



## Abnormal regulation of DDB2 gene expression in xeroderma pigmentosum group E strains

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**A damage-specific DNA binding protein (DDB) activity is absent from a subset (DDB<sup>-</sup>) of cells from individuals initially classified as group E of xeroderma pigmentosum (XP), a hereditary, photosensitive disease with a high incidence of skin malignancies. In these cases, mutations have been identified in the DDB2 gene (DDB2<sup>-</sup>) that codes for the small subunit, p48, of the DDB heterodimer. In four DDB2<sup>-</sup> strains, neither p48 nor DDB activity were observed before or after UV-irradiation, despite an unusually strong up-regulation of DDB2 mRNA levels after UV-irradiation. In a fifth strain, XP82TO, p48 was detectable and both DDB2 mRNA and p48 levels were more up-regulated after UV-irradiation than in normal primary cells. Moreover, DDB activity also became apparent after irradiation. XP82TO showed very mild clinical manifestations compared with the other DDB<sup>-</sup> patients. These results, coupled with our findings that most, if not all DDB<sup>+</sup> cells classified as XP-E were misclassified, suggests a direct correlation between DDB2 levels and the XP-E phenotype. *Oncogene* (2001) 20, 7041–7050.**

**Keywords:** DDB2; xeroderma pigmentosum; damage-specific DNA binding protein; unscheduled DNA synthesis; p48; skin carcinogenesis

### Introduction

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by a clinical and cellular hypersensitivity to ultraviolet (UV) light (Cleaver and Kraemer, 1995). Patients exhibit dermatological abnormalities and develop sunlight-induced malignancies at an early age. By cell fusion analyses, XP has been classified into seven genetic complementation groups (A–G), which were presumed to be defective in nucleotide excision repair (NER). A separate group, XP variant (XP-V), has proficient NER but is defective in post-replication bypass of pyrimidine dimers

(Lehmann *et al.*, 1975; Masutani *et al.*, 1999; Johnson *et al.*, 1999).

A damage-specific DNA binding protein (DDB) is a heterodimer of p127 (the product of the DDB1 gene) and p48 (the product of the DDB2 gene) (Keeney *et al.*, 1993; Dualan *et al.*, 1995). DDB preferentially and tightly binds to 6–4 photoproducts and only marginally to *cis-syn* cyclobutane pyrimidine dimers, for which the binding affinity is almost equal to that of the undamaged thymidines (Reardon *et al.*, 1993; Fujiwara *et al.*, 1999). Some, but not all, individuals initially classified as XP-E have mutations in DDB2 and have no detectable DDB activity (DDB<sup>-</sup>) (Chu and Chang, 1988; Kataoka and Fujiwara, 1991; Keeney *et al.*, 1992). Others (DDB<sup>+</sup>) have normal DDB, but we recently reported that at least three DDB<sup>+</sup> XP-E cells had been misclassified, and we suggested that it is necessary to reinvestigate the classification of other DDB<sup>+</sup> XP-E cells (Itoh *et al.*, 2000). We also reported that the lack of DDB activity in extracts from four strains of DDB<sup>-</sup> XP-E cells could be corrected with purified recombinant p48 (Nichols *et al.*, 2000). Therefore, it is feasible that mutations in the DDB2 gene are solely responsible for XP-E.

Expression of the DDB2 gene is regulated by UV irradiation. After an initial reduction in DDB activity, there is a 3–4-fold increase in DDB2 mRNA levels 38 h after UV-irradiation of a normal human primary fibroblast and p48 and DDB activity reached a similar elevation at 48 h after UV-irradiation (Nichols *et al.*, 2000). p127, though not increased after UV-irradiation, is transported from the cytoplasm to the nucleus upon the increase of p48 (Shiyanov *et al.*, 1999; Liu *et al.*, 2000). Since NER is all but completed 48 h after UV-irradiation (Friedberg *et al.*, 1995), and since recent NER reconstitution studies have shown that DDB is not required *in vivo* (Aboussekhara *et al.*, 1995; Mu *et al.*, 1995; Kazansev *et al.*, 1996), the function, if any, of DDB in NER or DNA repair in general is unclear.

XP-E has been reported to have the highest level of unscheduled DNA synthesis (UDS) among XP groups A–G (Cleaver and Kraemer, 1995). Recently, three DDB<sup>-</sup> patients (GM01389, XP23PV, and XP25PV), have been (re)assigned as XP-E and the cells from these patients were reported to have 40–60% of normal levels of UDS (Otrin *et al.*, 1998). (GM01389

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was formerly misassigned to XP-V.) In contrast, we reported that three DDB<sup>-</sup> XP-E cell strains, Ops1 (Itoh *et al.*, 1999) and XP2RO and XP82TO (Itoh *et al.*, 2000), show normal UDS levels. In sum, a total of seven DDB<sup>-</sup> cell strains are known.

In this study we have studied three issues: whether a defect in the DDB2 gene affects DNA repair synthesis; how mutant DDB2 gene(s) are regulated before and after UV-irradiation; and whether the XP phenotype, including skin malignancies, correlates with the severity of defects in DDB activity or DDB2 response to UV-irradiation. We have therefore investigated levels of DDB2 mRNA, p48 protein, and DDB activity before and after UV-irradiation of five DDB2<sup>-</sup> cell strains which were available to us and compared these to the clinical manifestations of the individuals. The results provide an important insight into the correlation between post-UV regulation of the DDB2 gene and the XP phenotype.

## Results

*Like other DDB<sup>-</sup> strains, GM01389 cells show normal DNA repair markers*

Recently GM01389, formerly assigned as XP-V, was reassigned as a DDB<sup>-</sup> XP-E with 40–60% UDS levels compared to a normal control (Otrin *et al.*, 1998). We have found that these relative measurements vary with the individual experiment, and have developed a simple method for measuring relative UDS, RRS, or RDS after UV-irradiation by simultaneously plating two cell strains on the same coverslip (Itoh *et al.*, 1994, 1996). We checked these three DNA repair markers with GM01389 cells using this system, and observed that all three were indistinguishable from normal (Table 1), consistent with our observations with other DDB2<sup>-</sup> cells (Itoh *et al.*, 1999, 2000). These results also confirm that GM01389 does not belong to XP-V, as XP-V cells have normal UDS and RRS, but reduced RDS levels (Itoh *et al.*, 1996, 2000).

