INTRODUCTION

As the molecular factory for protein synthesis, the ribosome underlies the cell’s capacity to grow. It is estimated that in a rapidly growing yeast cell, 60% of total transcription is devoted to ribosomal RNA (rRNA), and 50% of RNA polymerase II transcription and 90% of mRNA splicing are devoted to ribosomal proteins (RPs) (1). This extraordinary use of resources makes it critical for the economy of the cell to tightly regulate ribosome biogenesis, the process of making ribosomes. Defects in the molecular components involved in ribosome biogenesis cause a range of pathologies in different organisms. In Drosophila, mutations in as many as 64 ribosomal protein genes are linked to Minute syndrome, a collection of haploinsufficient phenotypes characterized by prolonged development, short and thin bristles, and poor fertility and viability (2, 3). In humans, an assortment of inherited or sporadic disorders known as ribosomopathies are linked to mutations in either ribosomal protein genes or ribosome biogenesis factors (4–6). A number of studies have also established causal associations between inherited mutations affecting ribosome biogenesis and elevated cancer risk (7, 8). It is therefore expected that cells have developed a surveillance system to monitor the status of the translational machinery. For example, ribosomopathies lead to “ribosomal stress” signals that converge on the p53 signaling pathway in affected cells and tissues (9).

The eukaryotic ribosome consists of two subunits formed by the association of 79 ribosomal proteins with 4 distinct rRNAs. The small subunit (40S) comprises the 18S rRNA assembled to 33 ribosomal proteins, whereas the large subunit (60S) contains the 5S, 5.8S, and 28S (25S in yeast) rRNAs associated with 46 ribosomal proteins. Ribosome biogenesis is an ordered process that begins in the nucleolus, where RNA polymerase I (Pol I) transcribes rRNA gene repeats into long precursor rRNAs (pre-rRNAs). Each pre-rRNA contains three rRNA segments in the order of 18S, 5.8S, and 28S that are separated by external transcribed sequences at 5′ and 3′ ends (5′- and 3′-ETS) and separated by two internal transcribed spacers (ITS1 and ITS2). ETSs and ITSs are removed through a series of highly coordinated but poorly understood endonucleolytic cleavage reactions. Ribosomal proteins are deposited onto pre-rRNA during transcription and rRNA maturation to form nascent pre-ribosomes that are then cleaved into the precursors of 40S and 60S subunits in the nucleolus. Pre-60S and pre-40S are then exported through the nucleoplasm into the cytoplasm, where they join the pool of translationally competent 80S ribosomal subunits (10). This assembly process is very complex, involving an estimated 200 ribosome assembly factors in yeast and more than 300 in mammalian cells (11, 12). Most assembly factors were identified by genetic analyses or protein complex purification. Their biochemical function and regulation, and by extension the regulation of ribosome biogenesis, remain poorly understood.

The ubiquitin pathway plays a critical role in the regulation of most cellular processes via an enzymatic cascade, where E1 and E2 enzymes catalyze the activation and conjugation of ubiquitin, while E3s confer reaction specificity through substrate recruitment (13, 14). Comprising the largest family of E3 ligases is the cullin RING E3 ligase (CRL) complexes in which cullin serves as the scaffold to bind small RING finger protein ROC1 (RBX1/HRT1) through a C-terminal domain and a linker-substrate receptor dimer or a substrate receptor directly via an N-terminal domain. Mammalian cells express nine distinct cullins, including two cullin 4 (CUL4) proteins, CUL4A and CUL4B, that use DNA damage–binding protein 1 (DDB1) as the linker. DDB1 bridges the interaction between CUL4 and a subset of DDB1 binding WD40 repeat proteins (DWD or DCAFs for CUL4C and MEFs. We identify the ribosome assembly factor PWP1 as a substrate of the CRL4DCAF1 E3 ubiquitin ligase. DCAF1 loss results in PWP1 accumulation, impairing rRNA processing and ribosome biogenesis. Knockdown or overexpression of PWP1 can rescue defects or cause similar defects as DCAF1 loss, respectively, in ribosome biogenesis. DCAF1 loss increases free RPL11, resulting in L11-MDM2 association and p53 activation. Cumulatively, these results reveal a critical function for DCAF1 in ribosome biogenesis and define a molecular basis of DCAF1 function in cell proliferation and development.
function of the L11-MDM2-p53 checkpoint pathway. Also provide in vivo evidence supporting a physiological relevant cell proliferation and tissue, organ, and animal development. They observed these results help to explain the critical function of DCAF1 in cell proliferation and tissue, organ, and animal development. They also provide in vivo evidence supporting a physiological relevant function of the L11-MDM2-p53 checkpoint pathway.

RESULTS
Conditional brain-specific Dcaf1 knockout in mice results in perinatal death and defects in brain and lens development

We previously generated two Dcaf1/VprBP mutant mouse strains and found that whole-body deletion of Dcaf1 resulted in embryonic lethality (21). Conditional deletion analyses identified a function of Dcaf1 in oocyte survival (22), B cell development and V(DJ) recombination fidelity (23), and expansion of T cells (24). To determine the cellular mechanism of Dcaf1 in embryonic development, we established conditional brain-specific Dcaf1 knockout mice (CKO) by crossing the floxed Dcaf1 mice with Nestin-Cre mice that express Cre recombinase under the control of Nestin promoter in neuronal and glial cell precursors (fig. S1A). Dcaf1 gene deletion and protein depletion in neonatal mouse brains were confirmed by genomic polymerase chain reaction (PCR) and immunoblotting at day 0 (P0) (Fig. 1A). Although the conditional brain-specific Dcaf1 knockout mice (Dcaf1f/f; Nestin-Cre) are born alive, all CKO mice died within 40 hours after birth (Fig. 1B). The proportion of the Dcaf1 CKO mice in total neonates is 12.5%, less than 25% expected Mendelian ratio, suggesting that some embryos might have died from brain-specific deletion of Dcaf1 during embryogenesis. Hematoxylin and Eosin (H&E) staining revealed a thinner ventricular zone, smaller striatum, and hemorrhages from aberrantly grown capillary vessels in the brain cortex (Fig. 1C and fig. S1B). Furthermore, the lens in the eyes of Dcaf1 CKO mice were collapsed (Fig. 1D). These results suggested a crucial function of Dcaf1 in brain development that is essential for perinatal survival.

