

A novel DDB2 mutation causes defective recognition of UV-induced DNA damages and prevalent equine squamous cell carcinoma

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Abstract

Squamous cell carcinoma (SCC) occurs frequently in the human Xeroderma Pigmentosum (XP) syndrome and is characterized by deficient UV-damage repair. SCC is the most common equine ocular cancer and the only associated genetic risk factor is a UV-damage repair protein. Specifically, a missense mutation in horse DDB2 (T338M) was strongly associated with both limbal SCC and third eyelid SCC in three breeds of horses (Halfflinger, Belgian, and Rocky Mountain Horses) and was hypothesized to impair binding to UV-damaged DNA. Here, we investigate DDB2-T338M mutant's capacity to recognize UV lesions *in vitro* and *in vivo*, together with human XP mutants DDB2-R273H and -K244E. We show that the recombinant DDB2-T338M assembles with DDB1, but fails to show any detectable binding to DNA substrates with or without UV lesions, due to a potential structural disruption of the rigid DNA recognition β -loop. Consistently, we demonstrate that the cellular DDB2-T338M is defective in its recruitment to focally radiated DNA damages, and in its access to chromatin. Thus, we provide direct functional evidence indicating the DDB2-T338M recapitulates molecular defects of human XP mutants, and is the causal loss-of-function allele that gives rise to equine ocular SCCs. Our findings shed new light on the mechanism of DNA recognition by UV-DDB and on the initiation of ocular malignancy.

Keywords: Xeroderma pigmentosum, Squamous cell carcinoma, Equine genetics, Disease mutations, UV-DDB, Localized UV irradiation, Chromatin association

1. Introduction

Ultraviolet (UV) irradiation induces covalent crosslinks between neighboring pyrimidines. If left unrepaired, error-prone replication of this damaged DNA leads to increased rate of mutagenesis and genome instability [1,2]. Specialized damage-surveillance factor UV-DDB recognizes diverse photolesions, crucial for the dynamic process of nucleotide excision repair (NER) [3]. Loss of function mutations in the gene encoding the DDB2 subunit of UV-DDB are associated with a rare autosomal recessive syndrome Xeroderma Pigmentosum (XP), which is characterized by sun sensitivity and severe risk for skin cancers [4].

Cellular UV-DDB activity requires two protein subunits, DDB1 and DDB2. DDB2's WD40 domain provides the sole recognition interface for diverse photo-damage [5], including those buried in nucleosomes [6], whereas DDB1 is needed to facilitate the folding and stability of DDB2 [7]. DDB1 also serves as a substrate adaptor for Cul4 E3 ubiquitination ligase complexes [8,9]. DDB2's role in UV-DDB seem to reflect a more recent evolutionary adaption to repair UV-lesions: while DDB1 is conserved from fission yeast to human, DDB2's homologs have only been previously reported in vertebrates [4]. Inactivating DDB2 mutants R273H and K244E have been found in XP complementation group E (XP-E) human patients, whereas no DDB1 mutation has been reported. In addition, UV-DDB is limited by the transcription level of DDB2 mRNA, which is activated by the transcription factor P53 in human cells, but not in rodents, where very low level of UV-DDB can be detected [4].