## *Levels of p48 protein and DDB activity in XP-E strains before and after UV-irradiation*

XP2/3RO cells have a single R273H substitution in p48 (Nichols *et al.*, 1996; Itoh *et al.*, 2000) and recombinant mutant XP2/3RO p48 (and recombinant wild type p48) migrates to the same position as native p48 during SDS–PAGE analysis (Nichols *et al.*, 2000). Ops1 cells have an R313ter mutation which results in a truncated p48 protein with a predicted molecular weight of 35 kD (Itoh *et al.*, 1999). When extracts from XP3RO or Ops1 were analysed by immunoblotting, no p48 was detected (Figure 1a). Upon longer exposure of the same membrane, several non-specific bands appeared, but no bands specific to XP3RO and Ops1 mutant p48 were detected (Figure 1b). To confirm that the rabbit polyclonal anti-p48 antibody can recognize the truncated p48 of Ops1, we transfected vectors harboring FLAG-tagged wild type or Ops1 mutant p48, immunoprecipitated both types of p48 with anti-FLAG antibody, and subsequently analysed the material with both anti-p48 antibody and anti-FLAG antibody after immunoblotting (Figure 1c). The absence of detectable p48 antigen in XP3RO and Ops1 strains implies that these mutant proteins are either not synthesized or are rapidly degraded in these two DDB2<sup>-</sup> cell strains. To attempt to control degradation of p48, XP2/3RO cell strains were incubated with the proteasome inhibitors lactacystin, NLVS, or proteasome inhibitor I. However, XP2/3RO p48 remained undetectable (data not shown).

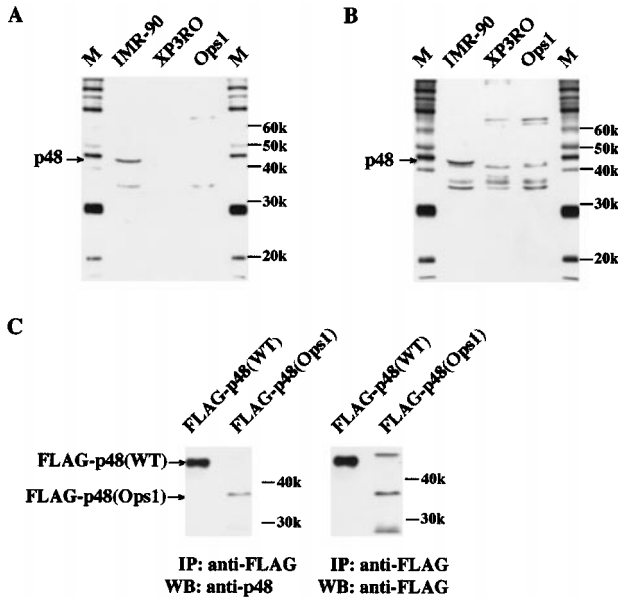
We recently reported that p48 in human normal primary fibroblasts increased roughly fourfold 48 h after UV-irradiation at a dose of 12 J/m<sup>2</sup> (Nichols *et al.*, 2000). We therefore assayed for p48 in four DDB2<sup>-</sup> strains before and after UV-irradiation. In XP2/3RO, GM01389, and Ops1 strains, p48 remained undetectable after the UV-irradiation (Figure 2).

In contrast to the above observations with XP2/3RO, GM01389, and Ops1, p48 was detectable at roughly half the normal level in the XP82TO cells before irradiation and this level increased approxi-

**Table 1** DNA repair levels *in vivo* in GM01389 cells

Cell strain <sup>a</sup>	Grains/nucleus		Range of relative repair rate (%) <sup>b</sup>	
UDS <sup>c</sup>				
GM01389/Mori	14.1 ± 1.1/14.0 ± 0.8		94–109	
GM01389/Turu	13.0 ± 0.6/12.4 ± 0.8		94–108	
RRS <sup>d</sup>				
GM01389/Mori	23.0 ± 1.1/19.6 ± 1.0		106–130	
RDS <sup>e</sup>				
GM01389/Mori	With caffeine 26.9 ± 2.1/27.1 ± 3.3	Without caffeine 25.4 ± 1.9/21.4 ± 2.4	With caffeine 82–118	Without caffeine 99–144

<sup>a</sup>Mori and Turu are normal primary fibroblast strains (Itoh *et al.*, 2000). <sup>b</sup>Range of relative repair rate is the range of UDS, RRS, or RDS values (grain numbers) of cell strains tested compared with those of the normal control (Mori or Turu) cells. <sup>c</sup>To measure UDS (unscheduled DNA synthesis), cells were UV-irradiated at a dose of 30 J/m<sup>2</sup>, and then immediately labeled with [<sup>3</sup>H]thymidine (50 μCi per ml) for 2.5 h. Exposure time was 24 h. Data are mean ± s.e.m. of 100 determinations. <sup>d</sup>To measure RRS (recovery of RNA synthesis), cells were UV-irradiated at a dose of 15 J/m<sup>2</sup>, incubated for 23 h, and then labeled with [<sup>3</sup>H]uridine (40 μCi per ml) for 1 h. The exposure time was 24 h. Data are mean ± s.e.m. of 50 determinations. <sup>e</sup>To measure RDS (recovery of replicative DNA synthesis), cells were UV-irradiated at a dose of 15 J/m<sup>2</sup>, incubated for 6 h, and then labeled with [<sup>3</sup>H]thymidine (15 μCi per ml) for 1 h. Exposure times were 24 h (with caffeine) or 13 h (without caffeine), respectively. Data are mean ± s.e.m. of 50 determinations