Deletion of Dcaf1 selectively eliminates proliferating brain and lens cells in vivo

To determine the cellular basis of the perinatal lethality and severe brain development defects caused by conditional Dcaf1 deletion, we examined cell proliferation and death in the brains of mouse embryos. Consistent with the intense Nestin expression pattern, Dcaf1 protein began to decrease at E12.5 and was depleted after E14.5 (fig. S1C) in brain tissues from Dcaf1f/f; Nestin-Cre mice. H&E staining of E13.5 Dcaf1 CKO brain revealed noticeable phenotypes, including enlarged ventricles in the cortex and collapse of ganglionic eminences (fig. S1D). Immunohistochemical (IHC) staining was performed to determine the Dcaf1 expression pattern and the phenotypes in Dcaf1 CKO brain. In the control mouse brain, the area of Dcaf1 expression was very similar to Nestin-positive and Ki67 (a proliferation marker)–positive area (Fig. 1E, upper panel). Dcaf1 depletion in Nestin-positive cells in Dcaf1 CKO mouse brain was confirmed.
(lower panel). Notably, Ki67-positive cells were markedly decreased in Dcaf1<sup>f/f</sup>;Nestin-Cre brain when compared with the control Dcaf1<sup>f/+</sup> brain, indicating that deletion of Dcaf1 results in loss of proliferating cells. IHC using antibodies to cleaved caspase-3 and TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) revealed apoptosis at the edge of collapsed ganglionic eminence and in the middle layer of brain cortex of Dcaf1 CKO brain (Fig. 1E). These results link the loss of Dcaf1 to increased apoptosis of active proliferating cells in the brain.

Nestin is also expressed in the lens epithelium, which are the parental cells responsible for growth and development of the entire ocular lens (25). In the lens of E14.5 mouse eyes, Dcaf1 depletion was detected in the lens epithelium, accompanied by a marked decrease of Ki67-positive cells (Fig. 1F, lower). These defects likely caused the collapse of the lens in Dcaf1 CKO neonates. Collectively, these results show that loss of Dcaf1 inhibits cell proliferation and increases cell apoptosis in the brain and lens, resulting in the damage of brain progenitor cells and leading to brain hemorrhages and atrophy, providing a plausible cellular mechanism for neonatal lethality after conditional Dcaf1 deletion in brain. Apoptotic markers were positive only in the Ki67-positive area, suggesting that deletion of Dcaf1 preferentially eliminated the proliferating, but not non-dividing, cells in the brain and lens. Notably, both brain and lens defects caused by the conditional deletion of Dcaf1 phenocopy those reported in the brain and lens after conditional deletion of Ddb1 (26), including selective elimination of proliferating cells, increased apoptosis, neuronal and lens degeneration, and perinatal lethality. These phenotypical similarities are consistent with the fact that DCAF1 is the major binding partner of DDB1 (21).

**Inducible disruption of Dcaf1 in adult mice results in marked thymic atrophy and bone marrow defect.**

To further explore the in vivo function of Dcaf1, we established inducible Dcaf1 knockout mice by crossing the floxed Dcaf1 mice with Cre-ERT2 mice that express tamoxifen-inducible Cre recombinase under the ubiquitin C promoter (fig. S2A). Dcaf1 protein depletion after tamoxifen administration was observed in multiple tissues (fig. S2B). In the course of analyzing these mice, we noted a marked thymic atrophy in tamoxifen-treated Dcaf1<sup>f/f</sup>;Cre-ERT2 mice (Fig. 2A). Dcaf1 protein depletion after tamoxifen administration was confirmed in thymus (fig. S2C). In addition to reduced thymic size, total thymocyte cell counts were significantly reduced in thymus from tamoxifen-treated Dcaf1<sup>f/f</sup>;Cre-ERT2 mice (1.36 ± 0.67 × 10<sup>7</sup> in knockout versus 15.52 ± 1.37 × 10<sup>7</sup> in control animals, P = 0.0005; Fig. 2B). Histological analysis of thymus from tamoxifen-treated Dcaf1<sup>f/f</sup>;Cre-ERT2 mice revealed a clear lack of typical corticomedullary architecture (Fig. 2C). These results suggested a critical function of Dcaf1 in thymus and T cell development.

Having observed a clear requirement for Dcaf1 in T cell development, we sought to determine whether Dcaf1 also played a role in myeloid cell development by examining the effect of Dcaf1 disruption in bone marrow. The efficiency of Dcaf1 disruption was assessed by quantitative reverse transcription PCR (RT-qPCR) of isolated bone marrow cells (Fig. 2D). The total number of bone marrow cells from femur was reduced (7.76 ± 1.60 × 10<sup>6</sup> in knockout versus 13.94 ± 1.41 × 10<sup>6</sup> in control animals; Fig. 2E), although this difference was not statistically significant. Histological analysis of bone marrow tissue supported the notion that bone marrow cellularity decreased following tamoxifen administration in Dcaf1<sup>f/f</sup>;Cre-ERT2 mice (Fig. 2F), suggesting that Dcaf1 also plays a critical function for bone marrow tissue development.

**Deletion of Dcaf1 selectively eliminates proliferating T and B cells in vivo.**

We determined the cellular basis of severe immune cell development defects caused by Dcaf1 deletion. T cell precursors migrate to the thymus as double negative (CD4<sup>+</sup>CD8<sup>+</sup>, DN) cells, mature into double-positive (CD4<sup>+</sup>CD8<sup>+</sup>, DP) cells, and subsequently commit to become single-positive (CD4<sup>+</sup> or CD8<sup>+</sup>, SP) cells. Active cell division...
occurs during T cell development, especially as DN cells progress during their development toward DP cells. In the Dcaf1 knockout thymus, CD4/CD8 staining notably revealed a near-complete loss of the DP cells, with a corresponding relative increase in the percentage of DN and SP cells (Fig. 2, G and H). While this could suggest a block in the DN to DP transition (with residual SP cells developed before tamoxifen treatment), both the absolute numbers of DN and SP cells were also greatly reduced (Fig. 2I). One explanation is that Dcaf1 deletion results in the loss of proliferating DN T cells, leading to the loss of DP and SP cells. Furthermore, we also noted a marked increase of apoptotic and dead cells in Dcaf1 knockout DP population compared with control cells (Fig. S2D). We conclude that the marked decrease of thymocytes is due to a combination of impaired DN proliferation and increased apoptosis of DP population.