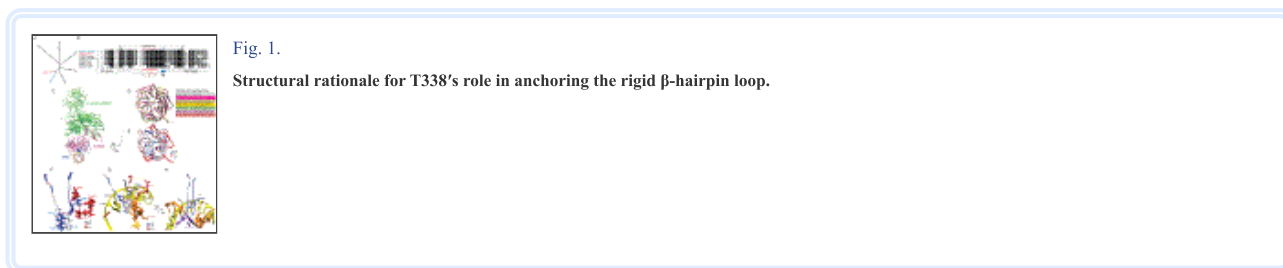
Squamous cell carcinoma (SCC) is frequently detected among XP patients [10], and is also the most common ocular cancer in horses [11]. We previously studied the Halfflinger horse breed with high SCC occurrence of the limbus, and found an autosomal recessive mode of inheritance [12]. A genome-wide association study (GWAS) followed by candidate gene sequencing identified a major genetic risk locus, where a missense mutation T338M of DDB2 gene was found as a candidate SCC allele [13]. Later, the same variant was found to be a risk factor for SCC initiated from the third eye lid, also known as the nictitating membrane [14], and from additional horse breeds [15,16]. This work led to the speculation that this was a loss-of-function variant and a causal risk factor for ocular SCC as computational modeling predicted that this mutation altered conformation of the β loop involved in photolesion recognition [13].

Here, we provide direct functional evidence indicating that the T338M mutant indeed is a loss-of-function allele that abolishes UV-DDB recognition of damaged DNA. We present a structural rationale for T338 to anchor the critical β -hairpin loop of DDB2 for proper DNA-engagement. Our biochemical and cellular characterizations of the T338M and human XP-E mutants prompted us to propose a 2-step damage-scanning model that critically depends on the β -hairpin loop anchored by the T338 residue.

2. Results

2.1. The conserved T338 of DDB2 tightly anchors the DNA-displacing loop of UV-DDB

We constructed a phylogenetic tree based on sequence of DDB2 homologs, and found the human and horse DDB2 protein are more related than the murine counterpart (Fig. 1A). While previously only reported in vertebrates, a blast analysis allowed us to detect an apparent DDB2 homolog in *Arabidopsis thaliana* (Q6NQ88), a photosynthesis organism. Thus, alignment of these evolutionarily diverse DDB2 reveals site-specific conservation and divergence within the β -hairpin loop, a crucial DNA-contacting loop connecting the 5th and 6th propeller domains of the WD40 fold of DDB2 (Fig. 1B and C). The tri-residues, F334, N335, and H336, directly contact undamaged DNA bases, forming a DNA-displacing finger [5,17] that are conserved across all species evaluated. While divergence in the rest of loop residues is observed, the T338 is one of the most conserved residues, with 100 % conservation across all seven diverse species examined (Fig. 1B).



The T338 locates spatially at the geometric center of the narrower plane of DDB2's WD40 barrel, surrounded by other propeller loops (Fig. 1C–E). The T338 tops the DNA-displacing finger protruding downward into the DNA minor groove (colored in blue, Fig. 1C). We hypothesize that the T338 may play an anchorage role for the DNA-displacing finger given its unique positioning. To describe the structural dynamics of T338, the DNA-displacing finger, and other WD40 loops under different biological context, we superimposed available X-ray crystallographic [5,8] or Cryo-EM structures [6] of UV-DDB complexes. Our structure superimposition includes those obtained with diverse origins (zebrafish fish vs. human) and composition (dimer vs. the CRL4-E3 complex), and with various type of damaged substrates (THF vs. 6–4pp) that are embedded in either naked or nucleosomal DNA (Fig. 1D). We found that the β -hairpin loop, including the T338 and the DNA-displacing finger, are spatially constraint in imposed structures, resulting in an invariant conformational consensus. This is unique among other WD40 loops, which show higher levels of conformational heterogeneity. The RMSD (root-mean-square derivation) of every α -carbon atom is rendered onto the superimposed structures in Fig. 1E. The comparison of the average RMSD score for each WD40 loops confirms that T338 resides in the hairpin loop with the highest structural conservation, indicating the DNA-displacing finger maintains high level of structural rigidity irrespective of the complex composition and substrates.

