DDB1 is essential for genomic stability in developing epidermis

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The mammalian epidermis is maintained by proliferation and differentiation of epidermal progenitor cells in a stereotyped developmental program. Here we report that tissue-specific deletion of the UV-damaged DNA-binding protein 1 (DDB1) in mouse epidermis led to dramatic accumulation of c-Jun and p21Cip1, arrest of cell cycle at G_2/M, selective apoptosis of proliferating cells, and as a result, a nearly complete loss of the epidermis and hair follicles. Deletion of the p53 tumor suppressor gene partially rescued the epithelial progenitor cells from death and allowed for the accumulation of aneuploid cells in the epidermis. Our results suggest that DDB1 plays an important role in development by controlling levels of cell cycle regulators and thereby maintaining genomic stability.

apoptosis | cell cycle | p53-dependent | ubiquitin ligase

Mammalian skin is formed by the continuous proliferation and differentiation of the basal layer of epidermal progenitor or transient amplifying cells, which are progeny of the self-renewing epidermal stem cells (1). The progenitor and stem cells have to cope with environmental damage such as UV exposure while executing normal developmental programs controlled by intracellular signals. Defects in the machinery for DNA repair and maintenance of genomic stability can cause abnormal proliferation of the progenitor cells and eventually lead to skin cancer (2, 3).

The damaged DNA-binding protein complex (DDB), consisting of DDB1 and DDB2, recognizes some UV-damaged DNA lesions and initiates the nucleotide excision repair (NER) process (4). Mutations in DDB2 account for the E group of xeroderma pigmentosum (XP), a repair-deficient disease characterized by a high risk of skin cancer in areas exposed to sunlight (5). Mice with deleted DDB2 exhibit increased skin tumorigenesis after UV-irradiation (6) and develop spontaneous tumors at a high rate when aged (7). DDB1 is evolutionarily conserved from yeast to humans and is likely to play a very fundamental role in cell physiology.

Recent work demonstrates that DDB1 functions as an obligatory subunit of the Cullin 4A (Cul4A) E3 ubiquitin ligase and facilitates NER by targeting NER factors such as DDB2 and Cockayne syndrome B protein (CSB) for ubiquitination and degradation (8–10). The DDB1–Cul4A ligase has been shown to target a variety of substrates. These substrates include the DNA replication licensing factor Cdt1 (11, 12) via additional adaptors PCNA (13) and Cdt2 (14, 15), protooncoprotein c-Jun via hDET1 and Cop1 (16), cell-cycle inhibitor p27Kip1 (17), and several histones (18, 19), all highlighting the importance of DDB1 in regulating cell cycle and DNA metabolism. Proteomic studies of proteins that interact with DDB1–Cul4A reveal a family of WD40-repeat proteins as substrate-recruiting adaptors for the E3 ligase (15, 20–22). These adaptors bind to the double β-propeller fold of DDB1 and are positioned to present associated substrates to Cul4A, which binds to the third β-propeller (20, 23).

The myriad substrates of the DDB1–Cul4A ligase suggest that this E3 ligase plays multiple roles beyond NER. In support of this notion, the deletion of DDB1 causes growth defects and changes in nuclear morphology in yeast (24) and lethality early in the development of the fruit fly (25). Recently, we showed that a null mutation of the *DDB1* gene in mice leads to early embryonic lethality, and conditional inactivation of the gene in brain and lens eliminates almost all proliferating cells via p53-mediated apoptosis (26). Given the established role of the DDB complex in the NER of UV-damaged DNA lesions in mammalian skin, we attempted to determine the effects of the conditional loss of DDB1 in the epidermis. We generated mice with epidermal deletion of DDB1 and investigated the molecular and cellular abnormalities by using primary cells. Here we show that loss of DDB1 resulted in genomic instability, aberrant cell cycle, and specific depletion of all epidermal progenitor cells. Analysis of the potential substrates of the DDB1–Cul4A E3 ligase suggests that abnormally accumulated c-Jun and p21Cip1 may contribute to the striking defects in the DDB1-deficient skin.