Development of pre- and pro-B cells to immature B cells in bone marrow is associated with active cell proliferation. The B220^IgM^- cells, which represent pre- and pro-B cell populations, were reduced in bone marrow of tamoxifen-treated Dcaf1^[f/+]; Cre-ERT2 mice (8.3% in knockout versus 25.1% in control, Fig. 2J), as were the B220^IgM^ cells, which represent immature B cells (3.8% in knockout versus 6.8% in control). Concomitantly, a relative increase in the percentage of recirculating, mature B cells marked as B220^IgM^- was found in bone marrow of tamoxifen-treated Dcaf1^[f/+]; Cre-ERT2 mice (38.6% in knockout versus 18.1% in control). This suggests that B cells that matured before tamoxifen injection in Dcaf1^[f/+]; Cre-ERT2 mice were largely unaffected by Dcaf1 disruption, but naive B cell development in bone marrow was inhibited. Collectively, we conclude that deletion of Dcaf1 selectively eliminates proliferating T cells and B cells in thymus and bone marrow.

We also examined mature lymphocytes in the spleen. Flow cytometric analysis of B and T cell populations in spleen showed no change in the relative percentage of lymphocytes following Dcaf1 disruption (fig. S2E, F and G). This suggests that developing B and T cells are more severely affected by Dcaf1 disruption than mature lymphocytes, possibly reflecting the G0 state of naive, mature T and B cells.

**Deletion of Dcaf1 selectively eliminates proliferating cells in vitro**

We hypothesized that the developmental defect present in T cells was likely due to a failure of thymocytes to proliferate, rather than a requirement for Dcaf1 in pathways specific to T cell development [e.g., V(D)J recombination, or positive or negative selection]. To test for a requirement of Dcaf1 in cell proliferation independent of its role in T cell development, we stimulated mature T cell proliferation in vitro and subsequently monitored cell divisions. We isolated mature lymphocytes from tamoxifen-treated Dcaf1^[f/+]; Cre-ERT2 and control Dcaf1^[f/+]; Cre-ERT2 mice and confirmed a decrease in Dcaf1 by immunoblotting (Fig. 3A). To follow cell divisions, lymphocytes were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester), a fluorescent dye that forms stable adducts on intracellular proteins and provides a quantitative measurement of cell division by using flow cytometry as it is diluted by half following each cell division. T cell proliferation was activated by anti-CD3 and anti-CD28 costimulation followed by in vitro culture. We collected cells at 42, 54, and 66 hours after stimulation or mock treatment and monitored the proliferation of T cells by flow cytometry. Whereas control cells showed continued, robust proliferation in response to activation as seen by the discrete CFSE peak resulting from replication dilution, we found that very few knockout T cells proliferated in response to activation at 42 and 54 hours and less than half had undergone any proliferation at 66 hours (Fig. 3B). The total cell number following T cell stimulation was decreased in Dcaf1^-disrupted primary lymphocytes compared to control primary lymphocytes (Fig. 3C). Notably, the ratio of costimulated versus mock-treated cell number is less than 1 in Dcaf1^-disrupted lymphocytes at 2 days after stimulation, suggesting that Dcaf1 disruption results in cell death after T cell stimulation (Fig. 3C). Therefore, we conclude that Dcaf1 is required for the proliferation of T cells during costimulation and suggest that loss of proliferative capacity may account for the developmental defects observed in T cells.

To further demonstrate that Dcaf1 functions in proliferating cells, we generated tamoxifen-inducible Dcaf1^[f/+]; Cre-ERT2 and Dcaf1^[f−]; Cre-ERT2 conditional mouse embryonic fibroblast (MEF) lines. We arrested both Dcaf1^[f/+]; Cre-ERT2 and Dcaf1^[f−]; Cre-ERT2 MEFs in G0-G1 quiescence by serum deprivation and then treated with 4OHT (4-Hydroxytamoxifen) to induce Dcaf1 deletion, followed by fluorescence-activated cell sorting (FACS) analysis of cell cycle phase distribution (Fig. 3D). This experiment showed that compared to the marked (10%; Fig. 3D, left panels) sub-G1 population detected in proliferating cells, deletion of Dcaf1 caused very little death of quiescent cells (1.9%; Fig. 3D, right panels). We further confirmed this finding by

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*Fig. 3. Deletion of Dcaf1 selectively eliminates proliferating cells in vitro.*

(A) Immunoblotting of primary lymphocyte lysates derived from Dcaf1^[f/+]; Cre-ERT2 and Dcaf1^[f−]; Cre-ERT2 mice 6 days after tamoxifen injection. (B) Primary lymphocytes were labeled with CFSE and followed by CD3/CD28 stimulation or mock. Cells were cultured in vitro for 42, 54, or 66 hours before collection. Flow cytometric analysis for CFSE was used to determine the proliferation, gated by the 7-AAD-negative T cells. (C) Primary T cells were costimulated with CD3/CD28 or mock-treated and then plated at equal cell numbers. The total cell numbers following activation, expressed relative to the numbers of mock-treated cells, are reported at 2 and 3 days after stimulation. (D) MEFs were cultured in 10 or 0.2% FBS 1 day before 4OHT treatment for 3 days. DNA content was analyzed by PI staining followed by flow cytometry. Red arrow indicates sub-G1 population, representing apoptotic cells. (E) MEFs were cultured in 10 or 0.2% FBS 1 day before 4OHT treatment for 4 days. One group of MEFs was restimulated by 10% FBS after 2-day serum starvation. Cell death and apoptosis analysis was carried out by staining with 7-AAD and annexin V, respectively.
annexin V (apoptotic) and 7-AAD (7-aminoactinomycin D, necrotic) staining and FACS analyses (Fig. 3E). This assay showed that apoptotic cells in control Dcaf1CreERT2 MEFS, as measured by positive staining to annexin V, ranged between 9.6 and 15.1% and was not markedly affected by either 4OHT treatment or whether cells were proliferating [cultured in the presence of 10% fetal bovine serum (FBS)] or in a quiescent state (cultured in 0.2% FBS). The Dcaf1CreERT2 MEFS showed similar apoptosis as the control Dcaf1 WT;CreERT2 MEFS when cultured in 10% FBS, and untreated by 4OHT (13.6%) or treated with 4OHT, but cultured in 0.2% FBS (12.1%). In contrast to the control MEFS, however, 4OHT treatment of Dcaf1CreERT2 MEFS resulted in a marked increase of apoptotic cells (26.8%), indicating that loss of Dcaf1 preferentially eliminates proliferating MEFS. Furthermore, switching of 4OHT-treated Dcaf1CreERT2 MEFS from 0.2% FBS (quiescent) to 10% FBS (proliferating) for 2 days increased annexin V–positive cells to 19.7%, indicating that loss of Dcaf1 potently killed cells once they started proliferating but had little effect on quiescent nondividing cells. Together, these results demonstrate that Dcaf1 plays a critical role in controlling cell cycle and, when it is deleted, selectively eliminates the proliferating cells both in vivo during embryonic brain development and T cell and B cell development and in vitro in anti-CD3/CD28–stimulated T cells and cultured MEFS.