**Figure 1** Detection by immunoblotting of wild type and mutant p48 protein. (a,b) Fifty  $\mu\text{g}$  of cell extracts were analysed by immunoblotting as described in Materials and methods. The filter was incubated in a 200-fold diluted anti-p48 antibody with 5% milk for 3.5 d at 4°C, and then incubated for 1 h at room temperature in goat anti-rabbit IgG (Amersham-Pharmacia) diluted 1 : 5000. The signal was enhanced by the chemiluminescence reagent (NEN Life Science Products, Inc.) and visualized by autoradiography. Exposure times were 10 s (a) or 1 min (b). IMR-90 cells were at PDL36. Ops1 was at P14. Since PDL or P are unknown for all of other DDB2<sup>-</sup> XP strains, we used the passage number (+P) after our frozen vial stock was thawed for these strains. XP3RO was at +P4. (c) U2OS cells were transiently transfected with 8  $\mu\text{g}$  of FLAG-p48 (wild type) or FLAG-p48 (Ops1) in pCMV-Tag2. Four hundred  $\mu\text{g}$  of each cell extract was immunoprecipitated with 20  $\mu\text{g}$  of anti-FLAG M2 antibody and the immunoprecipitated proteins were resolved by 10% SDS-PAGE and analysed by immunoblotting with the anti-p48 antibody (left panel) or anti-FLAG M2 antibody (right panel). IP, immunoprecipitating antibody; WB, detection antibody used for the Western blotting

mately fourfold 48 h after UV-irradiation (Figure 2). In fact, in this experiment the degree of induction of p48 was higher than that of the normal IMR-90 cells. To determine whether the p48 levels observed in XP82TO were reflected in DDB activity, the same extracts used in the experiments in Figure 2 were assayed for DDB activity by EMSA. In unirradiated cells, DDB activity was only detectable in the extracts from the normal primary IMR-90 cells, consistent with previous reports (Chu and Chang, 1988; Keeney *et al.*, 1992; Itoh *et al.*, 1999). However, DDB activity was clearly observed in XP82TO cells after UV-irradiation (Figure 3). This activity was roughly 10% that of the unirradiated IMR-90 cells or 6% that of the UV-irradiated IMR-90 cells. These results indicate that DDB activity is present in UV-irradiated XP82TO cells, although it is considerably lower than one would predict based upon the amount of p48 antigen present in these cells.

#### Levels of DDB2 mRNA before and after UV-irradiation of XP-E strains

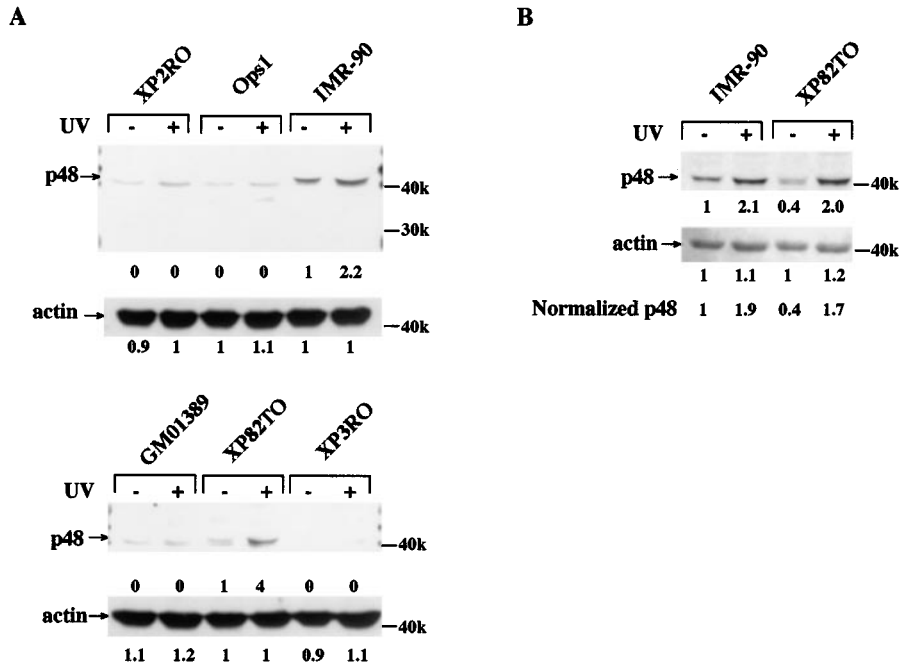
To determine DDB2 mRNA levels before and after UV-irradiation of DDB2<sup>-</sup> cells, we utilized QC-RT-PCR (Figure 4).  $\beta$ -Actin mRNA levels were used to normalize the amount of mRNA among the strains. Levels of DDB2 mRNA varied among the cells examined. This variation could have been due in part to differences in passage number of the cells utilized. For example, for IMR-90 cells, DDB2 mRNA levels at PDL33 were approximately twofold those of PDL44 and threefold those of RDL50 (Figure 4a). Unfortunately, this variable could not be controlled for due to the unavailability of early passage samples of most DDB2<sup>-</sup> strains. In spite of this complication, it is clear that with four DDB2<sup>-</sup> cell strains (XP2/3RO, GM01389, and XP82TO) uninduced p48 mRNA levels equaled or exceeded those of the normal IMR-90 cells; only in the Ops1 cells might the levels have been lower (Figure 4a).

After UV-irradiation of normal cells, DDB2 mRNA increases, reaching a maximum of 2–3-fold the basal level after 38 h (Figure 4), and then it decreases back to the basal level (Figure 4b, Nichols *et al.*, 2000). On the other hand, the mRNA levels in each of the DDB2<sup>-</sup> strains XP2/3RO, GM01389 and XP82TO increased nearly 10-fold after irradiation (Figure 4a), and in XP3RO and XP82TO they continued to increase for up to 72 h after UV-irradiation (Figure 4b). Clearly in these cases the regulation of DDB2 mRNA was abnormal. While a defect in a direct p48 feedback mechanism in these cells would be an obvious explanation for the observed continuing increase, the existence of normal levels of the mutated p48 in the XP82TO cells after UV-irradiation (Figure 2b) would necessitate an expanded comparison of the properties of the mutant p48 and the normal p48 properties. In Ops1 cells, DDB2 mRNA levels were also induced by UV-irradiation with a pattern similar to that of normal cells, but the basal levels were quite reduced (Figure 4a). This lower level could be related to the fact that the Ops1 cells were cultured from a skin biopsy taken when the subject was 62 years old.