To explore T338's contribution to the structural rigidity of the hairpin loop, we compared the RMSD value and hydrogen bonding among loop residues. T338 has the lowest RMSD value among all loop residues (Fig. 1F). Furthermore, T338 can form three salt bridges simultaneously with residues in surrounding propellers. The hydroxyl group of T338 interacts with both Arg113 *via* electrostatic interaction (2.9 Å), and with A144 or V114 *via* a hydrogen bond (2.0 Å), as previously predicted [13]. In addition, the backbone oxygen of T338 engages in another hydrogen bond (2.6 Å) with the amid hydrogen of S396's side chain (Fig. 1F). The DNA-displacing finger is additionally stabilized by an inter-backbone hydrogen bond between L337 and F334 (2.3 Å).

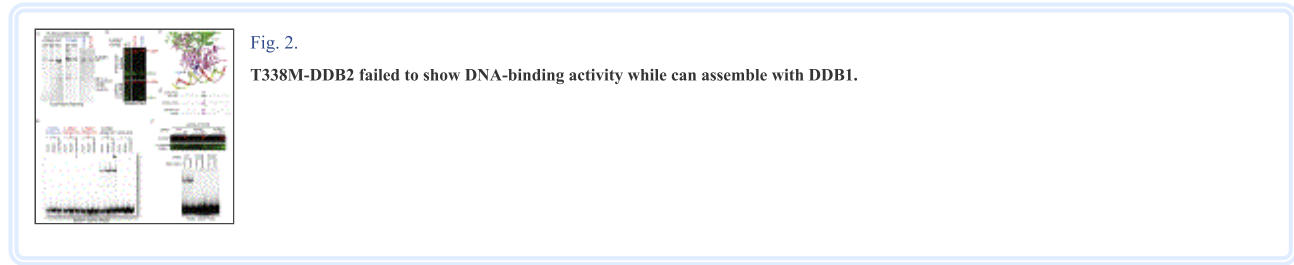
The high affinity, sequence-independent recognition of photodamaged DNA by UV-DDB depends on insertion of the DNA-displacing finger into the DNA minor groove, coupled with simultaneous flipping-out of the damaged base (D2, abasic) and its 3' neighbor (D1, Fig. 1G and H) [5]. The imidazole ring of H336 becomes coplanar with the undamaged base “Orphan2” (marked by an arrow in Fig. 1H) replacing D2. H336 also pi-stacks with the “D3” base on the damaged strand. The orphan2 is further stabilized by pi-stacking of its deoxyribose against F334 (Fig. 1H). Furthermore, the carboxamide of Q335 is paired with the base of Orphan1 by hydrogen-bonding (Fig. 1G).

As the immediate adjacent residue with tight spatial constraints, T338 is positioned at the anchor point for this precisely positioned DNA-displacing finger, which is required for damage recognition. Thus, our structural modeling predicts that the T338M mutant might interfere with DNA binding due to loss of hydrogen bonding potential and structural rigidity that are necessary to anchor DNA-displacing residues.

2.2. DDB2-T338M mutant can bind to DDB1 but is defective in DNA-binding

To assess the functional impact of the T338M substitution as predicted by our conservation and computational modeling, we reconstituted human UV-DDB complex containing several DDB2 variants (including the equine T338M) using multi-Bac expression system [18]. DDB2 folds poorly in the absence of DDB1 [9], therefore cDNAs encoding both subunits were cloned into a bi-cistronic targeting vector for baculovirus recombineering [7]. The DDB1 cDNA is fused with an N' terminal FLAG epitope tag that enables the purification of DDB2 variants that are pre-assembled with DDB1 in

insect cells (Hi-Five). We indeed detected in FLAG-purified fractions a prominent 110 kDa protein band, and a ~45 kDa species with lower abundance (Fig. 2A).