Results

Epidermal Deletion of DDB1 Leads to Complete Loss of Proliferating Cells. We first examined the pattern of expression of DDB1 in mouse skin by immunohistochemistry and determined that DDB1 protein was ubiquitously and abundantly expressed in mouse epidermis and predominantly localized in the nucleus of epidermal cells (Fig. 1*C*). To examine the consequence of epidermal deletion of DDB1, we generated mice carrying floxed *DDB1* alleles (designated as $DDB1^F$) (26) and expressing the Cre recombinase under the control of the *keratin 14* promoter (*K14-Cre*; see ref. 27; Fig. 1*A*). DDB1 expression in the epidermis and hair follicles was lost in mutant $DDB1^{F/F}$ *K14-Cre* mice starting at embryonic day 15.5 (E15.5), revealed by immunostaining (Fig. 1*C*), and in keratinocytes isolated from E17.5 mutant embryos, revealed by Western blotting (Fig. 1*B*). The mutant mice were born live with thin, fragile, and transparent skin and died within hours, probably because of transdermal water loss (Fig. 1*D*). The skin of the mutant embryos was intact, with no tearing *in utero* (data not shown), although it was frequently torn apart during birth (Fig. 1*D*).

By histological and immunohistochemical analyses, the mutant skin exhibited an extremely thin layer of epidermis and almost complete loss of hair follicles (Fig. 1*E*). The basal and

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Abbreviations: DDB, UV-damaged DNA-binding complex; NER, nucleotide excision repair; Cul4A, Cullin 4A; $DDB1^F$, floxed *DDB1* allele; *K14-Cre*, Cre recombinase controlled by keratin 14 promoter; E*n*, embryonic day *n*.

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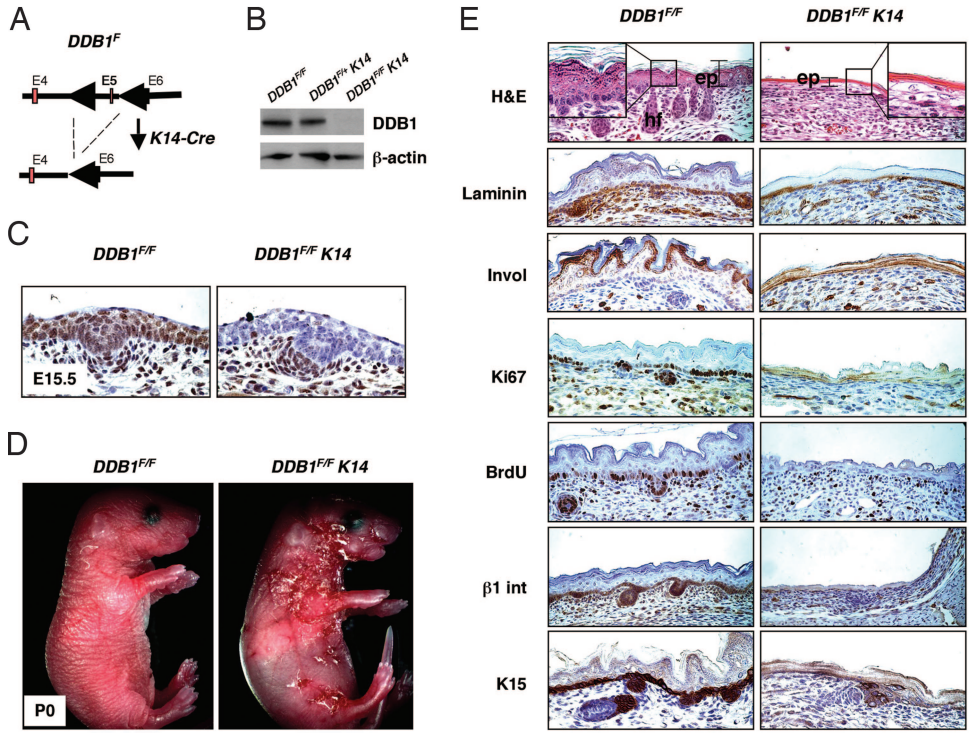

Fig. 1. Characterization of mouse epidermis with deleted DDB1. (*A*) Schematic representation of the floxed DDB1 locus (*DDB1*^{F}) before and after excision by the Cre recombinase protein, which is under the control of the *keratin 14* promoter (K14-Cre). (*B*) Western blot analysis of the keratinocytes isolated from E17.5 embryos. (*C*) Immunostaining of the skin sections of E15.5 embryos for DDB1 protein. (*D*) Phenotype of newborn mice. (*E*) Histology and immunohistochemistry of newborn mouse skin. ep, epidermis; hf, hair follicle; Invol, involucrin; β1 int, β1 integrin; K15, keratin 15.

