucleolus—the cellular compartment in which ribosome biogenesis initiates—and functions in the transcription and processing of pre-ribosomal RNA (pre-rRNA). Interestingly, defects in ribosome biogenesis underlie three other bone marrow failure syndromes, raising the question, Do defects in ribosome biogenesis contribute to FA pathogenesis? Our results expand the functions of FANCI to encompass ribosome biogenesis and DNA repair, highlighting the importance of future investigations into the role of other FA proteins in synthesizing ribosomes.

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for global protein synthesis by ribosomes. FANCI is predominantly in the deubiquitinated state in the NO, and maintenance of its nucleolar deubiquitinated state relies on both of the DUBs USP1 and USP36. Our results support a model for FANCI having a dual role in DNA repair and ribosome biogenesis, suggesting that defects in making ribosomes may also contribute to the molecular pathogenesis of FA in individuals with FANCI mutations.

Materials and Methods

Detailed procedures are given in *SI Appendix, Supplementary Materials and Methods*.

Affinity Purification and Mass Spectrometry Analysis of FANCI Interacting Partners. To identify FANCI-interacting proteins by coimmunoprecipitation and mass spectrometry, we followed a previously published protocol for identifying interacting partners of DNA repair proteins (45), with modifications. Mass spectrometry analysis of the TCA-precipitated peptides was performed by the Gygi laboratory at Harvard Medical School with eluates of either protein G beads alone, as a negative control, or anti-FANCI/protein G beads.

Cell Culture and Manipulation. HeLa, SH-SY5Y, and RKO cells were purchased from the American Type Culture Collection, which routinely analyzes cell lines by short tandem repeat analysis. HEK293FT cells were a generous gift from P. Glazer. Flp-In T-REx HEK293 cells were a gift from P. Gallagher. All cell lines were grown in DMEM (Cat. No. 11965118; Gibco) with 10% FBS and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

To deplete proteins of interest, cells were transfected twice with Dharmacon siRNA SMARTpools or with individual Dharmacon siRNAs using Lipofectamine RNAiMax (Cat. No. 13778075; Life Technologies) per the manufacturer's instructions.

Lentiviruses were packaged in the HEK293FT cell line by cotransfecting the pVSV-G, psPAX2, and modified pLX vectors (46) containing the cDNAs of interest in a 1:9:10 ratio (1 μg:9 μg:10 μg for a 10-cm plate) with Lipofectamine 2000 (Cat. No. 11668019; Life Technologies) per the manufacturer's instructions.

Protein Quantitation and Western Blotting. Protein concentration for samples was calculated with the Bradford Assay (Cat. No. 5000006; Bio-Rad). Samples were loaded onto Tris-glycine gels, separated by SDS/PAGE, and transferred to PVDF membranes (Cat. No. 1620177; Bio-Rad) for detection of proteins by Western blotting. Quantitation of Western blot signals was performed with ImageJ software, with three technical replicates for each biological replicate.

Subcellular Fractionation. We modified a previously published protocol to subcellular fractionate HeLa cells to isolate NO (47).

Coimmunoprecipitation. Antibodies were conjugated to 3 mg Protein A Sepharose CL-4B beads (Cat. No. 17-0780-01; GE Healthcare Life Sciences) as in ref. 48. Cell lysates were prepared by sonication followed by centrifugation to remove insoluble debris. Whole-cell extracts (WCEs) were incubated alongside the immunoprecipitations to control for any changes that occurred during the incubation period. After the incubation, the beads were washed five times, and proteins bound to the beads were eluted with 2× Laemmli sample buffer.

Immunofluorescence, Image Acquisition, and Analysis. Immunofluorescence staining of HeLa cells was performed as in ref. 34. The 3D image stacks were collected at 0.3-μm z increments on a DeltaVision Elite workstation (Applied Precision) based on an inverted microscope (IX-70; Olympus). Image preparation and analysis was done using ImageJ. Colocalization analysis was performed using the Coloc module in the Imaris (Bitplane) software (version 9.2.1).

Dual Luciferase Assay. HeLa cells were transfected with siRNAs and, 48 h later, transfected with 400 ng of pHrD-internal ribosome entry site (IRES)-luciferase plasmid (49) and 1 ng of *Renilla* plasmid (48) using Lipofectamine 3000 (Cat. No. L3000015; Life Technologies). The ratio of pHrD-IRES-luciferase/*Renilla* activity was calculated to control for transfection efficiency.

qRT-PCR Analysis. Seventy-two hours after the initial siRNA transfection, total RNA was extracted and purified with TRIzol reagent (Cat. No. 15596026; Life Technologies) per the manufacturer's instructions. cDNA was synthesized using the iScript gDNA Clear cDNA Synthesis Kit (Cat. No. 172-5035; Bio-Rad) following the manufacturer's instructions. Reactions for qPCR were set up on ice according to the manufacturer's instructions using the iTaq Universal SYBR Green Supermix (Cat. No. 172-5121; Bio-Rad). Amplification of the 7SL RNA was used as an internal control, and relative expression between samples was calculated with the comparative C_T ($2^{-\Delta\Delta Ct}$) method.

Northern Blotting and Bioanalyzer Analysis. Northern blot analysis was performed as described previously (50). Ratio Analysis of Multiple Precursors (RAMP) was performed as described (51). To measure the ratio of mature 28S to 18S rRNAs, total RNA that was prepared as described above was run on an Agilent Technologies 2100 Bioanalyzer at the Yale Center for Genome Analysis.

Protein Synthesis Assay. We assessed the rate of global protein synthesis using puromycin to label nascent peptides as in ref. 52.

Results

FANCI Is a Nucleoplasmic and Nucleolar Protein. We took an unbiased approach to discover FANCI-interacting proteins. Using an antibody against FANCI (53–58), we immunoaffinity-purified FANCI from HeLa nuclear extracts and identified the copurifying proteins by mass spectrometry. Surprisingly, some of the proteins with the highest peptide counts were nucleolar proteins (Dataset S1), including RNA helicases and all of the members of the PeBoW complex, a complex required for maturation of the LSU (59). Using Western blotting as an alternative readout, we confirmed that the PeBoW complex members PES1 and BOP1 are coimmunoprecipitated with FANCI (*SI Appendix*, Fig. S1*A*). The association of FANCI with proteins required for ribosome biogenesis led us to hypothesize that FANCI has a nucleolar function.

Other high-throughput proteomic evidence points to a role for FANCI in the NO. FANCI is listed in the nucleolar proteomes of HeLa (60) and T cells (61), two disparate human cell types. FANCI is the only FA protein in both nucleolar proteomes. Furthermore, FANCI copurifies with USP36, a known human nucleolar DUB (62). Therefore, four separate proteomic reports, including ours (Dataset S1), provide evidence for a nucleolar localization of FANCI.

To test the extent to which FANCI is localized to the NO, we turned to the well-established technique (47, 60, 63) of subcellular fractionation to isolate nucleoplasm (NP) and NO from HeLa cells. Equal masses of total protein from WCE, NP, and NO were separated by SDS/PAGE and analyzed by Western blotting. We employed eight controls to ensure the rigor of our subcellular fractionation technique (Fig. 1*A*). The cytoplasmic proteins α-tubulin and vinculin were present only in WCE, whereas the cytoplasmic and nucleoplasmic protein β-actin, the nuclear envelope protein SUN2, the nuclear lamina protein lamin B1, and the nuclear pore complex protein NUP98 were found in WCE and NP, but not in NO. The nucleolar proteins PES1 and RPA194 were enriched in the NO fraction and were almost undetectable in WCE. Taken together, these controls demonstrate the purity of our isolated NO.