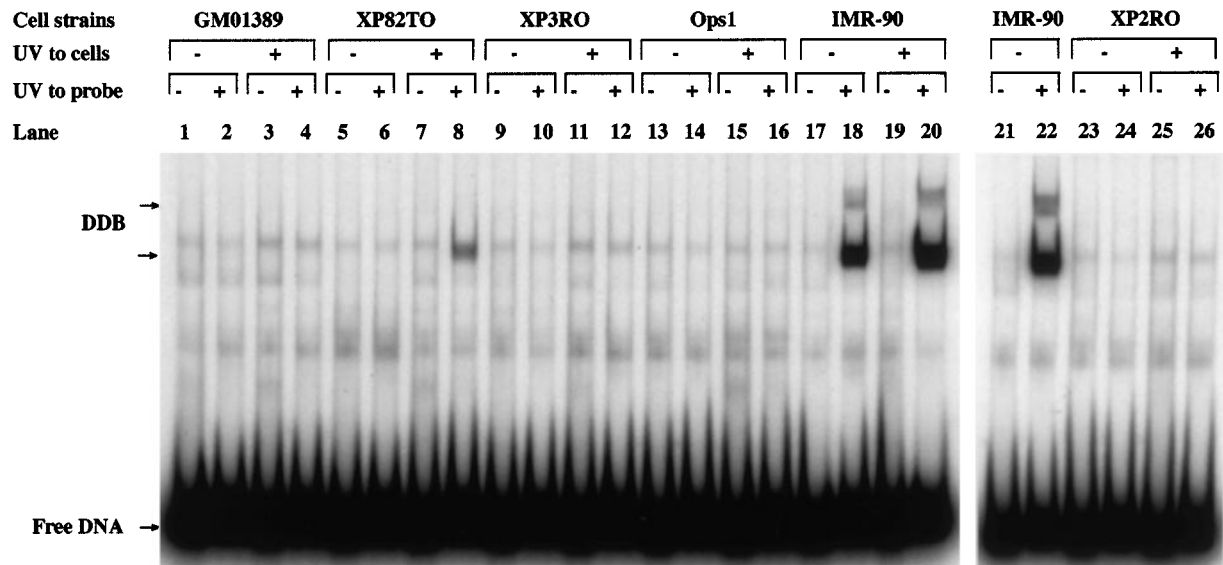
Since the DDB2 mRNA levels of XP2RO and XP82TO cells were up-regulated for at least 72 h after UV-irradiation (Figure 4b), we further examined p48 protein levels after UV-irradiation for up to 72 h in XP82TO cells or 96 h in XP2RO cells. The levels of p48 in the XP82TO cells increased up to 48 h then remained constant corresponding to DDB2 mRNA levels (Figure 5a). DDB activity has behaved similarly (Figure 5c,e). However, neither p48 antigen nor DDB activity were detected in XP2RO cells (Figure 5b,d,e), despite the overproduction of the DDB2 mRNA (Figure 4).

#### p48 peptide level is decreased during replicative senescence

To investigate whether the decrease in mRNA levels during replicative senescence of the normal fibroblasts



**Figure 2** p48 levels before and after UV-irradiation. (a,b) Extracts were prepared before and 48 h after UV-irradiation ( $12 \text{ J/m}^2$ ) and  $50 \mu\text{g}$  of each extract was analysed by immunoblotting. Filters were incubated in 200-fold diluted anti-p48 antibody with 5% milk for 4 h at room temperature (a) or for 3.5 d at  $4^\circ\text{C}$  (b), and then incubated with goat anti-rabbit IgG as in the legend to Figure 1. Each filter was then stripped and incubated in a 200-fold diluted anti-actin antibody for 1 h at room temperature. IMR-90 cells, PDL 36; XP2RO, +P4; Ops1, P16; GM01389, +P4; XP82TO, +P7; XP3RO, +P5. The intensity of signals was calculated by ImageQuaNT 1.2, and signals relative to those with unirradiated IMR-90 are given below the upper panels. The relative actin signals are given directly below the lower panels and p48 levels normalized by  $\beta$ -actin levels are given at the very bottom of (b)



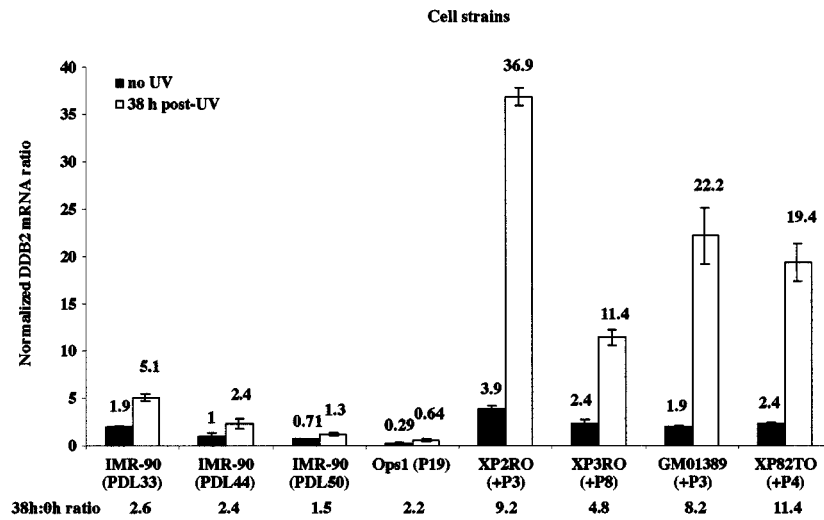
**Figure 3** DDB activity before and after UV-irradiation. The same extracts used in the experiment of Figure 2 were used herein. Three  $\mu\text{g}$  ( $\text{DDB}2^-$ ) or 1.5  $\mu\text{g}$  (wild type) extract were used in each EMSA as described in Materials and methods. The autoradiogram film was exposed for 5 d at  $-80^\circ\text{C}$ . The upper DDB band contains two molecules of DDB per duplex oligonucleotide. The lower DDB band, containing 1 DDB molecule, migrates just below the non-specific band that is present with each of the extracts

is reflected in p48 levels, we analysed p48 and mRNA levels in IMR-90 cells at PDL38, 45, and 56. The levels of p48 did decrease in proportion to the decreasing amounts of DDB2 mRNA (Figure 6).