spinous layers, located between the laminin-stained basement membrane and involucrin-stained fully differentiated granular layer, were absent in the mutant. The proliferating basal keratinocytes were completely lost in the mutant epidermis, as judged by the lack of Ki67 staining and BrdU incorporation (Fig. 1*E*). Epidermal stem cells, which express high levels of β1-integrin and keratin 15 (K15), were not detected in the epidermis or hair follicles of the mutant newborn mice (Fig. 1*E*). We conclude that epidermal deletion of DDB1 led to a near complete elimination of regenerating epidermis by the time of birth. This phenotype is reminiscent of the skin defects of mutant mice lacking p63, a homologue of the tumor suppressor p53 (28, 29).

Epidermal Progenitor Cells Deficient in DDB1 Undergo Apoptosis During Embryogenesis. We examined the histology of the epidermis at various stages during embryonic development after DDB1 deletion. DDB1 protein was not detectable in the mutant epidermis or hair follicles at E16.5, when no gross abnormality was apparent (data not shown). At E17.5, cells from the basal layer of epidermis or the outer root sheath of hair follicles of the mutant embryo detached from the basement membrane and from the neighboring cells (Fig. 2*A*). Most cells exhibited enlarged nuclei and many were undergoing apoptosis as indicated by condensed pyknotic nuclei (Fig 2*A Inset*, see arrows), TUNEL assay (Fig. 2*B*), and caspase-3 staining (Fig. 2*C*). At E18.5, the mutant epidermis and hair follicles lost their structural integrity, with only a few scattered Ki67-positive proliferating cells remaining (Fig. 2*D*). These results indicate that DDB1 is essential for the survival of epidermal progenitor cells and the depletion of progenitor cells by apoptosis during embryogenesis accounts for the loss of the epidermal layers in the newborns.

Activation of the DNA Damage Response and Cell-Cycle Arrest on DDB1 Deletion. DDB1 is reported to participate in DNA damage repair and regulate several factors involved in DNA metabolism. We tested whether loss of DDB1 in the epidermis led to an activated DNA damage response. Skin sections from E17.5 embryos were immunostained with antibodies specific for Ser-139-phosphorylated histone H2AX (γ-H2AX), a DNA damage marker induced by a wide array of insults including ionizing irradiation, UV, and chemical carcinogens. Indeed, γ-H2AX was detected throughout the mutant epidermis and hair follicles, and was more prominent in the proliferating cells (Fig. 3*A*). H2AX is a substrate of ataxia telangiectasia mutated (ATM), and ATM was also activated in the DDB1-deficient epidermal cells as demonstrated by immunohistochemical detection of phosphorylated Ser-1981 ATM (Fig. 3*A*). As another indication of DNA damage, the tumor suppressor p53 protein accumulated to high levels in most of the mutant keratinocytes (Fig. 3*A*). These results suggest that the loss of DDB1 triggered a broad activation of the DNA damage response.

To explore the cell-cycle status of the cells in the absence of DDB1, we isolated keratinocytes from control and mutant embryos at E17.5, the time when epidermal defects in the mutants were first detected. Flow cytometry analysis of the DNA content of DDB1-deficient cells revealed a significant increase in cells arrested in the G_2/M phase of the cell cycle compared with the controls (Fig. 3*B*). A small population of mutant cells contained less than the normal diploid DNA content, consistent with the apoptotic cells observed in the skin sections. The G_2/M checkpoint is usually activated to prevent cells from entering mitosis when DNA is damaged (30), and the increase in G_2/M cells is consistent with the activated DNA damage response in the mutant skin.