Similarly, subcellular fractionation revealed that FANCI and FANCD2 are localized to the NP and NO (Fig. 1*B*). We quantitated the enrichment of FANCI and FANCD2 in each fraction from three independent biological replicates using ImageJ (Fig. 1 *C* and *D*). Interestingly, FANCI is significantly enriched in the NO fraction relative to the NP (Fig. 1*C*), whereas there was no significant difference in the enrichment of FANCD2 in the NP and NO (Fig. 1*D*). The significantly greater signal for FANCI relative to FANCD2 in the NO (*SI Appendix*, Fig. S1*B*), but not in the NP (*SI Appendix*, Fig. S1*C*), suggests a FANCD2-independent role for FANCI in the NO.

We used immunoprecipitation of PES1 (59), a nucleolar protein (Fig. 1*A*) that is associated with FANCI (*SI Appendix*, Fig. S1*A* and Dataset S1), followed by Western blotting as an orthogonal method to confirm the association of FANCI with

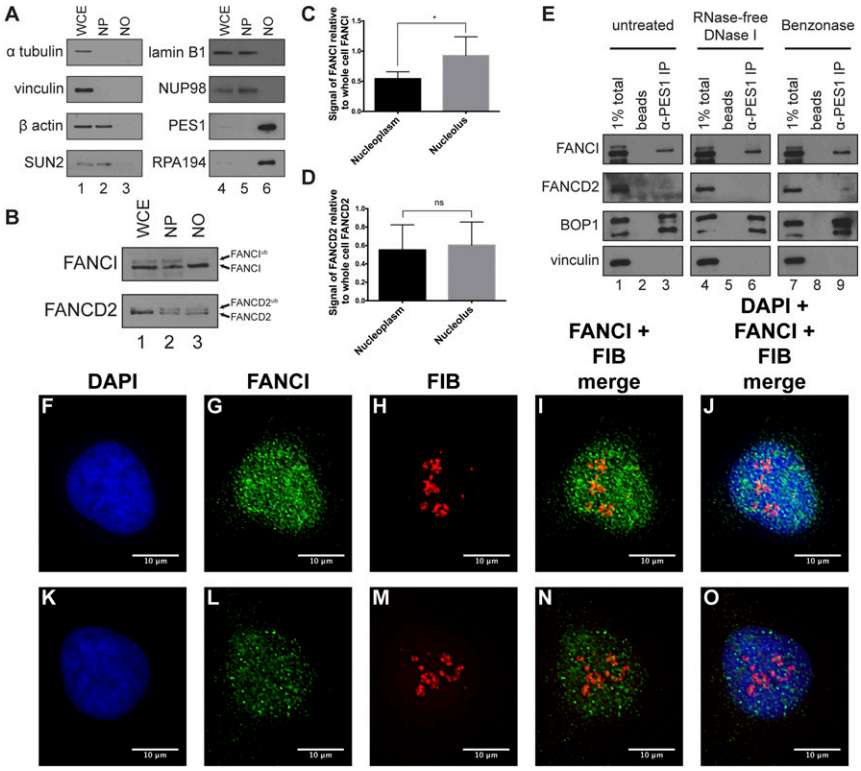

Fig. 1. FANCI is a nucleolar and nucleoplasmic protein. (*A*) Western blots for subcellular fractionation controls indicate productive separation of NP and NO. (*B*) FANCI and FANCD2 are localized to both NP and NO. Western blots for FANCI and FANCD2 show both monoubiquitinated and deubiquitinated forms, indicated by arrows. (*C* and *D*) FANCI, but not FANCD2, is enriched in NO. Densitometric quantitation of Western blots from three biological replicates for NP and NO. FANCI (*C*) and FANCD2 (*D*) were measured and set relative to the level present in WCE. Statistical significance was calculated using a two-tailed unpaired Mann–Whitney *U* test (mean ± SD). ns, not significant, $*P \leq 0.05$. (*E*) The nucleolar protein, PES1, coimmunoprecipitates FANCI. Shown are Western blots for FANCI and FANCD2 for WCE (1% total; lanes 1, 4, and 7) and α-PES1 immunoprecipitates (α-PES1 IP; lanes 3, 6, and 9). BOP1 is a known direct physical interactor of PES1 and is a positive control; vinculin was used as a negative control. Unconjugated protein A beads (lanes 2, 5, and 8) also served as a negative control. (*F*–*O*) FANCI is colocalized with the nucleolar protein fibrillarin by immunofluorescence in fixed HeLa cells. (*F* and *K*) DAPI. (*G* and *L*) Anti-FANCI. (*H* and *M*) Anti-fibrillarin (FIB). (*I* and *N*) Merge of anti-FANCI and anti-fibrillarin. (*J* and *O*) Merge of DAPI, anti-FANCI, and anti-fibrillarin. (Scale bars: 10 μm.) A single Z plane is shown.

nucleolar proteins and to confirm the localization of FANCI to the NO. HeLa cell extracts were untreated and incubated at 4 °C for 3 h (Fig. 1*E*, lanes 1 to 3; for IgG negative control immunoprecipitation, see *SI Appendix*, Fig. S1*D*), treated with RNase-free DNase I for 3 h at 4 °C (Fig. 1*E*, lanes 4 to 6), or treated with Benzonase for 3 h at 4 °C, which degrades both DNA and RNA (Fig. 1*E*, lanes 7 to 9). Degradation of RNA was confirmed by phenol–chloroform extraction from extracts, followed by analysis on an agarose gel (*SI Appendix*, Fig. S1*E*) (64). Under all of these conditions in which RNA is degraded, PES1 coimmunoprecipitated FANCI, but FANCD2 was not enriched to the same degree (Fig. 1*E*, lanes 3, 6, and 9), adding further support to the hypothesis that FANCI has a FANCD2-independent role in the NO. Additionally, since degradation of nucleic acids did not abrogate the interaction of FANCI with PES1, we conclude that large nucleic acids are not required for this interaction. Furthermore, PES1 coimmunoprecipitated FANCI in three other human cell lines, HEK293FT (*SI Appendix*, Fig. S1*F*), RKO (*SI Appendix*, Fig. S1*G*), and SH-SY5Y (*SI Appendix*, Fig. S1*H*). However, another nucleolar protein, fibrillarin, did not coimmunoprecipitate FANCI in HeLa cells (*SI Appendix*, Fig. S1*I*). Therefore, FANCI physically interacts with the nucleolar LSU biogenesis factor PES1 (59), but not the nucleolar SSU biogenesis factor fibrillarin.

As another orthogonal approach to test the hypothesis that FANCI localizes to the NO, we stained for FANCI and the commonly used nucleolar marker protein fibrillarin (34, 65, 66) by immunofluorescence. As previously seen for FANCI localization by multiple laboratories using multiple different anti-FANCI antibodies, including the anti-FANCI antibody we used (14, 54, 55, 58, 67), FANCI exhibits a pan-nuclear punctate nuclear localization (Fig. 1*F* for DAPI and Fig. 1*G* for FANCI) that does not exclude NO. Fibrillarin stains the dense fibrillar center (68, 69) of NO (Fig. 1*H*) and can be found colocalized with FANCI (Fig. 1 *I*–*O* and Movies S1 and S2). The Pearson correlation coefficient for colocalization of FANCI and fibrillarin ranged from 0.47 to 0.53, indicating a moderate positive linear relationship between these two proteins and nucleolar localization of FANCI. Thus, using three independent, orthogonal approaches, we have shown that FANCI is localized to the NO in human cells.