Ribosome assembly factor PWP1 is a substrate of CRL4DCAF1 E3 ubiquitin ligase

Several CRL4DCAF1 substrates have been reported (16), but none of them can explain the essential function of DCAF1 during early development or cell proliferation. To identify additional CRL4DCAF1 substrates, we performed two immunoprecipitations of endogenous DCAF1 complexes and mass spectrometry analyses (IP–mass spec) to search for DCAF1 interacting proteins. One used the anti-DCAF1 antibody to immunopurify DCAF1 complex from HeLa cells. The other was to knock-in three tandem FLAG epitopes (3xFLAG) to the N terminus of the endogenous DCAF1 gene in HeLa cells, followed by immunopurification of 3xFLAG-DCAF1 complex using the anti-FLAG antibody. Successful in-frame knock-in of 3xFLAG tag into endogenous DCAF1 was verified by DNA sequencing (fig. S3A), immunoblotting (Fig. 4A), and immunofluorescence (IF) staining (fig. S3, B and C). Both immunopurifications were stained with SYPRO Ruby (Fig. 4B) and subjected to mass spectrometry analyses (Fig. 4C).

These two IP–mass spec analyses identified combined 626 putative DCAF1-interacting proteins with at least two unique peptides for each individual protein (table S1). The statistically significant label-free quantification (LFQ) ratios that are proportional to the relative DCAF1 binding of individual proteins were averaged over three technical replicates. A stringent analysis was performed to identify the high-confidence DCAF1-interacting proteins with greater than fourfold abundance change compared to control immune complexes. This led to the identification of 43 and 32 proteins in DCAF1 or FLAG-DCAF1 immune complexes, respectively, including 17 proteins that were identified in both immune complexes (Fig. 4, C and D, and table S1). Of these 17 proteins, DCAF1 and 15 known components of CRL4 E3 complex, including both CUL4A and CUL4B, DDB1, DDA1, ROC1, NEDD8, and 9 subunits of COP9 signalosome complex (Fig. 4E), were identified. The only previously unknown protein identified in both DCAF1 and FLAG-DCAF1 immune complexes was the ribosome assembly factor Pwp1.

We confirmed the binding between DCAF1 and Pwp1 by the coimmunoprecipitation assays and demonstrated binding between ectopically (Fig. 4F) and endogenously (Fig. 4G) expressed DCAF1 and Pwp1 proteins. Furthermore, we found that deletion of Dcaf1 in MEFS prevented Pwp1 from binding with CUL4a (Fig. 4H), demonstrating that Dcaf1 bridges Pwp1 to CUL4a. To determine whether Pwp1 is a substrate of the CRL4DCAF1 E3 ligase, we performed
knockout and knockdown of DCAF1 and individual components in CRL4<sup>DCAF1</sup> E3 complex in MEF and HeLa cells. Deletion of Dcaf1 resulted in marked accumulation of Pwp1 in MEFs in a time-dependent manner (Fig. 4I). Moreover, depletion of DCAF1, DDB1, and ROC1 individually and in combination with both CUL4A and CUL4B all resulted in accumulation of PWP1 in HeLa cells (Fig. 4J). To demonstrate the ubiquitylation of PWP1 by the CRL4<sup>DCAF1</sup> E3 ligase, we performed both in vivo and in vitro ubiquitylation assays. We found that endogenously expressed PWP1 is actively ubiquitylated in HeLa cells and that knockdown of either DDB1 or DCAF1 individually or in combination with CUL4A and CUL4B resulted in substantial reduction of PWP1 ubiquitylation (Fig. 4K). An in vitro ubiquitylation assay demonstrated that the CRL4<sup>DCAF1</sup> E3 immuno-complexes caused robust ubiquitylation of PWP1, which is dependent on the addition of E1, E2, and E3 complex and substrate PWP1 (Fig. 4L). Together, these results demonstrate that the ribosome assembly factor PWP1 is a substrate of the CRL4<sup>DCAF1</sup> E3 ligase.

Loss of function of DCAF1 results in defects in ribosome biogenesis

PWP1 is a highly conserved protein in eukaryotes and is widely expressed across different tissues in mice and humans (27, 28). It was initially identified in budding yeast as a protein that contains multipletryptophan (W) residues defining a WD40 repeat and, when deleted, results in severe growth retardation and marked reduction of protein synthesis (29). In yeast, Pwp1 is a component of a subcomplex that associates with the ITS2 of the rRNA gene, and deletion of Pwp1 results in accumulation of 35S rRNA precursor and reduction in other rRNA precursors, as well as mature 25S and 18S rRNA (27, 30, 31). In Drosophila, Pwp1 (dPwp1) mutants result in substantial reduction in 5.8S, 18S, and 28S rRNA and develop the Minute phenotype linked to mutations in many individual ribosomal protein genes (2, 32). These studies suggest a conserved function of PWP1 for a critical step during ribosome biogenesis such as rRNA processing.