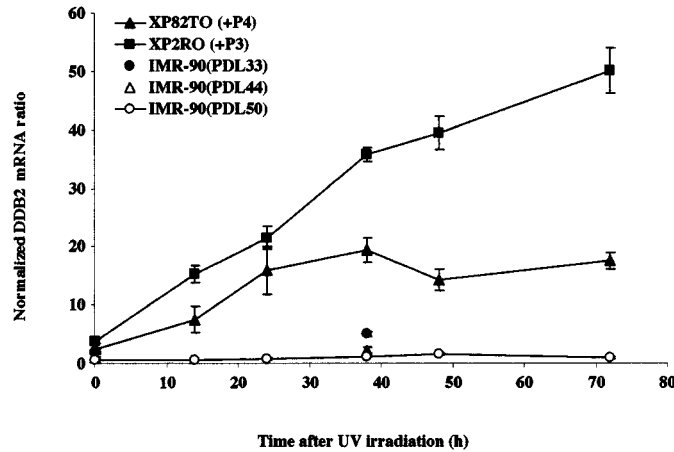
## Discussion

Recent *in vivo* studies showed that human  $\text{DDB}2^-$  cell strains are defective in global genomic repair of

A



B

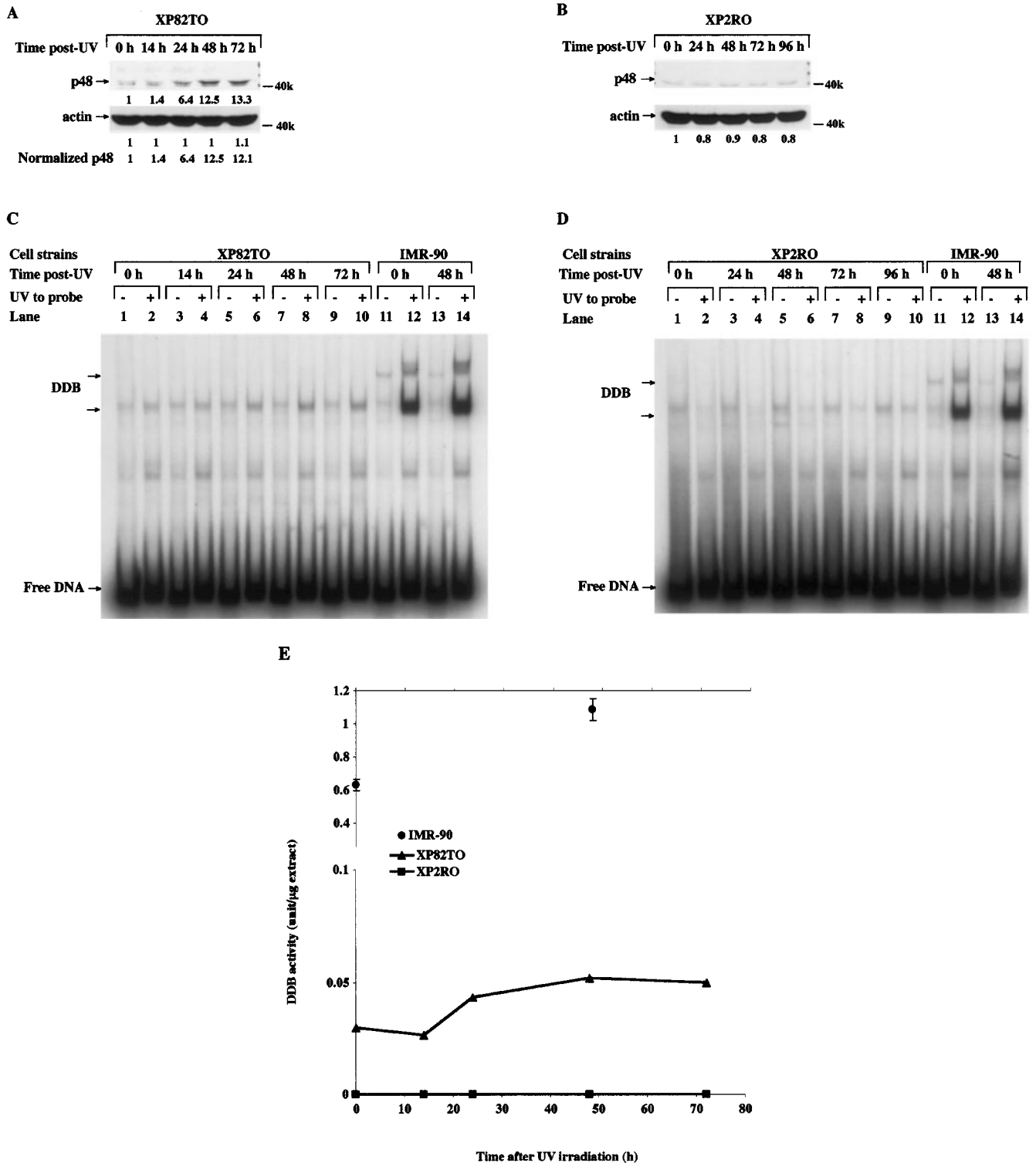


**Figure 4** DDB2 mRNA levels before and after UV-irradiation. (a,b) Total RNA was extracted at times indicated after 12 J/m<sup>2</sup> UV-irradiation and analysed by QC-RT-PCR using DDB2 primers between nucleotide +872 to +890 and nucleotide +1152 to +1171. Each sample was also analysed for  $\beta$ -actin message by competitive PCR to facilitate normalization of DDB2 mRNA levels. PCR products were resolved on a 3% super-resolution agarose gel in Tris-borate/EDTA buffer and quantified with a PhosphorImager and ImageQuANT 1.2. PDL, P, and +P are indicated and are defined in Materials and methods. Values are normalized to that of the PDL44 unirradiated IMR-90 cells

cyclobutane pyrimidine dimers (Hwang *et al.*, 1999) and 6–4) photoproducts (Hwang *et al.*, 1999; Itoh *et al.*, 1999). More recently, transfection of the DDB2 cDNA into Chinese hamster ovary cell lines that lack DDB activity enhanced global genomic repair and suppressed UV-induced mutagenesis (Tang *et al.*, 2000). Therefore, p48 seems to regulate both mutation frequency and mutagenesis after UV-irradiation.