FANCI Is Functionally and Physically Tied to the Transcription of Pre-rRNA. To test the hypothesis that FANCI functions in ribosome biogenesis, we asked whether FANCI is required for the transcription of rDNA into pre-rRNA. We employed a well-established dual-luciferase reporter system to assay pre-rRNA transcription by RNAPI (48, 49). In this system, one construct contains an IRES followed by the firefly luciferase gene downstream from the human rDNA promoter. The other construct, used to control for differences in transfection efficiency, contains the *Renilla* luciferase gene under the control of a constitutively active RNAPII promoter. In agreement with previous studies, depletion of NOL11, an SSU processome factor, decreased RNAPI transcription (48) (Fig. 2*A*). Depletion of FANCI with a pool of siRNAs or two individual, independent siRNAs (16, 70) also decreased rDNA transcription (Fig. 2*A*). However, depletion of FANCD2 did not affect the transcription of rDNA (Fig. 2*A*). To confirm defects in pre-rRNA transcription, we measured levels of the primary pre-rRNA transcript (47S/45S/30S) by qRT-PCR using previously published primers (71–73). Consistent with the luciferase assay results, depletion of FANCI or NOL11 decreased the levels of the primary pre-rRNA, while depletion of FANCD2 did not significantly change its levels (Fig. 2*B*). Since depletion of FANCI, but not FANCD2, leads to decreased rDNA transcription, we conclude that FANCI has a FANCD2-independent role in promoting rDNA transcription. We can also conclude that since the constructs used for the luciferase assay lack an origin of replication and are not replicated in cells, FANCI promotes rDNA transcription independent of DNA replication.

In addition to the functional connection between FANCI and RNAPI transcription, we asked if FANCI is physically associated with RNAPI. Using an antibody against the large subunit of RNAPI (RPA194), we immunoprecipitated RPA194 from WCEs

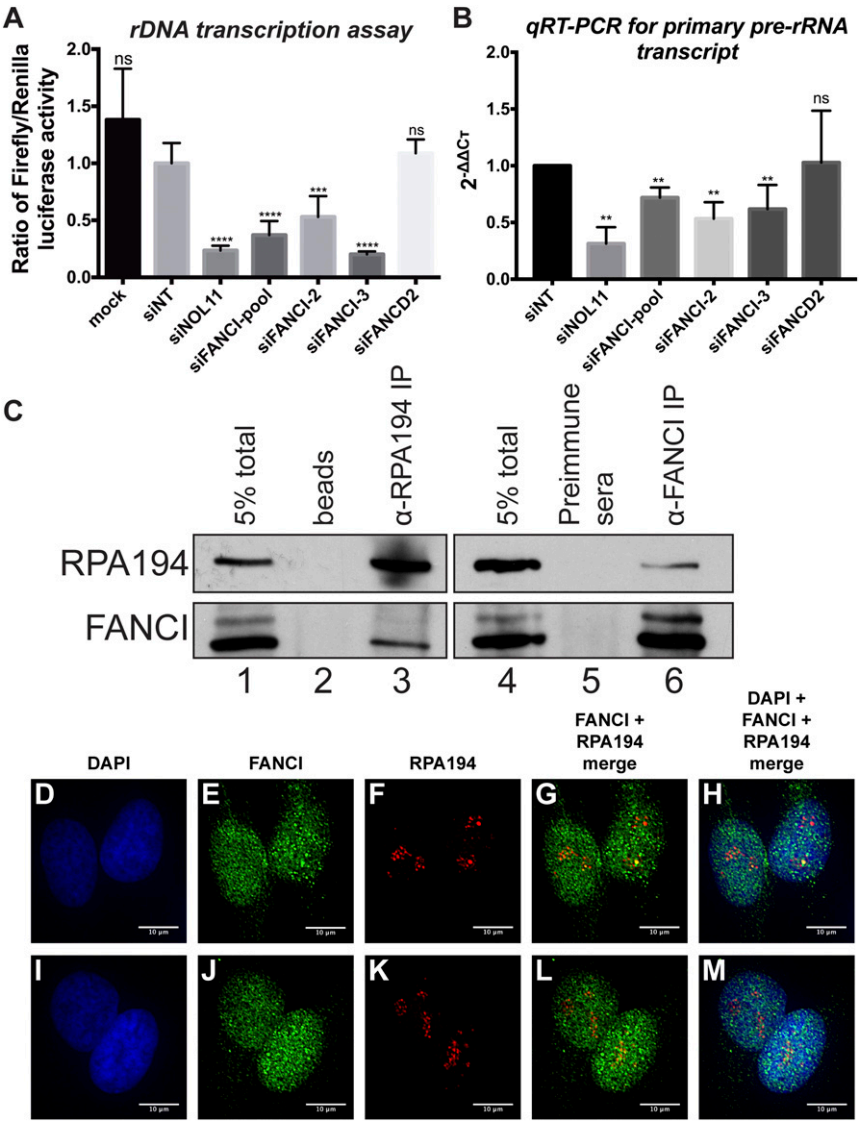

Fig. 2. FANCI is physically and functionally associated with pre-rRNA transcription. (*A*) FANCI is required for RNAPI transcription. Cells were transfected with the indicated siRNAs and, after 48 h, transfected again with plasmids containing firefly luciferase (under the control of the rDNA promoter) and *Renilla* luciferase (under the control of a constitutive promoter). Luminescence was quantitated 24 h later. Statistical significance for nine biological replicates was calculated using a two-tailed Mann–Whitney *U* test (mean ± SD). All comparisons are relative to siNT. ns, not significant, $***P \leq 0.001$, $****P \leq 0.0001$. (*B*) Depletion of FANCI decreases the levels of the primary pre-rRNA. qRT-PCR was used to quantitate the levels of the primary pre-rRNA after depletion of the indicated proteins using the 7SL RNA as an internal control. Relative expression values were calculated with the comparative C_T method (mean ± SD). Statistical significance for three biological replicates was calculated using the Wilcoxon signed-rank test with a hypothetical value of 1.0. All comparisons are relative to siNT. ns, not significant, $**P \leq 0.01$. (*C*) FANCI physically interacts with the large subunit of RNAPI (RPA194). HeLa WCEs were immunoprecipitated (IP) with α-RPA194 and α-FANCI antibodies. Unconjugated protein A beads and preimmune sera were used as negative controls. RPA194 and FANCI were detected by Western blotting. (*D–M*) FANCI colocalizes with RPA194 by immunofluorescence in fixed HeLa cells. (*D* and *I*) DAPI. (*E* and *J*) Anti-FANCI. (*F* and *K*) Anti-RPA194. (*G* and *L*) Merge of anti-FANCI and anti-RPA194. (*H* and *M*) Merge of DAPI, anti-FANCI, and anti-RPA194. (Scale bars: 10 μm.) A single Z plane is shown.

and Western blotted for FANCI. Indeed, RPA194 coimmunoprecipitated FANCI (Fig. 2*C*, lane 3). The reciprocal coimmunoprecipitation also demonstrated that FANCI physically interacts with RPA194 (Fig. 2*C*, lane 6). Since RPA194 is not shared with RNAPII or RNAPIII (37), we can conclude that FANCI physically associates with RNAPI. Importantly, this interaction is conserved in HEK293FT, RKO, and SH-SY5Y cell lines (*SI Appendix*, Fig. S2), consistent with FANCI having a direct role in ribosome biogenesis in the NO through its association with RNAPI by promoting rDNA transcription.