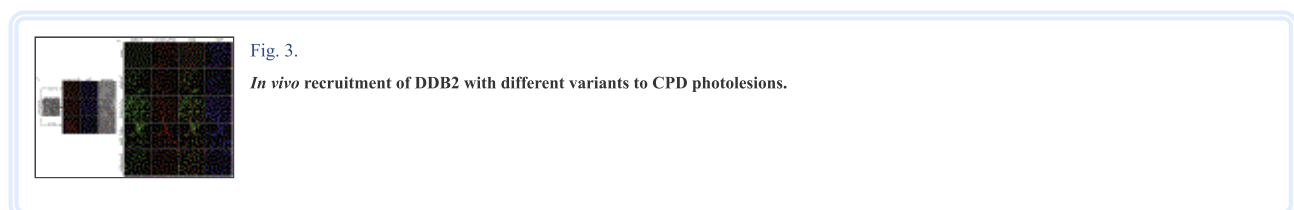
Several human XPE mutations, including K244E, are mapped to the DDB2-DNA interface [19,20], while others, including R273H, are mapped to the DDB1-DDB2 interface [21,22]. To assess the ability of the T338M DDB2 to assemble with DDB1, we included the XPE mutant R273H as a negative control, which is defective in DDB1-binding [23]. Indeed, DDB2 R273 locates near the larger plain of the DDB2 WD40 fold, but is spatially away from the helix-loop-helix motif of DDB2 that inserts into DDB1 (Fig. 2C). Nonetheless, R111, a loop residue of DDB1, is positioned within 4 Å of DDB2's R273.

Western blotting of the baculovirus-infected cell lysates confirmed that both DDB2 mutants, T338M and R273H, accumulated in insect cells to a comparable level as WT DDB2 (upper panel, Fig. 2B). We detected similar level of FLAG-DDB1 in all three FLAG-purified fractions. Only WT and T338M DDB2, but not R273H, co-purified with FLAG-DDB1 (lower panel, Fig. 2B). Consistent with the loss of R273H in the complex, the ~45 kDa band in the Coomassie-stained gel is no longer present (far right panel, Fig. 2A). Our result indicates the T338M DDB2 retains normal molecular interaction with DDB1, unlike the XPE mutant R273H.

The reconstitution of T338M-containing UV-DDB allows us to determine T338's role in DNA binding and UV damage-sensing. We performed EMSA experiments using 30mer DNA containing lesions at the two central thymidine, including a synthetic abasic site, and mismatches (Fig. 2D), which were previously shown to be substrates for UV-DDB [24]. As a negative control for the DNA binding ability, we additionally generated UV-DDB carrying K244E, another XPE mutants known to compromise DDB2's DNA binding and specificity for UV-damage [25]. To test whether a substitution of K244 to a bulky residue would also interfere with DNA binding, we also constructed the K244 F mutant. We showed that K244E and K244 F mutants both can co-purify with F-DDB1 at a similar level as for WT (Fig. 2F, upper). Results from the EMSA experiments suggest that WT UV-DDB can readily bind to undamaged DNA, and showed preferential binding to the abasic and mismatched probes (Fig. 2E and F). The UV-DDB-R273H showed no binding activity due to the lack of DDB2. To our surprise, equal or 3x excessive amount of the T338M mutant failed to exhibit any detectable binding activity to all DNA probes tested, phenocopying the K244 F mutant and the XPE mutant K244E (Fig. 2F, lower). Therefore, we demonstrated a severe DNA-binding defect of the T338M-containing DDB2 mutant, which is apparently folded and assembled properly with DDB1.