Although mutations in the DDB2 gene are directly responsible for the defect of the DDB activity *in vitro* (Nichols *et al.*, 2000), the relation between the effects on DDB activity and the clinical phenotype is unclear. Profiles of DDB<sup>-</sup> XP patients are shown in Table 2. XP2RO was 14 years old when the first skin tumors developed. Both XP2RO and XP3RO were clinically classified as ‘classical XP, light to moderately severe’

(Bootsma *et al.*, 1970; Kleijer *et al.*, 1973; de Weerd-Kastelein *et al.*, 1974). Unfortunately, we could not obtain any clinical information regarding the patient, GM01389. However, this patient was diagnosed as XP at the age of 16, which was the youngest among the five patients; she also seemed to have a ‘typical XP phenotype’ (Dr M Swift and Dr JE Cleaver, personal communications). Ops1 had a relatively severe XP phenotype and multiple skin neoplasias (five malignant melanomas, 14 basal cell carcinomas, and two squamous cell carcinomas *in situ* (Itoh *et al.*, 1999)). On the other hand, XP82TO only exhibited pigmented and depigmented macules and patches on especially exposed skin, and had no skin neoplasias (Kondo *et al.*, 1988). Thus, XP82TO had a mild XP phenotype despite the fact that her cells lack detectable DDB



**Figure 5** Time courses of p48 peptide level and DDB activity after UV-irradiation of XP82TO and XP2RO cells. (a,b) Cells were UV-irradiated ( $12 \text{ J/m}^2$ ) and harvested at the times indicated. Fifty  $\mu\text{g}$  of each cell extract was analysed by immunoblotting as described in Materials and methods. The filters were incubated in a 200-fold diluted anti-p48 antibody with 5% milk for 4 h at room temperature. The same filter was stripped and incubated in a 200-fold diluted anti-actin antibody for 1 h at room temperature. The signal intensities of were calculated by ImageQuaNT 1.2 and the relative values are indicated below each gel image. p48 and  $\beta$ -actin levels before UV-irradiation (0 h) were taken as unity and p48 levels were normalized against  $\beta$ -actin levels as shown. (c,d) The same extracts used in (a) and (b) were tested for DDB activity by EMSA as described in Materials and methods using 3  $\mu\text{g}$  from XP82TO and XP2RO cells or 1.5  $\mu\text{g}$  (c) or 1  $\mu\text{g}$  (d) from IMR-90 cells. The gel was exposed to X-ray film for 3 days (c) or 4 days (d) at  $-80^\circ\text{C}$ . (e) The DDB activity was calculated from (c) and (d) by ImageQuaNT 1.2 software. IMR-90 was at PDL 36; XP82TO and XP2RO were at +P4 and +P3, respectively

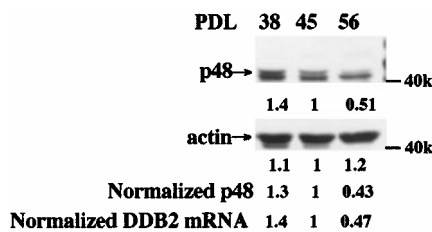
activity in the absence of UV-irradiation, as did those of the other four XP patients (Keeney *et al.*, 1992).

Levels of DDB2 mRNA, p48 peptide, and DDB activity of these five DDB2<sup>-</sup> cell strains are summarized in Table 3. In each case, mutations of DDB2 gene dramatically affected not only DDB activity, but also DDB2 mRNA and p48 peptide levels before and after UV-irradiation. Perhaps most dramatic is the abnormally high increase in DDB2 mRNA after UV-irradiation in all but the Ops1 cells, an increase which is accompanied by a drop in p48 protein level. While we expect the increase in mRNA to result from increased synthesis, it is possible that mRNA stability is enhanced. In addition, only XP82TO has residual DDB activity following UV-irradiation, and this small

amount of activity might explain the relatively mild phenotype of this individual.

Transfected wild type DDB2 cDNA and subsequent expression of p48 resulted not only in the accumulation of p48 in the nucleus, but also in the relocation of p127 from the cytoplasm to the nucleus. The mutant XP2/3RO and XP82TO p48 peptides were similarly expressed and were localized in the nucleus, but these were deficient in stimulating the nuclear accumulation of p127 (Shiyanov *et al.*, 1999; Nichols *et al.*, 2000; Liu *et al.*, 2000). By immunoprecipitation criteria, the mutant XP2/3RO p48 fails to form a complex with p127, but the mutant XP82TO p48 could bind to it (Shiyanov *et al.*, 1999). Therefore, there is an apparent discrepancy of p48 function between the ability to transport p127 to the nucleus and the coimmunoprecipitate p127. After UV-irradiation XP82TO cells have a low level of DDB activity for which both p48 and p127 are necessary in the nucleus (Shiyanov *et al.*, 1999; Nichols *et al.*, 2000). Therefore, mutant XP82TO p48 seems to be able to bind with p127 to at least some degree, but the binding ability may not be sufficient to efficiently transport p127 into the nucleus when the cells are unirradiated and p48 levels are low. Since the mutant XP82TO p48 increased very substantially after UV-irradiation, presumably some level of DDB complexes were formed that were able to bind to UV damaged DNAs as demonstrated by DDB activity (Figures 3 and 5c).