As FANCI is physically and functionally associated with RNAPI, we asked whether FANCI colocalizes with RPA194 by immunofluorescence. We can visualize distinct puncta that contain both FANCI and RPA194 (Fig. 2 *D–M* and Movies S3 and S4), consistent with colocalization of FANCI and RPA194. The Pearson correlation coefficient for colocalization of FANCI and RPA194 ranged from 0.46 to 0.74, indicating a moderate to strong positive linear relationship between these two proteins and nucleolar localization of FANCI. These results are consistent with FANCI functioning in pre-rRNA transcription by localizing to the fibrillar center (68) in NO (Fig. 2 *G* and *L*) through physical association with RPA194 (Fig. 2*C* and *SI Appendix*, Fig. S2). Together, fibrillarin staining adjacent to and overlapping with FANCI (Fig. 1 *F–O* and Movies S1 and S2) and the colocalization of FANCI to distinct puncta with RPA194 (Fig. 2 *D–M* and Movies S3 and S4) conclusively demonstrate that a portion of FANCI is localized to the NO.

FANCI Is Required for LSU Biogenesis and Global Translation. Given that FANCI physically interacts with PES1 (Fig. 1*E*), a factor required for LSU biogenesis (59), we asked whether FANCI is required for LSU pre-rRNA processing. The pre-rRNA is transcribed as a large, polycistronic precursor called the 47S pre-rRNA, which is quickly processed into the 45S pre-rRNA (38). These pre-rRNAs are detected as one band with a probe that detects defects in LSU pre-rRNA processing. Therefore, we will call this collection of 47S and 45S pre-rRNAs the "primary transcript plus" (PTP) as done in Wang et al. (51). The PTP contains the sequences for three of the mature rRNAs as well as external transcribed spacers (ETSs) and internal transcribed spacers (ITSs) that must be removed to generate the mature rRNAs (Fig. 3*A*) (38). Pre-rRNAs that are formed and further processed for the LSU include the 41S, 32S, and 12S pre-rRNAs (Fig. 3*A*).

To determine whether FANCI is required for LSU pre-rRNA processing, we depleted FANCI with siRNAs, harvested total RNA, and performed Northern blotting analysis on equal amounts of RNA with a probe complementary to ITS2 (Fig. 3*A*), which detects defects in LSU pre-rRNA processing. We also treated HeLa cells with siRNAs against PES1, NOL11, and FANCD2. We confirmed depletion of FANCI, FANCD2, NOL11, and PES1 by Western blotting (*SI Appendix*, Fig. S3).

Depletion of FANCI led to a noticeable decrease in the 12S pre-rRNA (Fig. 3*B*, lanes 3 to 5) compared with controls (Fig. 3*B*, lanes 1 and 2). We quantitated three biological replicates of Northern blots by RAMP (51) (Fig. 3*C*). RAMP for depletion of FANCI reveals that the 12S/32S, 12S/41S, and 12S/PTP ratios were all significantly decreased after depletion of FANCI with all siRNAs (Fig. 3 *D*–*F*). These same ratios were also decreased after depletion of PES1 (Fig. 3*G*), albeit to a greater extent, demonstrating that PES1 and FANCI are functionally linked in addition to their physical interaction. Unlike FANCI and PES1 depletion, depletion of NOL11, an SSU processome factor (48), did not decrease the 12S/32S, 12S/41S, and 12S/PTP ratios (Fig. 3*H*). Depletion of FANCD2 did not result in a similar processing defect compared with FANCI depletion (Fig. 3*I*), further suggesting that FANCI functions in a FANCD2-independent manner in ribosome biogenesis.

Consistent with a requirement for FANCI in efficient LSU pre-rRNA processing, depletion of FANCI led to a significant decrease in the ratio of the mature 28S/18S rRNAs (Fig. 3*J*), as measured by an Agilent 2100 Bioanalyzer. Decrease of the 28S/18S rRNA ratio indicates less LSU rRNA relative to SSU. Thus, FANCI is required for LSU pre-rRNA processing and for maintaining levels of the LSU rRNA, likely through a direct function in ribosome biogenesis with PES1.

Since FANCI is required for pre-rRNA transcription and processing, we tested whether FANCI is also required for global protein synthesis by ribosomes. This question is particularly salient because multiple other bone marrow failure syndromes lead to defects in ribosome function, including DBA, Shwachman–Diamond syndrome, and DC (74). We used a system that labels nascent peptides in live cells with puromycin (52). After depleting HeLa cells of FANCI, RPS19 (mutated in DBA), DKC1 (mutated in DC), and SBDS (mutated in Shwachman–Diamond syndrome) (*SI Appendix*, Fig. S4), we incubated with 1 μM puromycin [or 0.5 μM for the mock (1/2) control] for 1 h and then Western blotted for puromycin and β-actin as a loading control (Fig. 4*A*). Depletion of FANCI led to a significant decrease in global protein synthesis (Fig. 4*A*, compare lanes 4 to 6 with lanes 1 and 3 and Fig. 4*B*). Depletion of RPS19, DKC1, and SBDS