A mosaic analysis in Drosophila wing discs revealed that Dcafl/Mahljog<sup>−/−</sup> cells developed defects similar to the Minute mutants (20). Furthermore, a recent genome-wide RNA interference (RNAi) screen in human HeLa cells identified several components of the CRL4 E3 ligase, including CUL4A, CUL4B, DDB1, ROC1, and CSN subunits, that are required for the early steps of nucleolar biogenesis (33). The substrate receptor for the CRL4 E3 ligase involved in the ribosome biogenesis was not identified. These findings prompted us to determine whether the CRL4<sup>DCAF1</sup> E3 ligase, by ubiquitylating PWP1, is necessary for rRNA processing and ribosome biogenesis. We adapted procedures developed by Badertscher et al. (33) that used dynamic translocation of BYSTIN/ENP1, a 40S trans-acting factor, to monitor biogenesis in HeLa cells. Under steady-state conditions, BYSTIN predominantly localizes to nucleoli and shuttles into the cytoplasm along with newly synthesized 40S subunits. Blocking 40S nuclear export with leptomycin B (LMB) caused BYSTIN to accumulate in the nucleoplasm in unperturbed cells, but trapped in the nucleoli if an early step of ribosome biogenesis was blocked (Fig. 5, A to C). We found that either overexpression of PWP1 or knockdown of DCAF1, like knockdown of either DDB1, in combination with CUL4A and CUL4B or ribosomal assembly factor rRNA 2′-O-methyltransferase fibrillarin (FBL), resulted in a failure of BYSTIN translocating out the nucleoli, indicative of an early step defect in nucleolar ribosome biogenesis before the transport of ribosomal precursor subunits into the nucleoplasm (Fig. 5, A and B; overexpression and knockdown efficiency were shown in fig. S4, A and B). To demonstrate that depletion of DCAF1 impairs ribosome biogenesis by accumulating PWP1, we performed a double knockdown of DCAF1 and PWP1 in HeLa cells. We found that BYSTIN was mostly accumulated in the nucleoplasm of double knockdown cells after LMB treatment, with some portion remaining in the nucleoli, similarly as control cells (Fig. 5C; knockdown efficiency was shown in fig. S4C). This result indicates that depletion of DCAF1-induced nucleolar ribosome biogenesis defect is largely rescued by knockdown of PWP1.

**Fig. 5. Loss of function of DCAF1 results in defects in ribosome biogenesis.**

(A) HeLa cells stably expressing PWP1 or empty vector were treated with 20 nM LMB or solvent for 2 hours. The subcellular distribution of BYSTIN/ENP1 was determined by IF. (B and C) HeLa cells were transfected with siRNAs targeting indicated genes individually or in combination and then treated with 20 nM LMB or solvent for 2 hours. (D) Diagram illustrating the major steps of mammalian rRNA processing. Blue arrows indicate the major cleavage sites in human cells. (E and F) HeLa cells were transfected with siRNAs targeting DCAF1 or control siRNA and then followed by pulse-chase analysis of the [3H]uridine-labeled rRNAs. The newly synthesized and total rRNAs were determined by autoradiography and methylene blue staining, respectively. (G) The relative intensities of newly synthesized 41S, 28S, and 18S rRNAs compared to 45S precursors at 0 hours in (E) were quantified. The intensities of newly synthesized 5.8S rRNAs in (F) were quantified and normalized against 55S rRNAs. Error bars represent ±SD for duplicate experiments. (H) Dcaf1<sup>−/−</sup>;Cre-ERT2 MEFs were treated with 200 nM 4OHt or solvent for 3 days, followed by pulse-chase analysis of the 1-[methyl-<sup>3</sup>H]methionine–labeled rRNAs.
To provide additional evidence supporting the function of DCAF1 during ribosome biogenesis, we performed pulse-labeling analysis using [3H]uridine in HeLa cells after knocking down DCAF1. This experiment showed that depletion of DCAF1 resulted in substantial accumulation of 41S rRNA precursor with a concomitant decrease of newly synthesized 28S, 18S, and 5.8S rRNA (Fig. 5, D to G; knockdown efficiency was shown in fig. S4D). Analyzing low-molecular weight RNA by 7 M urea polyacrylamide gel electrophoresis (PAGE) confirmed the decrease of newly synthesized 5.8S rRNA after DCAF1 knockdown (Fig. 5, F and G). To further confirm the role of DCAF1 in rRNA processing, we treated Dcaf1+/--;Cre-ERT2 MEF cells with 4OHT for 3 days to induce Dcaf1 deletion and examined rRNA synthesis and processing by pulse-chase analysis. Because the uridine salvage pathway is not very active in MEF cells, we replaced [3H]uridine with [1-3H]-methyl methionine, which can be more rapidly incorporated because of the rapid turnover of the cellular methionine pool. 47S transcript (pre-rRNA) is extensively modified by methylation once it is transcribed. [1-3H]-methyl methionine is converted readily to S-adenosylmethionine, which donates the 3H-methyl in the methylation of rRNA precursors (34). Depletion of Dcaf1 did not substantially affect the total 28S and 18S rRNA, as measured by methylene blue staining, but decreased both newly synthesized 28S and 18S rRNA (Fig. 5H; knockout efficiency was shown in fig. S4E). Together, these results demonstrate that the CRL4DCAF1 E3 ligase is important for rRNA processing and ribosome biogenesis, and this function of CRL4DCAF1 is achieved, at least in part, by regulating PWP1.

Loss of Dcaf1 activates RP-Mdm2-p53 checkpoint pathway

We previously reported that impairment of ribosome biogenesis caused by the inhibition of RNA Pol I–dependent ribosomal DNA (rDNA) transcription via a low dose (5 nM) of actinomycin D accumulates free, unassembled ribosomal protein L11 that binds to MDM2 and inhibits MDM2-mediated p53 ubiquitylation, leading to an increase of p53 (35). To determine whether impairment of rRNA processing and ribosome biogenesis by loss of DCAF1, like the inhibition of Pol I–dependent rDNA transcription by actinomycin D, also activates the p53 pathway, we examined the histology of thymus sections from moderately affected Dcaf1Δp50;Cre-ERT2 or Dcaf1Δp50;Cre-ERT2 animals 6 days after tamoxifen administration, at which time there was robust deletion of the Dcaf1. Incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) was almost undetectable in thymus from knockout mice, indicating a strong inhibition of DNA replication (Fig. 6A). Concurrently, cleaved caspase-3 and p53 were more abundantly detected in knockout mice, suggesting a strong increase of apoptosis induced by p53 accumulation. Consistent with these in vivo observations, deletion of Dcaf1 in MEF cells resulted in substantial decrease of Ki67 signals and accumulation of p53 and PUMA and BAX, two proapoptotic p53 target genes (Fig. 6B).