Rescue of Apoptosis by Inactivation of p53. If the elevated p53 protein observed in the mutant animals is responsible for induction

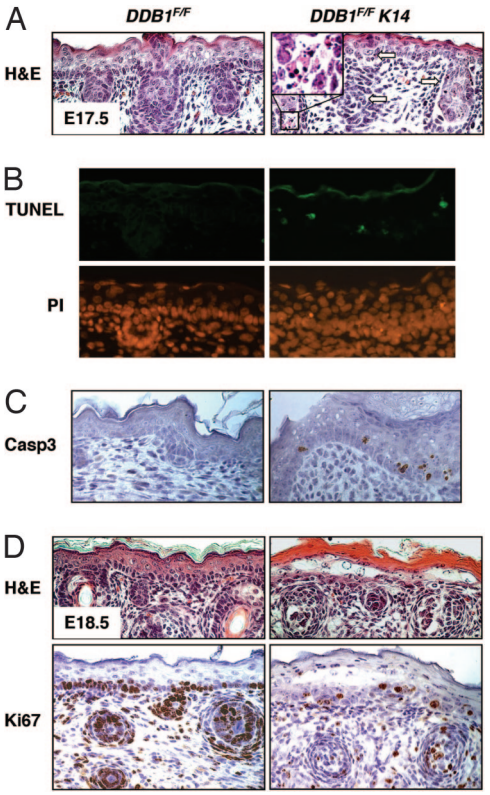

Fig. 2. Apoptosis of proliferating cells in *DDB1$^{F/F}$ K14-Cre* embryonic epidermis. (*A*) H&E staining of embryonic skin at E17.5, with pyknotic nuclei in the mutant indicated by arrows and in *Inset*. (*B*) TUNEL assays for DNA with nick ends, visualized by fluorescence microscopy. PI, propidium iodide. (*C*) Caspase 3 (Casp3) staining of E17.5 skin sections. (*D*) H&E and Ki67 staining of embryonic skin at E18.5.

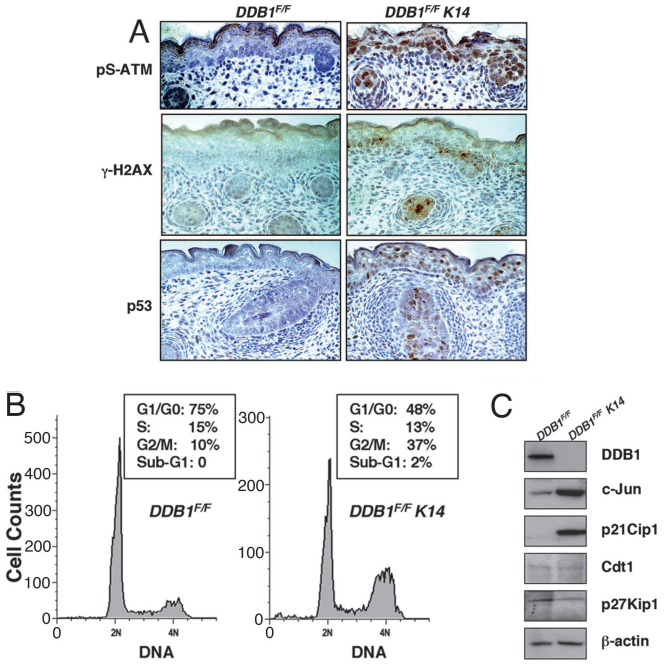

Fig. 3. Activated DNA damage response and misregulated cell cycle and in *DDB1$^{F/F}$* K14-Cre *keratinocytes.* (A) *Immunostaining of skin sections from E17.5 embryos for DNA damage response markers pS-ATM (Ser-1981-phosphorylated ATM), γ-H2AX (Ser-139-phosphorylated H2AX), and p53. (B* and *C*) Flow cytometry (*B*) and immunoblotting (*C*) analyses of keratinocytes isolated from E17.5 embryos.

of apoptosis and cell-cycle arrest in DDB1-deficient keratinocytes, then deletion of p53 might rescue or alleviate the epidermal defects of the mutant mice. Double mutant (*DDB1$^{F/F}$ p53$^{-/-}$ K14-Cre*) mice were generated by breeding, and indeed, newborns exhibited partial restoration of the epidermis and hair follicles and retention of proliferating keratinocytes with positive Ki67 staining (compare Fig. 4*A* with Fig. 1*E*). These recovered cells did not escape the *DDB1* gene deletion driven by *K14-Cre* because no DDB1 protein was detectable (Fig. 4*B Top*). The double mutant cells exhibited large nuclei and abnormal aggregation within the epidermal basal layers and hair follicles (Fig. 4*A Inset*). The DNA damage response was still activated in these cells, as shown by their positive staining for γ-H2AX (Fig. 4*B Bottom*), suggesting that p53-mediated apoptosis was not an immediate downstream event from DDB1 deletion. However, many areas of the double mutant epidermis were still very thin, with a few abnormally large Ki67-positive cells (data not shown), indicating the incomplete rescue of the epidermal growth defect by p53 deletion. The double mutant mice, like the *DDB1$^{F/F}$ K14-Cre* mice, died after birth, probably because of transdermal water loss.