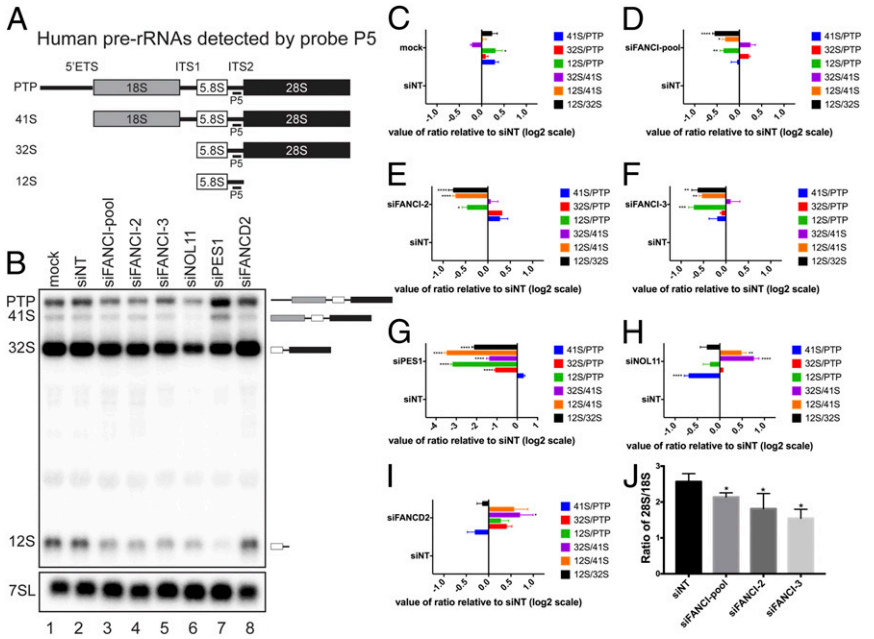

Fig. 3. FANCI functions in LSU pre-rRNA processing. (*A*) Human pre-rRNA processing schematic. The human pre-rRNA is transcribed as a large precursor, the 47S pre-rRNA, which is processed into the 45S pre-rRNA, both of which are detectable as a single band by Northern blotting, which we refer to as the PTP (51). The 41S, 32S, and 12S pre-rRNAs are detected by a probe (P5) that hybridizes with ITS2. (*B*) FANCI depletion results in LSU pre-rRNA processing defects. HeLa cells were transfected with the indicated siRNAs, and total RNA was harvested after 72 h of depletion. Pre-rRNAs were separated by gel electrophoresis, and Northern blots were performed using radioactively labeled P5 probe. Northern blotting with a probe against the 7SL RNA was used as a loading control. Small illustrations of the pre-rRNAs are to the right of their respective bands. (*C*–*I*) RAMP (51) profiles for Northern blotting from three biological replicates for mock (*C*), siFANCI-pool (*D*), siFANCI-2 (*E*), siFANCI-3 (*F*), siPES1 (*G*), siNOL11 (*H*), and siFANCD2 (*I*). Statistical significance was calculated using a two-way ANOVA with Sidak multiple comparisons test (mean ± SD). All comparisons are relative to siNT. The *x* axes of all graphs were equalized, except for siPES1 in *G*, which has larger RAMP values. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$. Unmarked bars are not significant. (*J*) FANCI depletion results in decreased mature 28S rRNA relative to 18S. The ratio of 28S/18S was calculated using an Agilent 2100 Bioanalyzer. Statistical significance was calculated using a one-tailed unpaired Mann–Whitney *U* test for three biological replicates (mean ± SD). All comparisons are relative to siNT.

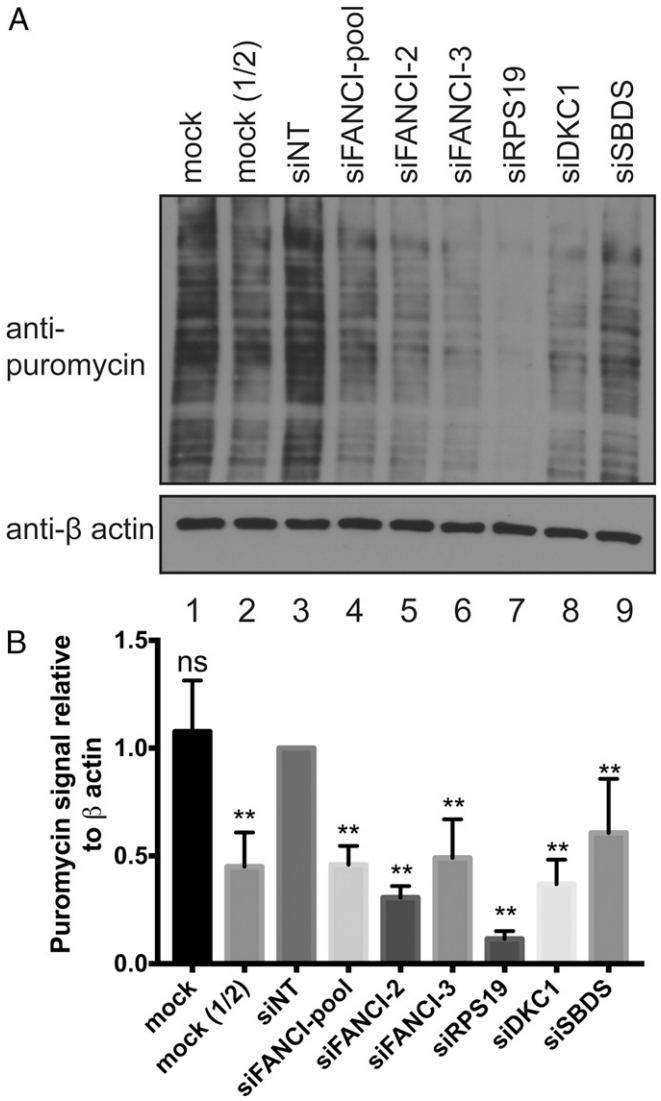

Fig. 4. FANCI is required for global protein synthesis. (*A*) FANCI is required to maintain normal levels of protein synthesis. HeLa cells were transfected with the indicated siRNAs and, after 72 h of depletion, were treated with 1 μM puromycin for 1 h to label nascent peptides. A control treated with 0.5 μM puromycin was included [mock (1/2)]. Nascent peptides labeled with puromycin were detected by Western blotting with an anti-puromycin antibody. Western blotting with an anti–β-actin antibody was used as a loading control. (*B*) Densitometric quantitation of Western blots from three biological replicates (mean ± SD). Statistical significance for three biological replicates was calculated using the Wilcoxon signed-rank test with a hypothetical value of 1.0. All comparisons are relative to siNT. ns, not significant, $^{**}P \leq 0.01$.

also significantly reduced protein synthesis (Fig. 4*A*, lanes 7 to 9 and Fig. 4*B*). Therefore, like other bone marrow failure genes, FANCI is required for maintaining levels of global protein synthesis. Thus, the defects in pre-rRNA transcription and processing due to depletion of FANCI also led to defects in the ultimate function of ribosomes: protein synthesis. This highlights the crucial nature of the localization and function of FANCI in the NO for ribosome biogenesis because it has far-reaching cellular effects that extend even to cytoplasmic ribosome function.

FANCI Is Predominantly in the Deubiquitinated State in the NO. FANCI and FANCD2 are activated for their role in DNA repair by monoubiquitination (14, 17, 18). We hypothesized that the deubiquitinated form of FANCI is primarily involved in ribosome biogenesis since a greater proportion of nucleolar FANCI is in the deubiquitinated form compared to nucleoplasmic FANCI (Fig. 5*A*, compare lanes 2 and 3). Subcellular fractionation of HeLa cells supports this hypothesis. Quantification of the signal of monoubiquitinated FANCI relative to deubiquitinated FANCI ($FANCI^{ub}$/FANCI) revealed a greater proportion of deubiquitinated FANCI in the NO compared with whole-cell or nucleoplasmic FANCI (Fig. 5*B*). In contrast, there was no significant difference between the ratio of monoubiquitinated to deubiquitinated FANCD2 ($FANCD2^{ub}$/FANCD2) in the NO compared with NP (Fig. 5*C*). We conclude that FANCI is preferentially maintained in the deubiquitinated state in the NO under normal growth conditions.

To further test the hypothesis that nucleolar FANCI is preferentially deubiquitinated in the NO, we induced the monoubiquitination of FANCI and FANCD2 and asked whether nucleolar FANCI is still predominantly in the deubiquitinated state. MMC and hydroxyurea (HU) are known to induce the monoubiquitination of FANCI and FANCD2 (14). Subcellular fractionation of HeLa cells treated with HU and MMC demonstrated that nucleolar FANCI is still predominantly in the deubiquitinated state under these DNA-damaging conditions, while nucleolar FANCD2 was not (HU-treated in Fig. 5 *D–F* and MMC-treated in Fig. 5 *G–I*; for controls for subcellular fractionation, see *SI Appendix*, Fig. S5 *A* and *B*). Therefore, under conditions that induce monoubiquitination of FANCI for DNA repair, FANCI, but not FANCD2, persists predominantly in the deubiquitinated state in the NO.