To explore the mechanism of p53 activation in the Dcaf1 loss, we separated unassembled ribosomes from ribosome-free proteins by ultracentrifugation and analyzed for free unassembled ribosomal proteins in the S100 fraction of Dcaf1Δp50;Cre-ERT2 or Dcaf1Δp50;Cre-ERT2 MEFs. Immunoblot analysis revealed that depletion of Dcaf1 results in the accumulation of free ribosomal protein RPL11, while the total amount of RPL11 protein was largely not affected (Fig. 6C). Knockdown of DCAF1 in U2OS cells increased the MDM2-L11 association, the levels of p53 and CDK inhibitor p21, a p53 transcriptional target (Fig. 6D). To provide genetic evidence linking the loss Dcaf1 to increased MDM2-L11 association and p53 activation, we knocked down Dcaf1 in wild-type or Mdm2C305F/C305F primary MEF cells. The C305F mutation was first identified in human osteosarcoma (36) and disrupts MDM2’s binding with L11 (37, 38). We found that compared with the wild-type MEFs, knockdown of Dcaf1 in Mdm2C305F/C305F MEFs similarly resulted in an increase in Pwp1 protein but failed to accumulate p53 and p21 (Fig. 6E), consistent with L11 not being able to bind Mdm2. Together, these results provide a molecular mechanism—increased free L11 and L11-MDM2 binding leading to activation of p53—for the G1 cell cycle arrest and apoptosis in cells with impaired ribosome biogenesis after loss of DCAF1 (Fig. 6F).

Fig. 6. Loss of Dcaf1 impairs ribosome biogenesis and activates RP-Mdm2-p53 checkpoint pathway. (A) IHC staining of BrdU, cleaved caspase-3, and p53 from sections of formalin-fixed, paraffin-embedded thymus tissue following tamoxifen treatment. Positive staining is indicated by brown color; sections were counterstained with hematoxylin. (B) Dcaf1Δp50;Cre-ERT2 and Dcaf1Δp50;Cre-ERT2 MEFs were treated with 200 nM 4OHT for 3 days. (C) 4OHT-treated Dcaf1Δp50;Cre-ERT2 and Dcaf1Δp50;Cre-ERT2 MEFs were homogenized in hypotonic buffer (designated as total cell lysates). Ribosomes were pelleted from lysates by ultracentrifugation at 100,000g for 3 hours at 4°C, and the ribosome-free supernatants were collected (designated as the S100 fractions). (D) U2OS cells stably expressing FLAG-tagged MDM2 or empty vector were transfected with siRNAs targeting DCAF1 or control siRNAs. The interactions between MDM2 and RPL11 proteins were determined by co-IP analysis. (E) Activation of p53 by loss of DCAF1 depends on intact RP-MDM2-p53 pathway. Primary Mdm2+/- or Mdm2C305F/C305F MEFs were transfected with siRNAs targeting Dcaf1 or control siRNAs. (F) Schematic model for the function of DCAF1-based E3 ubiquitin ligase in the regulation of PWP1 protein, ribosome biogenesis, cell proliferation, and development.
DISCUSSION

Much of our knowledge on the control of ribosome biogenesis were gained from extensive studies in yeast. In contrast, very little is known about this process in higher eukaryotes that are much more complex because of the elaborate signaling networks that control ribosome biogenesis and different nutrient responses. Previous studies using inhibitors of the proteasome and neddylation have implicated the ubiquitin-proteasome system and cullin E3 ligases in ribosome biogenesis in human cells (39, 40). Recent genome-wide screening directly linked a CUL4-DDB1-based E3 ligase in the regulation of ribosome biogenesis (33). Our study supports these previous findings and identifies the first ribosome assembly factor that is regulated by degradation by a CRL E3 ligase in mammalian cells (Fig. 4). DCAF1 binds directly with PWP1, is required for PWP1 ubiquitylation both in vivo and in vitro, and, when deleted or depleted, results in accumulation of PWP1 (Figs. 4 and 6). Functionally, overexpression of PWP1 causes a similar defect in nucleolar ribosome biogenesis as DCAF1 loss (Fig. 5, A and B). Simultaneous knockdown of PWP1 and DCAF1 prevented the defects of nucleolar ribosome biogenesis caused by DCAF1 loss (Fig. 5C). Together, these results demonstrate that PWP1 is a direct and major substrate of the CRL4DCAF1 E3 ligase that can regulate ribosome biogenesis (Fig. 6F).

In yeast, PWP1 is a component of a subcomplex that associates with ITS2 and plays an important role in pre-rRNA processing and creating stable pre-ribosomes (27, 30, 31). In Drosophila, Pwp1 (dPwp1) mutants develop a Minute phenotype that was originally identified as similar to haploinsufficient defects in ribosomal proteins in development and later linked to mutations in many individual ribosomal protein genes (2, 32). These studies suggest a conserved function of PWP1 in rRNA processing and early steps of ribosome biogenesis. The biochemical mechanism of PWP1 in rRNA processing in human cells is currently unknown. Loss of function of DCAF1, like knockdown of DDB1, ROC1, or combination of CUL4A and CUL4B, blocked nucleolar ribosome biogenesis (Fig. 5). DCAF1 loss resulted in PWP1 accumulation and defects in the 41S cleavage, suggesting that PWP1 may function in 41S cleavage in the nucleolus. This notion is supported by the finding that overexpression of PWP1 similarly blocked nucleolar ribosome biogenesis (Fig. 5A). These findings indicate that the level of PWP1 protein needs to be properly regulated and, when deleted or abnormally accumulated, impairs ribosome biogenesis, implying a checkpoint-like function for PWP1. The function of PWP1 has recently been extended beyond rRNA processing to Pol I-mediated rRNA transcription (32) and telomere maintenance (41). Whether these two processes are also regulated by DCAF1 and collectively contribute to defects in cell proliferation and development, and p53 activation is an interesting possibility that will require further study.

DCAF1 is an evolutionarily conserved gene. Deletion of Dcaf1 resulted in an early development block in plant, fly, and mouse and inhibition of cell proliferation (19–21). Mutation of Drosophila Dcaf1/Mahjong gene results in a “loser cell” phenotype that is shared, notably, with Drosophila cells harboring mutations in ribosomal protein genes collectively known as Minute phenotype (2, 20). A number of potential substrates of the CRL4DCAF1 ligase have been reported (16), but none of these reported substrates can explain an evolutionarily conserved and essential function of DCAF1 in development or ribosome biogenesis. The studies presented here provide a cellular and molecular mechanism explaining the essential function of DCAF1 in cell proliferation and development. We found that Dcaf1 deficiency in brain and lens caused increased apoptosis, neuronal and lens degeneration, brain hemorrhages, and neonatal death (Fig. 1). We also found that systemic inducible deletion of Dcaf1 in adult mice resulted in marked thymic atrophy and bone marrow 9

MATERIALS AND METHODS

Plasmids and reagents

Expression constructs for DCAF1 were previously described (44). Full-length human PWP1 was inserted into pcDNA3-3myc or p3FLAG-CMV vectors for transient expression, and PWP1, MDM2, and UBC were constructed into lentiviral vectors for stable transduction. Detailed information for antibodies, chemicals, oligonucleotides, and other key resources used in this study are reported in table S2.