2.3. DDB2-T338M mutant failed to be recruited to focally irradiated foci in cells

The physiological substrate for UV-DDBs is chromatinized DNA with higher order structure that can not be recapitulated by *in vitro* DNA binding assays [26]. Furthermore, structural insights on UV-DDB-nucleosome have further supported a role for UV-DDBs as a specialized sensor and recognizer to UV damages occluded by chromatin [6]. To further examine DDB2-T338M's ability to recognize photolesions in a cellular context, we utilized a localized UV irradiation assay, whereby focal DNA damages can be introduced to nuclei by UV irradiation through a polycarbonate microfilter [27,28]. Cyclobutane pyrimidine dimers (CPDs) are the major form of photolesions, and can be detected by immunofluorescence (IF)-staining using an anti-CPD antibody [29]. To test the specificity of the microfilter assay, we monitor the IF pattern of CPD staining when HeLa cells are irradiated by 200 J/m² UVC light under 3 conditions (Fig. 3A). While only background fluorescence is detected when cells were covered with a layer of aluminum foil during the irradiation, we detect a pan-nuclei pattern with ring-shaped nuclei periphery highlighted when cells were directly irradiated through air only. Importantly, cells that irradiated through a 3μm microfilter exhibited robust pattern of CPD foci (ranging from 1 to 8) in all cells examined (Fig. 3A).



We generated HeLa cell lines stably expressing C-terminal V5 tagged DDB2 variants, including pcDNA vector-only as a negative control, DDB2-WT as a positive control, and the T338M mutant, alongside with the two human XPE mutant R237H and K244E (Fig. 3B for V5-IF and Fig. 4 for WB).

Upon 200 J/m² irradiation through a 3μM microfilter without period of recovery, DDB2-WT show nuclear foci formation in all cells examined, while T338M, R237H, and K244E-expressing cells show these variant protein remain evenly distributed throughout nuclei (Fig. 3B). To test whether the focal UV-induced DDB2 foci colocalize with CPD foci, we performed sequential IF procedures that begins with anti-V5 staining, followed by a fixation step, and DNA denaturing by 2 M HCl, and lastly anti-CPD staining. The fixation step is necessary to preserve the IF signal, as the DNA denaturing step destroys most antigen epitopes, while a prolonged DNA denaturing step is necessary for detecting robust CPD IF signals [30,31]. Notably, we observe robust colocalization between the DDB2-WT foci and the CPD foci (Fig. 3B) that is not detected in the mutants. However, we did confirm that T338M, R237H, K244E variants-expressing cells have a pan-nuclear pattern of DDB2 expression and were indeed focally irradiated at similar efficiency as WT cells. Thus, DDB2-T338M, as well as human XPE mutants, failed to be recruited to focally damaged DNA sites, suggesting the structural integrity of the β-hairpin loop, the proper assembly with DDB1, and the positive-charged WD40 interface with DNA are all independently required for proper recruitment of DDB2 to photolesions *in vivo*.

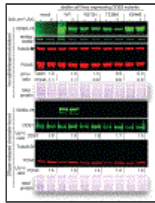


Fig. 4.

Biochemical fractionations to enrich chromatin-bound DDB2 variants with or without UVC irradiation.

2.4. DDB2-T338M and human XPE mutants show defect in chromatin association

To further test the cellular distribution of DDB2 variants, we biochemically fractionated cellular proteins into a soluble fraction under low-salt condition, and a chromatin-bound fraction released from high-speed pellets by DNaseI digestion. We also set up parallel experiments with or without UV radiation on all stable cell lines to monitor any UV-dependent alternations in protein abundance. While β-tubulin, an abundant component of cytoskeleton, is readily detected in the soluble fractions by WB (Fig. 4), we were not able to detect any trace of β-tubulin signal in the chromatin-bound fractions. In addition, soluble fractions do not show any UV-dependent alternation in protein level of endogenous UV-DDB and PCNA, an important scaffold protein that has been shown to transit from soluble nucleoplasmic form to chromatin-bound form in order to participate in DNA repair process [32]. However, we observe consistent, UV-dependent elevation of both DDB1 and PCNA level in all of our chromatin-bound fractions (Fig. 4). Meanwhile, DDB2's abundance in the soluble fractions appears to be downregulated upon UV, except for the K244E variant (Fig. 4). DDB2 has been shown to be subjected to UV irradiation-dependent proteasomal degradation [33]. However, the observation of K244E mutant to be resistant to UV-triggered degradation has been reported using independent methodologies [34]. Notably, only DDB2-WT proteins are detected at significant level in chromatin-bound fraction, while all variants do not appear to show comparable chromatin association with or without UV irradiation. In summary, we show that disruptions of DDB2's interfaces either with DNA in T338M and K244E, or with DDB1 in R237H compromise DDB2's ability to stably associate with chromatin, providing additional molecular rationale for the failure of these mutants to support UV-DDB repair.