Does FANCI require monoubiquitination to associate with nucleolar proteins? We immunoprecipitated RPA194 in cells expressing HA-tagged FANCI with a K523R missense mutation that prevents its monoubiquitination (14) and observed that FANCI does not require monoubiquitination for association with RNAPI (*SI Appendix*, Fig. S5*C*). Conversely, to test whether monoubiquitination precludes the association of FANCI with nucleolar proteins, we treated cells with HU and MMC to induce monoubiquitination of FANCI, immunoprecipitated PES1, and Western blotted for FANCI. Both monoubiquitinated and deubiquitinated FANCI were coimmunoprecipitated with PES1 (*SI Appendix*, Fig. S5 *D* and *E*), indicating that monoubiquitination of FANCI does not prevent its association with nucleolar proteins. Quantitation of $FANCI^{ub}$/FANCI in the whole cell and the PES1 immunoprecipitate for each treatment revealed that FANCI associated with PES1 is more deubiquitinated than that in whole cells (*SI Appendix*, Fig. S5 *F* and *G*). This is in agreement with our observation that nucleolar FANCI is more highly deubiquitinated than FANCI in the whole cell under DNA-damaging conditions (Fig. 5 *E* and *H*). Together, our results indicate that unlike in the NP where DNA repair occurs and where FANCI is predominantly monoubiquitinated, nucleolar FANCI is predominantly deubiquitinated.

FANCI Distribution Between the NP and NO Changes upon DNA Damage. Since FANCI, but not FANCD2, is enriched in the NO compared with the NP under normal conditions (Fig. 1 *C* and *D*), we asked if DNA damage changes the proportion of FANCI and FANCD2 in the NO and NP. We quantitated the fraction of nucleoplasmic versus nucleolar FANCI or FANCD2 in cells treated with HU or MMC (the same blots used for this quantitation were also used for Fig. 5 *E*, *F*, *H*, and *I*). HU led to no significant difference in the NP versus NO signal for either FANCI or FANCD2 (Fig. 6 *A* and *B*). Thus, inhibiting DNA synthesis lowers the nucleolar enrichment of FANCI, whereas treatment of cells with MMC leads to a significant enrichment of the NP versus NO signal for both FANCI and FANCD2 (Fig. 6 *C* and *D*). This suggests that the formation of interstrand cross-links results in a different localization of FANCI and FANCD2 between the NP and NO. For both HU (*SI Appendix*, Fig. S6 *A* and *B*) and MMC treatment (*SI Appendix*, Fig. S6 *C* and *D*), however, there is no significant difference

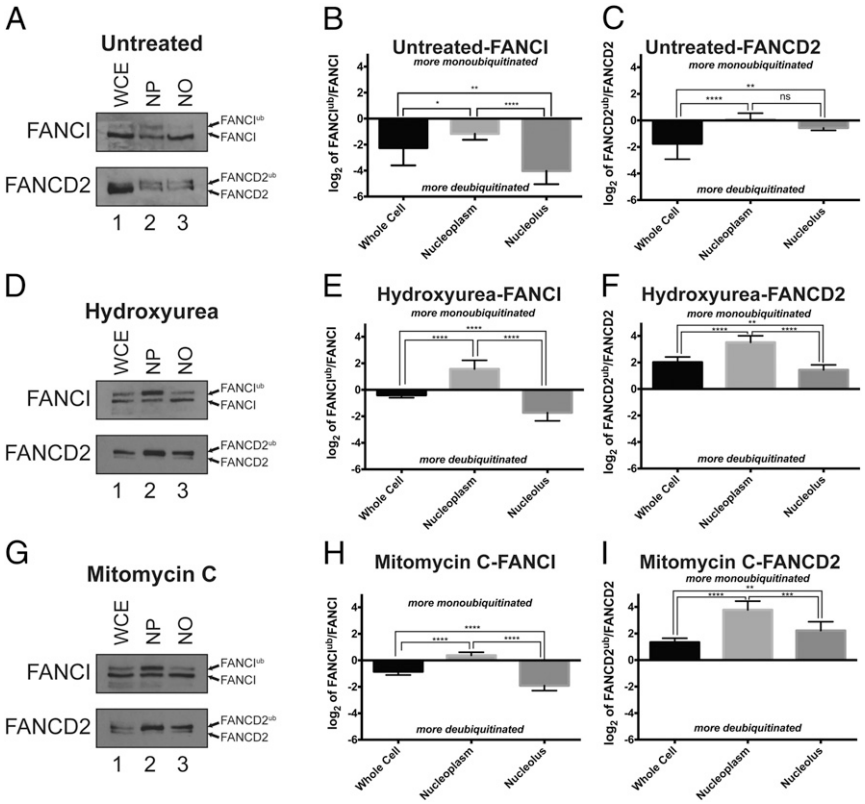

Fig. 5. FANCI is predominantly in the deubiquitinated state in the NO. (*A*–*C*) FANCI, but not FANCD2, is more deubiquitinated in the NO than in the NP. (*A*) Western blots for FANCI and FANCD2 detect both monoubiquitinated and deubiquitinated forms of each protein as indicated by the arrows. Densitometric quantitation of the ratio of monoubiquitinated relative to deubiquitinated FANCI (*B*) and FANCD2 (*C*) in untreated cells. Statistical significance for all quantitations in this figure was calculated using a Kruskal–Wallis test with an uncorrected Dunn's test for multiple comparisons (mean ± SD). ns, not significant, $*P \leq 0.05$, $**P \leq 0.01$, $****P \leq 0.0001$. All quantitations were performed using the same Western blots as in Fig. 1*B*. (*D*–*I*) Nucleolar FANCI persists predominantly in the deubiquitinated state under DNA damaging conditions. HeLa cells were treated with 2 mM HU (*D*) or 1 μM MMC (*G*) for ∼24 h and fractionated into WCE, NP, and NO. For controls, see *SI Appendix*, Fig. S5 *A* and *B*. Western blots for FANCI and FANCD2 detect both monoubiquitinated and deubiquitinated forms of each protein, as indicated by the arrows. (*E* and *F*) Quantitation of *D*. Densitometric quantitation of the ratio of monoubiquitinated relative to deubiquitinated FANCI (*E*) and FANCD2 (*F*) in HU-treated cells. (*H* and *I*) Quantitation of *G*. Densitometric quantitation of the ratio of monoubiquitinated relative to deubiquitinated FANCI (*H*) and FANCD2 (*I*) in MMC-treated cells. $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

between the signal of nucleoplasmic FANCI versus FANCD2 or nucleolar FANCI versus FANCD2. This is consistent with FANCI and FANCD2 functioning in a 1:1 stoichiometry in DNA repair under these conditions. In sum, DNA damage abolishes the nucleolar enrichment of FANCI, likely redistributing it throughout the NP to carry out its role in DNA repair.

USP1 and USP36 Are Required for Maintaining the Deubiquitinated State of FANCI in the NO. Since FANCI is maintained predominantly in the deubiquitinated state in the NO, we asked whether loss of the DUBs USP1 and USP36 leads to an increase in the nucleolar $FANCI^{ub}$/FANCI ratio. USP1 has been shown previously to be required for the deubiquitination of FANCI and FANCD2 (14, 21). USP36 has been shown to interact with FANCI (62). Additionally, USP1 is a nucleoplasmic protein, whereas USP36 is a nucleolar protein (75), and thus each may regulate the ubiquitination of FANCI in their respective compartments.