Cell culture and cell transfection

HeLa and U2OS cells were cultured at 37°C with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 medium, respectively, supplemented with 10% FBS (Gibco). 293F cells were adapted to suspension culture in FreeStyle 293 Expression Medium (Thermo Fisher Scientific). Cells were authenticated using short tandem repeat assays. Mycoplasma test results were negative. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) for plasmid DNAs and small interfering RNAs (siRNAs) following the manufacturer’s instructions. Stable cell lines were established by lentivirus transduction, selected, and maintained in medium containing puromycin (1 μg/ml; Amresco).

MEF cell lines were established from the tamoxifen-inducible conditional Dcaf1 knockout mouse embryos. E13.5 embryos were isolated by removing head, thoracic, and abdominal organs. The body tissue was minced by aspiration-ejection cycle three times using an 18-gauge needle with syringe in DMEM supplemented with 15% FBS and 1% penicillin-streptomycin (Corning), followed by incubation overnight at 37°C with 5% CO2. Mdm2(2C305F/2C305F) primary MEF was a gift from Y. Zhang (University of North Carolina at Chapel Hill).

Primary lymphocytes from mouse lymph nodes and spleen were obtained from 6-week-old tamoxifen-treated mice 6 days following...
the first injection. After lysis of red blood cells, cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin, 2 mM l-glutamine, 50 μM β-mercaptoethanol, and 100 nM 4OH T. Cells were cultured for 8 hours before CFSE labeling and T cell activation.

**RNAi and CRISPR-Cas9**

All siRNA oligonucleotides were synthesized with 3’ dTdT overhangs by Sigma-Aldrich in a purified and annealed duplex form. The sense sequences targeting human DCAF1, CUL4A, CUL4B, DDB1, ROC1, or FBL are reported in table S2 (33, 45). RNAi-mediated down-regulation was performed by transfecting siRNAs in accordance with the manufacturer’s instructions. A nontargeting control siRNA duplex (sense 5’-UUCUCCGAACGUGUCACGU-3’) was included as a negative control. The knockdown efficiency was assessed 48 to 72 hours after transfection by immunoblotting.

The procedures for CRISPR-Cas9-mediated knock-in followed the protocols previously published (46). The single guide RNA (sgRNA) targeting the start codon of human DCAF1 (exon 3) was designed by using the online CRISPR Design Tool (http://crispr.mit.edu/) and constructed into CRISPR-Cas9 vector pSpCas9(BB)-2A-GFP (PX458) (Addgene #48138). The single-stranded oligodeoxynucleotide (ssODN) template containing 3xFLAG sequence was synthesized by Integrated DNA Technologies. The sequence of sgRNA and ssODN was provided to report statistically significant protein abundance fold changes. 1 hour at 4°C. Immunoprecipitates were washed three times with lysis buffer, and proteins were eluted from beads with 1× Laemmli loading buffer. For immunoblotting, cell lysates were mixed with Laemmli loading buffer to 1× and heated at 99°C for 5 min. Proteins were resolved on SDS-PAGE and visualized by chemiluminescence. Images were taken by a ChemiDoc MP Imaging system (Bio-Rad). Protein bands were quantitated using Bio-Rad Image Lab 6.0.

**Mass spectrometry analysis**

The DCAF1 immune complexes were derived using either anti-DCAF1 or anti-FLAG antibodies as described in the text and validated by SYPRO Ruby staining (Thermo Fisher Scientific) and immunoblotting. The immune complexes were then subjected to in-solution tryptic digestion using a filter-aided sample preparation approach (47). Briefly, the immune complexes were eluted by incubating with the molar excess competing antigen peptides and transferred to Vivacon 500 ultrafiltration devices (10,000 MWCO, Hydrosart membrane, Sartorius). Protein samples were washed twice with a urea buffer (8 M urea, 0.1 M tris- HCl, pH 8.0) supplemented with 100 mM dithiothreitol (DTT) followed by centrifugation at 10,000g for 10 min at room temperature. After denaturation and reduction, proteins were then alkylated in urea buffer containing 50 mM iodoacetamide at room temperature for 30 min, shielded from light. Samples were washed twice with 50 mM ABC buffer (100 mM ammonium bicarbonate in ddH2O). Two hundred microliters of 30 mM ABC containing trypsin (5 μg/ml) was added to the samples and incubated at 37°C overnight. The digested peptides were collected by centrifugation with ABC buffer and desalted over homemade C18 tips. The clean peptide mixtures were separated on nano-C18 reversed-phase columns connected to an EASY-nLC 1000 system (Thermo Fisher Scientific) and analyzed with a Q Exactive mass spectrometer. Three technical liquid chromatography–mass spectrometry replicates were obtained for each sample.

Mass spectra were processed, and peptide identification was performed using the Andromeda search engine found in MaxQuant software version 1.5.7.4 (Max Planck Institute, Germany). All protein database searches were performed against the UniProt human protein sequence database (UP000005640). Peptide inference was made with a false discovery rate (FDR) of 1%, and peptides were assigned to proteins with a protein FDR of 5%. Peptide identifications are reported by filtering of reverse and contaminant entries and assigning to their leading razor protein. LFQ was performed on the basis of peak area. The measured area under the curve of mass/charge ratio and the retention time-aligned extracted ion chromatogram of a peptide were performed via the LFQ module. Data processing and statistical analysis were performed on Perseus (version 1.6.0.7). Protein quantitation was performed on three technical replicates, and two-sample t test was used with a P value of 5% to report statistically significant protein abundance fold changes.