Upon examination of Table 3, one might conclude that the degree of defectiveness of p48 function in DDB2<sup>-</sup> patients might correlate with their XP phenotype and their skin carcinogenesis. However, the limited sample size and patient history would make



**Figure 6** p48 peptide and DDB2 mRNA levels during replicative senescence. Fifty  $\mu$ g of cell extracts from IMR-90 cells at indicated PDL were analysed by immunoblotting as described in Materials and methods. The filter was incubated with 200-fold diluted p48 antibody and 5% milk for 4 h at room temperature. The same filter was stripped and incubated with 200-fold diluted  $\beta$ -actin antibody for 1 h at room temperature. Total RNA extracted at the same time that cell extracts were prepared, was analysed for both DDB2 and  $\beta$ -actin mRNA levels as described in Materials and methods. Both p48 and DDB2 mRNA were normalized vs their  $\beta$ -actin counterparts

**Table 2** Clinical and genetic phenotypes of five DDB2<sup>-</sup> individuals

Strain	Sex	Age <sup>a</sup>	Skin tumor(s)	Clinical Phenotypes	Mutation of DDB2 gene	Genotype
XP2RO <sup>b</sup> (GM02415)	F	34	+	Moderate	c818G>A	R273H <sup>c</sup> Homozygote
XP3RO <sup>b</sup>	F	29	-	Mild-moderate	c818G>A	R273H <sup>c</sup> Homozygote
XP82TO <sup>d</sup>	F	41	-	Very mild	c730A>G	K244E <sup>c</sup> Homozygote
GM01389 <sup>e</sup>	F	16	ND	ND	del(A1045-C1047)	delN349 <sup>f</sup> L350P <sup>f</sup> Compound heterozygote
Ops1 <sup>g</sup>	F	62	++	Moderate-severe	c937C>T	R313ter <sup>g</sup> Homozygote

<sup>a</sup>Age of the patient at diagnosis. <sup>b</sup>Bootsma *et al.*, 1970, Kleijer *et al.*, 1973, de Weerd-Kastelein *et al.*, 1974. XP3RO is the second cousin of XP2RO. <sup>c</sup>Nichols *et al.*, 1996; Itoh *et al.*, 2000. <sup>d</sup>Kondo *et al.*, 1988. <sup>e</sup>No clinical information is available. <sup>f</sup>Nichols *et al.*, 2000. <sup>g</sup>Itoh *et al.*, 1999. She was strongly suspected to be XP from approximately age of 20

**Table 3** Summary of DDB2 expression parameters and clinical severity of DDB2<sup>-</sup> strains

Strain	DDB2 mRNA <sup>a</sup>		p48 protein <sup>a</sup>		DDB activity <sup>a</sup>		Clinical severity
	-UV	+UV <sup>b</sup>	-UV	+UV <sup>c</sup>	-UV	+UV <sup>c</sup>	
XP2RO	3.9	36.9	0	0	0	0	moderate
XP3RO	2.4	11.4	0	0	0	0	mild-moderate
XP82TO	2.4	19.4	0.4	1.7	0.05	0.1	very mild
GM01389	1.9	22.2	0	0	0	0	ND <sup>d</sup>
Ops1	0.3	0.6	0	0	0	0	moderate-severe
IMR-90	0.7-1.9	1.3-5.1	1.0	1.9-2.2	1.0	1.7	-

<sup>a</sup>Data are from Figures 2, 3, 4 and 5. <sup>b</sup>mRNA levels were measured 38 h after UV irradiation (12 J/m<sup>2</sup>). <sup>c</sup>p48 peptides and DDB activity (unit/ $\mu$ g extract) were qualified 48 h after UV irradiation (12 J/m<sup>2</sup>). <sup>d</sup>No clinical information is available

the correlation tentative. Such a correlation does exist for patients with ataxia-teleangiectasia (AT), an autosomal recessive disorder characterized by cerebellar, degeneration, immunodeficiency, chromosomal instability, radiosensitivity, and cancer predisposition (Shiloh, 1997; Lavin and Shiloh, 1997). The typical AT phenotype is caused by defects of the ATM gene (Savitsky *et al.*, 1995) that give rise to truncated or severely destabilized ATM protein (Lakin *et al.*, 1996; Watters *et al.*, 1997). However, AT variants, that have milder manifestations of the clinical and cellular characteristics of the disease, have reduced levels of normal ATM protein compared with normal cells (Gilad *et al.*, 1998).

The regulation of the DDB2 gene before and after UV-irradiation is clearly complex and is likely important in the prevention of the XP phenotype and UV-induced skin cancers. Hence, to truly understand DDB and its anti-carcinogenic function, we must clarify its regulation, its activity and its role (if any) in DNA repair, none of which are currently understood.

## Materials and methods

### Cells and culture conditions

The DDB2<sup>-</sup> cell strains used in this study are shown in Table 2. XP2RO (GM02415) and GM01389 cells were purchased from the Coriell Institute cell repository (Camden, NJ, USA). XP3RO cells (derived from the second cousin of XP2RO) were a generous gift from Dr JHJ Hoeijmakers (Erasmus University, Rotterdam). Ops1, a DDB2<sup>-</sup> primary fibroblast strain, and Mori and Turu, normal human primary fibroblast strains, were established in Kumamoto (Itoh *et al.*, 1999, 2000); IMR-90, normal human fibroblasts (CRL1262) and U2OS, a human osteosarcoma cell line, were from the American Type Culture Collection (Rockville, MD, USA). All cells were cultured in Dulbecco's modified Eagle's minimum essential medium (Life Technologies, Inc.) supplemented with 20% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Inc.) at 37°C in a humidified 5% CO<sub>2</sub> incubator. The IMR-90 cells had been tracked by population doublings (PDL) and the Ops1 cells had been tracked by passage number (P). Neither PDL nor P had been tracked for the other DDB2<sup>-</sup> cell strains, so that we tracked P from when our frozen vial stocks were thawed for these studies (+P).