To confirm the association of FANCI with USP1 and USP36, we made tetracycline-inducible cell lines that express either N-terminally FLAG-tagged USP1 or USP36 and used cells transfected with an empty vector as a negative control. We induced expression of the tetracycline-inducible promoter for 24 h and performed immunoprecipitation with anti-FANCI antibodies and with preimmune sera as a negative immunoprecipitation control. Western blotting for the FLAG epitope revealed that USP1 and USP36 physically associate with FANCI (Fig. 7*A*, lanes 6 and 9). Thus, we confirmed that FANCI associates with both its known DUB (USP1) and a nucleolar DUB (USP36).

To test whether USP1 and USP36 regulate the ubiquitination state of FANCI in the NO, we treated cells with a nontargeting siRNA or with siRNAs against USP1 or USP36. After 48 h, 40 nM MMC was added for ∼24 h to induce monoubiquitination of FANCI. We harvested whole-cell lysates, immunoprecipitated with anti-RPA194, Western blotted for FANCI (Fig. 7*B*), and quantified the ratios of $FANCI^{ub}$/FANCI in WCEs (Fig. 7*C*) versus the RPA194 immunoprecipitate, which represents a subset of nucleolar FANCI (Fig. 7*D*).

For WCE, depletion of USP1 increased $FANCI^{ub}$/FANCI (Fig. 7*C*), as previously reported (14, 19), with no significant difference seen for depletion of USP36 (Fig. 7*C*). In the RPA194 immunoprecipitates, depletion of USP1 increased $FANCI^{ub}$/FANCI (Fig. 7*D*). Thus, USP1 is required for deubiquitinating FANCI that localizes to both the NP and the NO. Interestingly, depletion of USP36 also led to an approximately twofold increase in nucleolar $FANCI^{ub}$/FANCI (Fig. 7*D*). Unlike USP1, USP36 is required for maintaining FANCI in the deubiquitinated form only in the NO. Overall, we have discovered that two DUBs, USP1 and USP36, are required for maintaining FANCI in the deubiquitinated state.

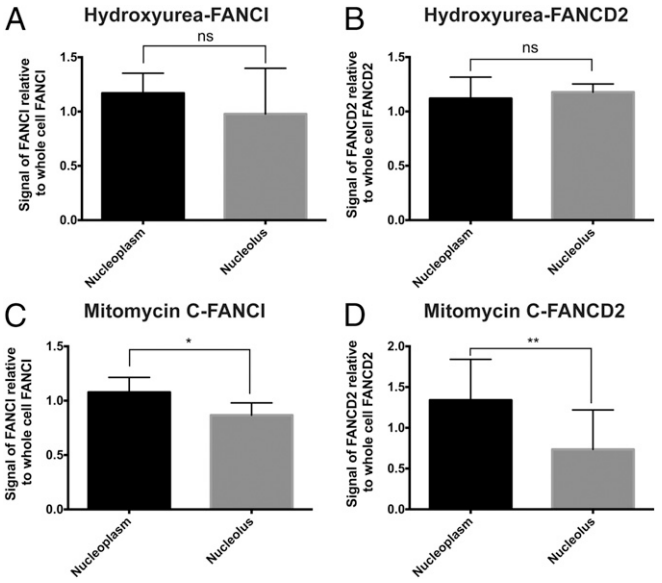

Fig. 6. DNA damage decreases the enrichment of FANCI in the NO. (*A* and *B*) HU treatment results in equal distribution of FANCI between NP and NO. Densitometric quantitation of Western blots from three biological replicates for nucleoplasmic and nucleolar FANCI (*A*) and FANCD2 (*B*) relative to WCE from cells treated with 2 mM HU for 24 h. All quantitations were performed using the same Western blots as in Fig. 5*D*. ns, not significant. (*C* and *D*) MMC treatment results in increased distribution of FANCI and FANCD2 to the NP relative to the NO. Densitometric quantitation of Western blots from three biological replicates for nucleoplasmic and nucleolar FANCI (*C*) and FANCD2 (*D*) relative to WCE from cells treated with 1 μM MMC for 24 h. All quantitations were performed using the same Western blots as in Fig. 5*G*. Statistical significance for all results in this figure was calculated using a two-tailed unpaired Mann–Whitney *U* test (mean ± SD). $*P \leq 0.05$, $**P \leq 0.01$.

Discussion

We demonstrate that the FA protein FANCI is a nucleolar protein that is required for ribosome biogenesis. FANCI copurifies with nucleolar proteins in multiple cell types by nucleolar proteomics (60–62), by affinity purification, and by subcellular fractionation. Furthermore, FANCI coimmunoprecipitates nucleolar proteins required for pre-rRNA transcription and pre-LSU rRNA processing in multiple cell lines. In addition to its physical connection to these nucleolar processes, knockdown of FANCI results in decreased pre-rRNA transcription, defective LSU pre-rRNA processing, and decreased levels of mature LSU rRNA (28S), indicating that FANCI functions directly in ribosome biogenesis. Nucleolar FANCI is predominantly deubiquitinated, unlike FANCI involved in DNA repair (14), and requires the DUBs USP1 and USP36 for maintaining its nucleolar deubiquitinated state. These results support a model in which FANCI has a dual role in DNA repair and in ribosome biogenesis, bringing known observations together with our own into a comprehensive picture. We propose that FANCI functions in cellular metabolism when it is not needed for DNA repair.

Alternative Functions for FA Proteins Are Crucial for Understanding Disease Pathogenesis. FANCI's function in the NO is one of three recent observations that indicate FA proteins have roles in processes other than DNA repair (33, 76, 77). Sumpter et al. (76) discovered a cytoplasmic role for the FANCC protein in selective autophagy of viruses (virophagy) and mitochondria (mitophagy). Recently, BRCA1 [also known as FANCS (32)] was shown to interact with RNAPI and regulate its function (33). Depletion of BRCA1 decreased levels of rRNA synthesis (33), in agreement with the reduction in pre-rRNA transcription we observe after depletion of FANCI. Importantly, Johnston et al. (33) showed a connection between depletion of an FA protein (BRCA1/FANCS) and defects in ribosome biogenesis. These studies imply that there may be multiple contributory pathways to the pathogenesis of bone marrow failure in FA, including defects in ribosome biogenesis (33). Indeed, such a connection between FA and cellular pathways outside DNA repair was first hypothesized over 10 y ago (3). It was recently argued that clinical insight and management of FA would be better served by a "shared axiom" incorporating the DNA repair functions of FA proteins with their other functions (77). Our work and that of Johnston et al.