**Histology**

Mouse tissues were fixed in PBS-buffered 4% paraformaldehyde (PFA) (VWR) overnight at 4°C, embedded in paraffin, cut into 4-μm sections, and attached on Superfrost Plus Micro Slides (VWR). The slides were deparaffinized with SafeClear II Xylene Substitutes (Thermo Fisher Scientific) and hydrophilized following standard protocol and stained with H&E dye. For IHC, the hydrophilized slides were boiled in 10 mM citrate buffer (pH 6.0) for 30 min for antigen retrieval and washed thoroughly with PBS. Sections were

**Immunoblotting and immunoprecipitation**

To prepare total cell lysates, mouse tissues were minced in cold radioimmunoprecipitation assay buffer supplemented with protease inhibitors, phosphatase inhibitors, micrococcal nuclease (2 U/ml), and 1 mM CaCl2. Culture cells were washed with cold phosphate-buffered saline (PBS) once and lysed in 1× Laemmli loading buffer for immunoblotting or 0.5% NP-40 lysis buffer supplemented with protease inhibitors and phosphatase inhibitors for immunoprecipitation. For immunoprecipitation, cell lysates were incubated with specific antibody for 3 hours at 4°C, followed by addition of protein A–agarose for
incubated with specific antibodies overnight at 4°C. After PBS wash, the primary antibodies were detected by diaminobenzidine (DAB) with EnVision+ kit [Dual Link System–HRP (DAB+) kit, DAKO] according to the manufacturer’s instruction, followed by hematoxylin counterstaining. Both H&E and IHC slides were dehydrated and mounted with Permount Mounting Medium (Thermo Fisher Scientific). **Flow cytometry**

All assays were performed on the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific), and data were analyzed using FlowJo v10.0.7 software. To analyze DNA content by propidium iodide (PI) staining, cells were fixed in 70% cold ethanol overnight at 4°C, washed two times with PBS containing 1% bovine serum albumin (BSA), and then incubated for 30 min at 37°C with ribonuclease A (RNase A) (0.1 mg/ml) in PBS. Cells were then stained with PI (50 μg/ml) in PBS for 20 min at room temperature, shielded from light. For cell death and apoptosis analysis, cells were harvested by trypsinization, and staining was carried out using the annexin V Apoptosis Detection Kit (BD Biosciences). Briefly, cells were resuspended in 1× binding buffer and incubated with fluorochrome-conjugated annexin V and 7-AAD for 15 min in darkness at room temperature.

For thymocyte, splenocyte, and bone marrow cells, single-cell suspensions were prepared in PBS containing 2% FBS (FACS buffer) after lysis of red blood cells in four parts 0.8% NH₄Cl, 0.1 mM suspensions were prepared in PBS containing 2% FBS (FACS buffer) and incubated with fluorochrome-conjugated annexin V and 7-AAD for 15 min in darkness at room temperature.

**Pulse-chase analysis of rRNA synthesis**

For monitoring rRNA processing, pulse-chase experiments using [5,6-³H]uridine and 1-[methyl-³H]methionine were carried out in HeLa and MEF cells, respectively. HeLa cells were transfected with siRNA targeting DCAF1 for 48 hours, pulsed with [5,6-³H]uridine (10 μCi/ml) for 30 min, and then chased with unlabeled uridine (0.5 mM)–containing medium for the indicated time points. For the pulse-chase analysis in MEFs, 4OHT or solvent-treated cells were cultured in methionine-cysteine–free medium supplemented with dialyzed serum for 30 min and labeled with 1-[methyl-³H]methionine (50 μCi/ml) for additional 30 min. Cold methionine (100 μM) was added to chase the label for various lengths of times. Total RNA was extracted by TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. To assay rRNA precursors and mature 18S and 28S rRNA, 5 μg of ³H-labeled RNA was separated on a 1% formaldehyde agarose gel at 35 V for 24 hours in 1× Mops buffer.

**Ribosome purification and isolation of ribosome-free ribosomal proteins**

 procedures for the preparation of ribosome-free ribosomal protein were modified as previously described (50). Briefly, MEF cells of different genotypes (Dcaf1⁺/⁻;Cre-ERT2 and Dcaf1⁺/⁻;Cre-ERT2) were grown on 10-cm plate and then treated with 200 nM 4OHT for 48 hours to induce Dcaf1 knockout. Cells were harvested using trypsin, rinsed with PBS, and then resuspended in hypotonic buffer [5 mM tris-HCl (pH 7.5), 2.5 mM MgCl₂, and 1.5 mM KCl] supplemented
with protease inhibitors. Cells were lysed by homogenization using a Dounce homogenizer. Ribosomes and polysomes were pelleted by ultracentrifugation at 100,000g for 3 hours at 4°C. Ribosome-free supernatants were carefully collected (100 sample), proteins were precipitated by trichloroacetic acid (15%), and pellets were dissolved in SDS sample buffer. Free ribosomal protein L11 and other proteins were analyzed by immunoblotting.

Statistical analysis
Statistical analyses were performed with a paired, two-tailed Student’s t test. The values of P < 0.05 were considered statistically significant. Data presented were means ± SD, unless otherwise indicated.

SUPPLEMENTARY MATERIALS
Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/1/eabd6078/DC1

View/request a protocol for this paper from Bio-protocol.

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Acknowledgments: We thank members of the Xiong and Marzluff laboratories for discussions and support throughout this study, in particular X. Yang, R. Meganck, and C. Holmquist for aid with pulse-labeling analysis and Northern blotting. Funding: This study was supported by NIH grants R01 CA212407 to Y.Z., GM29832 to W.F.M., GM067113 to Y.X., GM133107 to X.C., and UNC UCRF to X.C. Author contributions: Y.X. conceived the project and discussed with W.F.M. X.-R.H., N.S., S.C.J., P.W., and Z.L. performed the experiments and analyzed the data. M.D.S. assisted with VprBP/Dcaf1 mouse genetic analyses, L.X. and X.C. carried out the mass spectrometry analysis, and Y.Z. planned the experiment involving Mdm2C305F/C305F MEFs with Y.X., X.-R.H., N.S., S.C.J. W.F.M. wrote the manuscript, and all authors reviewed and/or edited the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 1 July 2020
Accepted 27 October 2020
Published 18 December 2020
10.1126/sciadv.abd6078

Citation: X.-R. Han, N. Sasaki, S. C. Jackson, P. Wang, Z. Li, M. D. Smith, L. Xie, X. Chen, Y. Zhang, W. F. Marzluff, Y. Xiong, CRL4[DCAF1/VprBP] E3 ubiquitin ligase controls ribosome biogenesis, cell proliferation, and development. Sci. Adv. 6, eabd6078 (2020).