Because the inactivation of p53 only partially rescued the epidermal defects resulting from DDB1 deletion, we examined two p53-regulated events, apoptosis and cell-cycle arrest, in the double mutant epidermis. Unlike the epidermis of the *DDB1$^{F/F}$ K14-Cre* embryos at E17.5, the epidermis of the *DDB1$^{F/F}$ p53$^{-/-}$ K14-Cre* embryos at the same developmental stage exhibited no significant increase of apoptosis compared with that of the *DDB1$^{F/F}$ p53$^{-/-}$* control embryos (data not shown), suggesting that p53 deletion rescued the DDB1-deficient cells from apoptosis. Flow cytometry analysis of the keratinocytes isolated from the double mutant embryos revealed cells with a wide range of DNA contents, and a major population of cells with >4N DNA content (Fig. 4*C*). Thus, DDB1 loss resulted in profound failure to faithfully execute chromosome segregation, leading to polyploidy and aneuploidy, as revealed on the elimination of p53-dependent apoptosis.

p53-Independent Accumulation of c-Jun and p21Cip1 on DDB1 Deletion. DDB1 has been recently appreciated as having functions independent of its direct role in DNA damage repair. The DDB1–Cul4A E3 ubiquitin ligase targets for degradation several proteins that regulate cell-cycle progression, including c-Jun, Cdt1, and p27Kip1 (11, 12, 16, 17). We assessed the levels of these proteins by immunoblotting whole-cell extracts of primary keratinocytes isolated from control and DDB1-deficient E17.5 embryos and found a dramatic elevation in the level of c-Jun, but no significant change in Cdt1 and p27Kip1, in the absence of DDB1 (Fig. 3*C*). Strikingly, p21Cip1, another cell-cycle inhibitor, also accumulated dramatically (Fig. 3*C*), which could account for the G_2/M arrest of the mutant epidermal cells (Fig. 3*B*).

The increase in c-Jun protein levels in the DDB1-deficient keratinocytes was found to be p53-independent (Fig. 4*D*). This finding is compatible with the observation that c-Jun is directly ubiquitinated and degraded by the DDB1–Cul4A ligase (16). Unexpectedly, p21Cip1 protein, a well established transcriptional target of p53, also remained high in the absence of p53 (Fig. 4*D*). The p53-independent increase of p21Cip1 raised an intriguing possibility that the DDB1–Cul4A E3 ligase might regulate the turnover of p21Cip1. We deleted the *DDB1* gene from *DDB1$^{F/F}$* mouse embryonic fibroblasts (MEFs) by infection with an adenoviral vector expressing Cre (31) and followed the degradation of p21Cip1 by immunoblotting after a chase in the presence of cycloheximide. The half-life of p21Cip1 increased dramatically in the absence of DDB1, whereas that of p27Kip1 did not change significantly (Fig. 4*E*), suggesting that p21Cip1,