3. Discussion

Our findings provide direct functional evidence supporting that the T338M missense mutation is the causal risk allele for squamous cell carcinomas from multiple horse breeds and ocular locations [13,15,16, 35]. In addition, our biochemical observations indicate that the proper orientation and rigidity of the DNA-binding hairpin loop of DDB2 is not only critical for recognizing damaged DNA lesions, but also required for basal-level DNA engagement involving contacts with undamaged DNA. We therefore propose a 2-step sequential model to explain how DDB2 utilizes its rigid DNA-displacing loop to gain access to photodamaged DNA (Fig. 5).

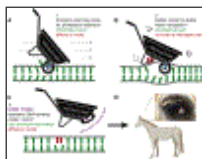


Fig. 5.

A 2-step sequential model explaining how DDB2 depends on its rigid DNA-displacing loop to stably recognize photo-lesions.

In the first damage-scanning step, DDB2 inserts its rigid DNA-displacing loop into the duplex, constantly displaying undamaged bases outward by competing with existing base-pairings. The resulted widening and/or unwinding of the DNA favorably brings the negatively charged phosphate-backbone in closer proximity to the bottom of the WD40 fold, where 16 basic residues reside. Thereby, DDB2 can sustain a low-affinity substrate-scanning mode in the absence of UV lesions (Fig. 5A), which correlates with DDB2's robust association with chromatin in absence of UV damages (

Fig. 4). This is further supported by the defective DNA-binding and chromatin association in the K244E mutant, whereby the charge swapping mutation could compromise the WD40-DNA interface.

In the subsequent damage-recognition step, once a damaged base is displayed by the DNA-displacing loop, an additional specialized damaging-sensing loops would capture the damaged base, contributing additional binding energy necessary to stabilize existing DNA contacts. The translocation of UV-DDB would in turn be stalled by the gain of stability, leading to a high affinity DNA-binding conformation that contributes to stable association to chromatin, and to efficient recruitment to UV-damaged chromatin sites, which enables subsequent recruitment of repair factors [27,36] (Fig. 5B).

The rigid DNA-displacing loop becomes mis-oriented in the T338M DDB2 mutant due to the loss of essential spatial constraints provided by the hydrogen bonding *via* T338's hydroxyl group, which is absent for Methionine (Fig. 5C). DDB2 with compromised DNA-displacing loop therefore becomes unable to initiate basal DNA-binding mode, resulting in failure to gain stable association with chromatin and to be recruited to the damaged sites as a critical initiator for the repair process, and eventually in carcinogenesis from UV-sensitive tissues in multiple horse breeds (Fig. 5D) [35]. Interestingly, the proposed 2-step model is in agreement with a single-molecule study on UV-DDB [25], where it was demonstrated that UV DDB inspects DNA for damages by a 3-dimensional scanning mode, and followed by a second phase of stable engagement to damaged sites.

UV-DDB is required to complement NER activity in cells derived from XP-E patients, but is not mechanistically required for *in vitro* reconstituted NER activity using naked DNA substrates [37], highlighting its specialized role in facilitating DNA repairs in chromatinized environment [6]. Our findings reveal a tight coupling between DDB2's *in vitro* DNA binding activity and its *in vivo* access to photolesions and chromatin among all variants tested, suggesting DDB2's cellular functions critically depend on its basal affinity toward naked DNA.