### Measurement of UDS, recovery of RNA synthesis (RRS) and, recovery of DNA synthesis (RDS) after UV-irradiation

UDS, RRS (Itoh *et al.*, 1994), and RDS (Itoh *et al.*, 1996) were measured as previously described. Twenty  $\mu$ l aliquots of test and control cell suspension (approximately  $5 \times 10^5$  cells/ml) were plated with a micropipette in two rows on a single coverslip (18  $\times$  18mm) for each experiment, since variations among coverslips was observed to interfere with comparisons between cell strains. For UDS, the cells were irradiated with UV-C (254 nm) at a dose of 30 J/m<sup>2</sup>, immediately labeled with <sup>3</sup>H-thymidine (40  $\mu$ Ci/ml) for 2.5 h, and fixed. Coverslips were mounted on glass slides, dipped in Kodak NTB-3 emulsion, and exposed for 24 h at 4°C. Grains above nuclei

were counted under a microscope. For RRS, cells were irradiated with UV-C (254 nm) at a dose of 15 J/m<sup>2</sup>. After UV-irradiation, they were incubated for 23 h, labeled with <sup>3</sup>H-uridine (40  $\mu$ Ci/ml) for 1 h, and then autoradiography was performed as above. For RDS, the cells were irradiated with UV-C (254 nm) at a dose of 15 J/m<sup>2</sup>. After UV-irradiation, they were incubated for 6 h, then labeled with <sup>3</sup>H-thymidine (15  $\mu$ Ci/ml) for 1 h, and then autoradiography was performed as above.

### Analysis of p48 and DDB activity before and after UV-irradiation

p48 was detected by immunoblotting as previously described (Nichols *et al.*, 2000).  $0.5-1.0 \times 10^6$  cells were seeded onto 100-mm dishes. When the cells reached exponential growth, they were washed once with phosphate-buffered saline (PBS) (Life Technologies, Inc.), and irradiated with 12 J/m<sup>2</sup> of 254 nm light and then cultured medium was readded and the cells incubated until harvest.

To prepare whole cell extracts, cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, and protease inhibitors (Complete; Roche Molecular Biochemicals)), and then lysed with a Dounce homogenizer. Two volumes of potassium glutamate buffer (250 mM Tris-HCl (pH 8.0), 10 mM MgCl<sub>2</sub>, 0.3 M potassium glutamate (pH 7.8), 5 mM DTT, 50% glycerol, and protease inhibitors) were added, and the extracts were incubated for 30 min at 4°C. The extracts were clarified by ultracentrifugation for 1 h at 106 000 g in a Beckman 100.3 rotor at 4°C and then dialyzed against 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 10% glycerol. Thirty to 100  $\mu$ g of extract were resolved by 8–10% SDS-polyacrylamide gel electrophoresis (PAGE) and peptides were transferred to a nitrocellulose filter, and a 200-fold dilution of purified rabbit polyclonal p48 antibody was used for immunoblotting. After the detection of p48, the same membrane was stripped and a 200-fold dilution of anti-actin antibody (Santa Cruz Biotechnology, Inc.) was used to provide an internal control. The gel images were analysed using ImageQuaNT 1.2 (Molecular Dynamics).

The proteasome inhibitors, lactacystin, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-vinylsulfone (NLVS), or proteasome inhibitor I (Calbiochem) were dissolved in dimethyl sulfoxide (Sigma-Aldrich), and were used at final concentrations of 10–20  $\mu$ M for 4–14 h, 10–50  $\mu$ M for 4–16 h, or 20  $\mu$ M for 14 h respectively. After exposure to the inhibitors, cell extracts were prepared as described above and then 50–100  $\mu$ g of cell extracts were resolved by 10% SDS-PAGE and then subsequently analysed by protein immunoblotting.

DDB activity in cell-free extracts was analysed by electrophoretic mobility shift assays (EMSA) as previously described (Nichols *et al.*, 2000). Samples of the same extract analysed by immunoblotting were incubated in 25 mM Tris-HCl (pH 8.0), 5% glycerol, 5 mM MgCl<sub>2</sub>, 60 mM KCl, 0.1 mg/ml bovine serum albumin, and 5 nmol of poly(dI-dC) (nucleotide residues) (Midland Certified Reagent) for 15 min at room temperature and then 8 fmol of <sup>32</sup>P-labeled 77-base pair duplex DNA which had been UV-irradiated with 6 kJ/m<sup>2</sup> was added and the reaction was incubated for an additional 15 min at 30–32°C. Samples were resolved by 5% PAGE in Tris-borate/EDTA buffer, and the dried gels were exposed to X-ray film. The film was scanned using a PhosphorImager (Molecular Dynamics) and the image was analysed by ImageQuaNT 1.2. One unit of DDB activity is defined as that amount forming 1 fmol of DDB/DNA complex.

### Transfection, immunoprecipitation, and Western blot

Expression vectors for FLAG-tagged wild type p48 and Ops1 mutant p48 were constructed in pCMV-Tag2 (Stratagene). Cells were seeded at 90% confluence in 100-mm dishes and transfected with the indicated plasmids using LipofectA-MINE 2000 (Life Technologies, Inc.). Cells were harvested 20 h post-transfection and cell extracts were prepared as described above and subjected to immunoprecipitation with anti-FLAG M2 IgG (Sigma-Aldrich). After incubation at 4°C for 16 h, antibody-antigen complexes were collected onto 40 µl of protein A/G plus-agarose (Santa Cruz Biotechnology, Inc.), and the beads were washed three times with PBS. Proteins were removed from the beads, resolved by 10% SDS-PAGE, and subsequently analysed by immunoblotting.

### Analysis of DDB2 mRNA levels by quantitative competitive reverse transcription-PCR (QC-RT-PCR)

Competitive RNA was prepared essentially as described (Nichols *et al.*, 2000) with some modifications. Total RNA was isolated from cells with a RNeasy Mini Kit (Qiagen) and QC-RT-PCR was carried out essentially as described by Borson *et al.* (1998). To maximize the signal, Thermoscript reverse transcriptase (Life Technologies, Inc.), and Ex Taq polymerase (Takara) were used. DNA competitor was made with a DNA competitor construction kit (Takara). cDNAs used for each PCR reaction were reverse-transcribed from 1–100 ng of total RNA plus 1000–100 000 copies of DDB2

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