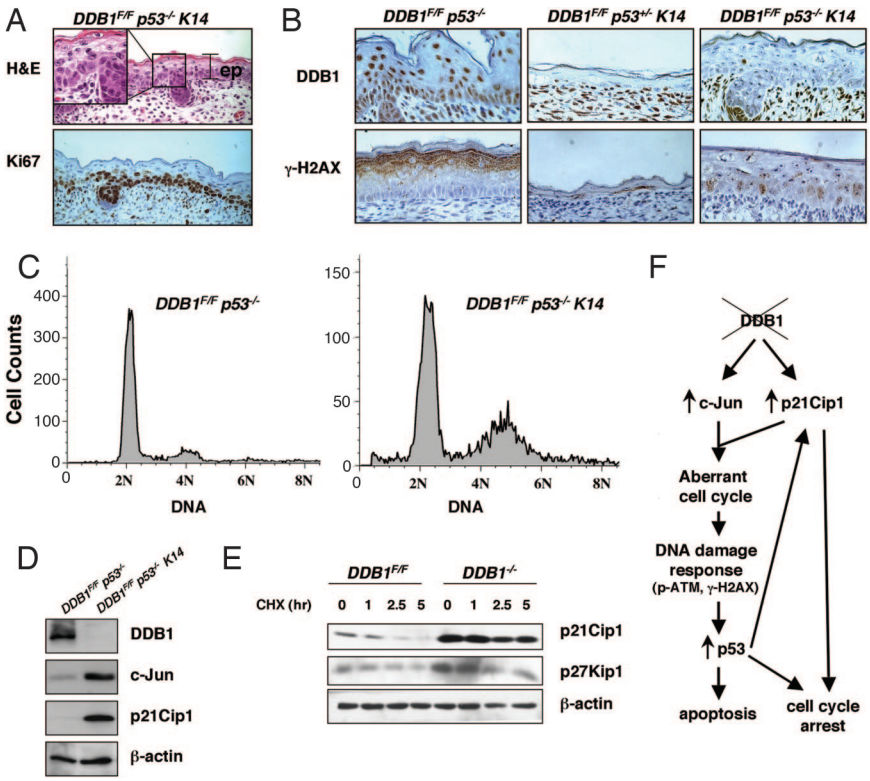

Fig. 4. Rescue of apoptosis, but not cell-cycle arrest, by inactivation of *p53*. (*A*) H&E and Ki67 staining of skin sections from newborn *DDB1*$^{F/F}$ *p53*$^{-/-}$ *K14-Cre* mice. (*B*) Immunostaining of newborn skin sections for DDB1 and γ-H2AX. (*C* and *D*) Flow cytometry (*C*) and immunoblotting analyses (*D*) of keratinocytes isolated from E17.5 embryos. (*E*) Turnover of p21Cip1 and p27Kip1 proteins in MEFs with *DDB1* undeleted (*DDB1*$^{F/F}$) or deleted (*DDB1*$^{-/-}$) *in vitro*, after cycloheximide (CHX) chase for the indicated time. (*F*) Model of the role of DDB1 in apoptosis and cell-cycle control.

and not p27Kip1, is probably a direct substrate of DDB1–Cul4A. At most, 2-fold increases in p21Cip1 mRNA levels were observed in *DDB1*$^{-/-}$ *p53*$^{-/-}$ as compared with p53$^{-/-}$ MEFs. An increase of p27Kip1 levels occurred very late after Cre expression in the MEFs (data not shown) and was not observed in DDB1-deficient primary epidermal cells (Fig. 3*C*). We speculate that p27Kip1 might not be a direct target regulated by DDB1–Cul4A but might accumulate as a late stress response to DDB1 deletion *in vitro*. Although p21Cip1 is thought to be degraded by the proteasome, the underlying mechanism has been debated (32, 33). The p53-independent increase and stabilization of p21Cip1 after DDB1 deletion suggest that p21Cip1 might be a previously unidentified target for the DDB1–Cul4A E3 ligase, or that p21Cip1 turnover might be indirectly controlled by DDB1–Cul4A.

Discussion

In an earlier study, we reported the consequence of DDB1 deletion in the development of mouse brain and lens (26). We demonstrated that the loss of DDB1 caused a selective elimination of almost all proliferating neuronal progenitor cells and lens epithelial cells by p53-dependent programmed cell death (26). Here we show that deletion of DDB1 in mouse epidermis, in a similar way, resulted in loss of dividing epidermal progenitor cells. Our data strengthen the ubiquitous requirement for DDB1 in maintaining genomic stability and survival of proliferating cells *in vivo* and further define the molecular and cellular defects resulting from DDB1 inactivation.

A number of substrate proteins of the DDB1–Cul4A E3 ligase are likely to become misregulated on DDB1 deletion. In some cell lines in which DDB1 was knocked down by RNA interference, increased levels of Cdt1 protein were reported and proposed to compromise genomic integrity (15, 34). Increasing numbers of Cdt1-positive cells were also observed in DDB1-deficient mouse brain sections (26). However, we did not detect significant changes in Cdt1 levels in the DDB1-deficient primary keratinocytes (Fig. 3*C*), consistent with reports that Cdt1 is also targeted for proteolysis by another E3 ubiquitin ligase, SCF-Skp2, during DNA replication (35). The results suggest that Cdt1 may not be a target of the DDB1–Cul4A ligase in the skin. In contrast, we detected dramatic increases in the level of c-Jun in the DDB1-deficient skin, similar to the increases seen previously in DDB1-deficient primary MEFs and DDB1-depleted cell lines (16, 26), consistent with the notion that c-Jun is an authentic substrate of the DDB1–Cul4A ligase (16). Here we propose that the accumulation of both c-Jun, which promotes proliferation (36), and p21Cip1, which halts cell division (37), could lead to the major phenotypes of DDB1 loss (Fig. 4*F*). The simultaneously increased c-Jun and p21Cip1 could result in aberrant cell division and replication stress, which then activate a DNA damage response to cause apoptosis and cell-cycle arrest in a p53-dependent mechanism (38, 39). In the absence of p53, the cells survive apoptosis, but still arrest at G_2/M with aneuploidy, because of p53-independent inhibition of the cell cycle by p21Cip1. It needs to be investigated whether deletion of p21Cip1 and/or c-Jun in mice with epidermal deletion of DDB1 would lead to a more complete rescue of the proliferation defects in the skin.