Although established as an inactivating XP-E mutation, how the R273H mutant fails to support UV-DDB is unclear [4]. Interfaces of DDB2 with DNA and DDB1 are both crucial for UV-DDB function. The observation that R273 does not participate directly in any of these two interfaces prompted the prediction that R273 contributes to DNA binding by stabilizing the structure of the 4th blade of WD40/DDB2 [5]. Our biochemical purification provides direct evidence that R273H mutant destabilizes the DDB1-DDB2 assembly. Due to the lack of DDB2-R273H from the fraction purified by DDB1, we could not directly study the DNA binding of DDB2-R273H [24]. Our results show that the R273H mutant failed to be localized to focal UV lesions, and that it failed to stably associate with chromatin, suggesting DDB2's cellular localization and UV-responsiveness rely on its ability to properly assemble with DDB1.

Different reports exploring binding affinity of UV-DDB toward different substrates reached apparently contradictory conclusions. For example, no significant binding was observed toward T-C mismatch, or naked DNA without damage [38], whereas basal level of DNA binding to undamaged DNA, and a preferential binding to mismatched DNA are evident from another study [24]. We do note the variation in DNA length, the type of UV lesion, and the molar ratio of competitive DNA used in these different studies. We did detect a basal level of binding affinity toward undamaged probes from our WT UV-DDB fraction, which is completely abolished with a single T338M mutation that disrupted the rigidity of the DNA-displacing loop. The fact that the T338M mutant assembles with DDB1 with normal efficiency argues against the possibility that the single amino acid substitution has led to a gross misfolding of the DNA-interface of DDB2. The physiological relevance of the displacing loop-dependent basal DNA affinity is supported by our observations that DDB2-WT stably associate with chromatin in the absence of UV irradiation, while T338M could not (Fig. 4).

Dysfunctional telomeres and the ensuing genome instability are major conduits to epithelial malignancy, but lab mice have long telomeres, and reduced level of chromosomal abnormality have been detected in most mice tumors [39]. As a result, epithelial cancers are rare in mice [40].

Additionally, UV-DDB is not detected in many rodent tissues. Perhaps it is not surprising that the adaptation of DDB2 protein for DNA-damage repair varies among species depending on behaviors, habitats, anatomical features, and metabolic demands of sun light. Valuable insights can be learnt from genetic and molecular dissection of tumors originated from additional model organisms, including the horse, as the horse shares similar telomere length and oncogenic exposures as humans.

4. Materials and methods

4.1. Plasmid constructs

The pFastBac-Dual plasmid expressing N-terminal FLAG epitope tagged human DDB1 cDNA and untagged human DDB2 cDNA was a generous gift from Kaoru Sugawara (Biosignal Research Center, Kobe University, Japan). The mammalian expression vector pcDNA3-DDB2-V5/His with Neocycin-resistance was obtained. [27]. T338M, R273H, K244E and K244 F were introduced into the DDB2 cDNA by site-directed mutagenesis reactions (QuikChange II, Agilent). All constructs in this study are in the process for Addgene deposition.

4.2. Baculovirus production and insect cell culture

To generate the recombinant bacmid, pFastBac-Dual plasmids was transformed into DH10BacY bacterial competent cells (Geneva) carrying a bacmid backbone with integrated YFP expression. The transformed cells were plated on blue-white screening plates containing 50 µg/mL Kanamycine, 7 µg/mL Gentamicin, 10 µg/mL tetracycline, 100 µg/mL X-gal, and 1 mM IPTG. White colonies were re-streaked on agar plates, confirmed by genotyping PCR, and inoculated in liquid culture for bacmid purification. Sf9 insect cells growing in 6-well plates (in SF900III medium, at 27°C) were transfected with purified DNA using FuGENE HD (Promega). Supernatant containing the viral stock was harvested 60 h post-transfected, and amplified in two consecutive passages at a MOI (Multiplicity of Infection) of 0.1 by infecting Sf9 cultured in suspension format. Cell density was maintained between 3×10^5 – 1.5×10^6 /mL in SF900III (Gibco). Cell morphology and percentage of YFP positivity were closely monitored. To express recombinant UV-DDBs, 15 mL of twice amplified baculovirus stock was used to infect 400 mL of HiFive insect cell growing in suspension at 1.0×10^6 /mL in SF900III medium. HiFive cells upon harvesting 48 h post infection are usually swelled, and 100 % YFP-positive.