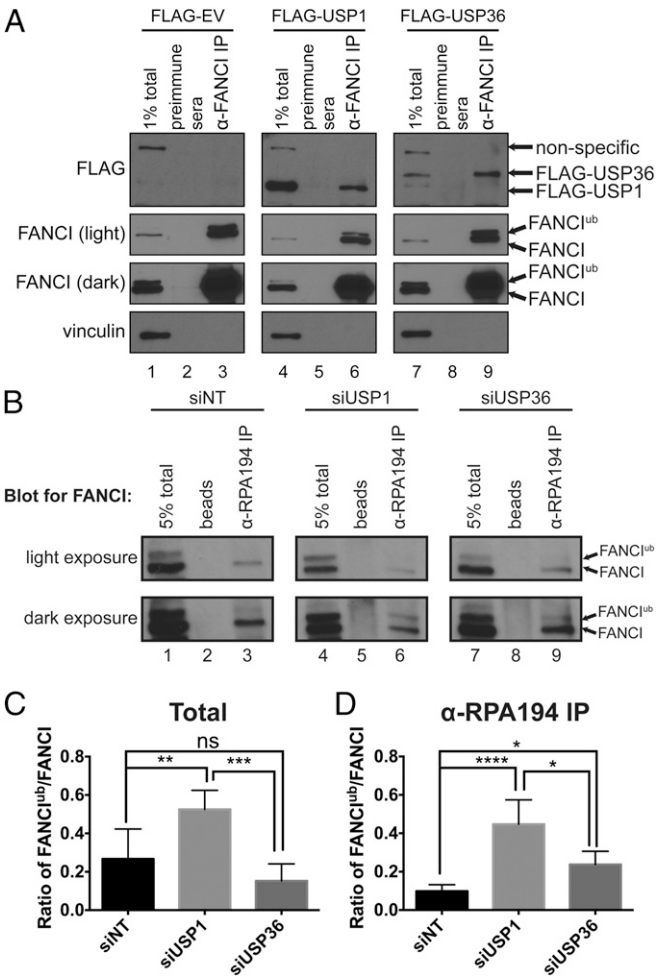

Fig. 7. The DUBs USP1 and USP36 maintain deubiquitinated nucleolar FANCI. (*A*) FANCI physically interacts with USP1 and USP36. Flp-In T-REx HEK293 cells with tetracycline-inducible expression of either FLAG-EV (empty vector), FLAG-USP1, or FLAG-USP36 were induced for ∼24 h with 1 μg/mL doxycycline hyclate. WCEs were prepared and immunoprecipitated (IP) with preimmune sera (negative control) or anti-FANCI antibody. Proteins with the N-terminal FLAG epitope, FANCI, and vinculin were detected by Western blotting. FANCI is a positive control for immunoprecipitation and vinculin is a negative control. (*B*) Deubiquitination of nucleolar FANCI requires USP1 and USP36. HeLa cells were transfected with the indicated siRNAs. After 48 h of depletion, cells were treated with 40 nM MMC overnight. After 24 h, cell extracts were prepared and immunoprecipitated with an anti-RPA194 antibody. Unconjugated protein A beads were used as a negative control. FANCI was detected by Western blotting. (*C* and *D*) Densitometric quantitation of the ratio of monoubiquitinated relative to deubiquitinated FANCI ($FANCI^{ub}$/FANCI) for WCEs (*C*) and anti-RPA194 immunoprecipitate (*D*). Statistical significance for all quantitations in this figure was calculated using a Kruskal–Wallis test with an uncorrected Dunn's test for multiple comparisons (mean ± SD). ns, not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

(33) extends that shared axiom to ribosome biogenesis, connecting FA to bone marrow failure ribosomopathies (24, 74). This raises the intriguing question, Is FA also a ribosomopathy?

FANCI Cannot Be Reliably Expressed for Complementation Experiments. Using genetic depletion and numerous biochemical assays as end points, we have provided strong evidence that FANCI is required for pre-rRNA transcription and LSU pre-rRNA processing, building a solid foundation for future studies on the details of how FANCI functions in ribosome biogenesis. However, we have not yet been able to test how patient mutations in FANCI affect nucleolar function. In our hands, using transient transfection, only a small percentage of cells express exogenous FANCI. Even lentiviral transduction followed by antibiotic selection resulted in very few cells expressing GFP-tagged FANCI. While this level of expression is sufficient for coimmunoprecipitation (*SI Appendix*, Fig. S5*C*), such low expression is not sufficient for the rescue of other biochemical end points. Furthermore, exogenous expression of FANCI extinguishes with time, preventing the construction of stably expressing FANCI cell lines.

Others have reported similar difficulties in expressing FANCI in human cells. Colnaghi et al. (67) reported that only one cell line from an individual with mutated FANCI could be successfully corrected by expression of exogenous FANCI (14). A decrease in expression of lentivirally expressed FANCI was also observed by Castella et al. (78). Other laboratories have found that while other FA proteins can be expressed and functionally complement their respective mutants, FANCI cannot be exogenously expressed in a FANCI-null cell line (79). Remarkably, there are no FANCI-null cell lines or cell lines from individuals with mutated FANCI that are stably complemented with exogenously expressed FANCI in the Oregon Health & Science University FA cell repository (https://apps.ohsu.edu/research/fanconi-anemia/celllines.cfm). These results have negative implications for the potential treatment of FA using lentiviral transduction as a therapeutic avenue (80) for FA patients with FANCI mutations.

DNA Repair Proteins and Nucleolar Function. Do other FA proteins have a role in ribosome biogenesis? While we find that FANCI has a FANCD2-independent role in ribosome biogenesis, depletion of FANCD2 did result in a significant increase in the ratio of the 32S/41S pre-rRNAs (Fig. 3*I*), suggesting that FANCD2 may also contribute to ribosome biogenesis. However, while we do find FANCD2 protein in the NO, unlike FANCI, FANCD2 is not enriched there (Fig. 1 *C* and *D*). Perhaps the role of FANCD2 in the NO is repairing the DNA that is found there, rather than in some specialized function.

In addition to FANCI and BRCA1/FANCS (33), several other DNA repair proteins also have nucleolar connections. APE1/Ref1 associates with the nucleolar protein nucleophosmin to promote quality control of rRNA that contains modified bases (81). Similarly, SMUG1 plays a role in rRNA quality control, as loss of SMUG1 results in increased levels of modified uridines in rRNA (82). The Bloom helicase, like FANCI, is required for rDNA transcription (83). Furthermore, Cockayne syndrome A and B proteins are required for transcribing rDNA (84, 85). Very recently, Lee et al. (86) demonstrated that the SHPRH protein, which polyubiquitinates proliferating cell nuclear antigen, associates with RNAPI and promotes rDNA transcription. The interplay between ribosome biogenesis and DNA repair is only beginning to be elucidated (87).

Possible Mechanisms for FANCI Function in Ribosome Biogenesis. Many hypotheses remain to be tested about the detailed mechanisms by which FANCI functions in the NO. One possible mechanism is through the resolution of R loops that form during RNAPI transcription (88), which has been tested for other FA proteins (88). Alternatively, FANCI may function in ribosome biogenesis by coordinating rDNA replication and transcription, because it has been shown that replication bubbles can sometimes form in actively transcribed rDNA repeats (89). Interestingly, the yeast ortholog of PES1, Yph1, is associated with the origin recognition complex (90), suggesting a possible association between PES1-bound FANCI and rDNA replication. Since FANCI is a DNA-binding protein, it may act as a transcription factor for RNAPI. Additionally, FANCI may act as an RNA chaperone either directly by binding RNA or as part of a complex. Interestingly, the crystallographic structure of FANCI could accommodate its binding to either single-stranded or double-stranded DNA (18). FANCI may bind RNA in a manner similar to single-stranded DNA, providing a mechanism by which FANCI may function as an RNA chaperone. Finally, FANCI may function as a protein complex stabilizer in the NO. These possibilities are not mutually exclusive and represent fruitful avenues for future investigation.

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