Our data indicate that DDB1 is particularly important for maintaining genomic stability and survival of cells that are actively dividing. Based on this model, we suggest that the molecular cascade leading to apoptosis would not be activated in DDB1-deficient cells that have permanently exited the cell cycle. Support for this idea is provided by a line of mice we have

generated with DDB1 deleted only in postmitotic neurons, which exhibited no apparent phenotype in early development (Y.C. and S.P.G., unpublished data). This model also indicates that the developmental requirement of DDB1 is probably independent of its role in DNA damage repair. Complete abolishment of all NER activities in several mutant mouse strains did not result in any developmental abnormalities of the skin similar to the abnormalities described here (5). These observations highlight the important role of DDB1 and its substrates in controlling genome integrity and cell survival, specifically in dividing cells.

Materials and Methods

Generation of *DDB1$^{F/F}$ K14-Cre* Mice. Mice carrying a floxed DDB1 allele (26) (*DDB1$^{F/F}$*) were crossed with transgenic mice expressing Cre recombinase under the control of the keratinocyte specific *keratin 14* promoter (*K14-Cre*) (The Jackson Laboratory, Bar Harbor, ME).

Histology and Immunohistochemistry. Whole embryos or newborn mice, after being killed, were fixed overnight at 4°C in PBS-buffered 4% paraformaldehyde and embedded in paraffin. Five-micrometer cross-sections through the body midline were prepared and stained with H&E. For immunohistochemistry, tissue antigens were unmasked, and sections were incubated with antibodies against the following proteins: ATM pS1981 (Rockland, Gilbertsville, PA), DDB1 (Zymed Laboratories, San Francisco, CA), laminin-1 L-9393 (Sigma, St. Louis, MO), phosphohistone H2AX (Ser-139) and caspase 3 (Cell Signaling Technology, Beverly, MA), p53 FL-393 and β1 integrin M-106 (Santa Cruz Biotechnology, Santa Cruz, CA), Ki-67 TEC-3 (Dako, Glostrup, Denmark), and Involucrin and keratin 15 (Covance, Berkeley, CA). For incorporation of BrdU, 100 mg of BrdU was injected i.p. into pregnant female mice, and the embryos were dissected 1 h later for processing and incubation with antibody against BrdU (PharMingen, San Diego, CA). A TUNEL apoptosis detection kit (Upstate, Lake Placid, NY) was used to detect the presence of DNA strand breaks in the tissue sections.

Analysis of Primary Keratinocytes. Primary epidermal keratinocytes were isolated by floating E17.5 embryonic skins on dispase (Roche, Mannheim, Germany) for 1 h at room temperature, followed by incubation in 0.25% trypsin for 0.5 h and filtration through 35-μm cell strainers. Cells were either fixed in ethanol for staining with propidium iodide and subsequent flow cytometry analysis of cellular DNA contents or lysed for whole-cell extracts used for Western blotting with antibodies against c-Jun, p21Cip1, p27Kip1 (all from Santa Cruz Biotechnology), Cdt1 (T. Nishimoto, Kyushu University, Fukuoka, Japan), and β-actin (Sigma).

Protein Stability Assay. MEFs were isolated from E13.5 *DDB1$^{F/F}$* embryos. Early passaged MEFs were infected for 3 days with an adenoviral vector expressing Cre or an empty vector (31). Cells were then treated with 10 μg/ml cycloheximide to inhibit new protein synthesis over the indicated periods, followed by lysis for Western blotting.

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