4.3. Purification of recombinant UV-DDBs from insect cells

To prepare for the protein extracts, fresh HiFive cell pellets were washed with PBS, and resuspended with 8x vol of Lys450 buffer, containing 20 mM HEPES-NaOH pH 7.9, 450 mM NaCl, 0.5 % Triton X-100, 10 mM KCl, 4 mM MgCl₂, 0.2 mM EDTA, 10 % Glycerol, freshly added 0.2 mM DTT, 200 mM PMSF, and Protease Inhibitor Cocktail (P8340 Sigma), on a nutator for 30 min at 4 °C. The extract was then cleared by centrifugation at 28,000 g for 30 min at 4 °C. 10 mL of such cleared extract was incubated with 100 µL bed-volume of anti-FLAG agarose beads for at least on a nutator for 4 h at 4 °C. The immunoprecipitated complexes were washed extensive with Lys450. To competitive elute the FLAG bait proteins, 0.25 µg/µl of 3xFLAG peptide was included in the elution buffer (10 mM HEPES-NaOH pH 7.9, 10 % glycerol, 50 mM NaCl, 1.5 mM MgCl₂, 0.05 % Triton X-100, and freshly added 0.2 mM DTT, 200 mM PMSF, and Protease Inhibitor Cocktail), and multiple sequential elutions was performed in a thermomixer shaking at 1400 rpm at room temperature. Finally, elutions were filtered through empty chromatography columns (BIO-RAD 7326204). Aliquots snap-froze on dry ice and stored in –80°C freezer.

4.4. Antibodies used in western blotting and immuno-stainings

Anti-human DDB2 was obtained from Cell Signaling Technology (CST, 5416 T); anti-FLAG mouse monoclonal antibody (M2) was obtained from Sigma (F1804); Anti-FLAG EZview FLAG agarose beads were also obtained from Sigma (E6779); Rabbit anti-V5 monoclonal antibody was obtained from CST (13202); Mouse anti-β-Tubulin (D-10) monoclonal antibody was from Santa Cruz (sc-5274); Mouse monoclonal anti-PCNA (PC10) was from CST (2586 T); Rabbit anti-DDB1 polyclonal antibody was from Invitrogen (PA5-34631); Mouse anti-CPD (TDM-2) monoclonal antibody was obtained from Cosmo Bio. Secondary antibodies against Rabbit IgG (Green-colored, IRDye800, cat# 926-32211) and Mouse IgG (Red-colored, IRDye680, cat#926-68070) IgGs were obtained from LiCoR-Odyssey. Donkey anti-mouse IgG conjugated with Alexa Fluor 594, and donkey anti-rabbit IgG conjugated with Alexa Fluor 488 were obtained from the Jackson ImmunoResearch.

4.5. Electrophoretic mobility shift assay (EMSA)

Synthesized DNA oligos (sequence shown in Fig. 2D) were annealed using the following PCR program: 95 °C for 2 min, gradually cooled to 25 °C over 45 min, and separated by 10 % polyacrylamide gel electrophoresis (PAGE). The double stranded DNA probe was gel-purified and eluted in gel elution buffer, containing 20 mM Tris-HCl (pH 7.5), 0.25 M sodium acetate (pH 5.2), 1 mM EDTA (pH 8.0), 0.25 % SDS, overnight at 4 °C with end-to-end mixing. DNA probe was then purified by isopropanol precipitation. 2 pmol of DNA probe was end-labeled by T4 polynucleotide kinase in presence of γ-32 P ATP. The labeled probe was purified using Qiagen PCR purification column and stored in –20 °C for up to one month.

10 µL of EMSA reactions were set up on ice containing a buffer of 20 mM HEPES-NaOH (pH 7.9), 50 mM NaCl, 0.02 % NP-40, 0.02 % Triton X-100, 5% Glycerol, 5 mM MgCl₂, 0.1 mg/mL BSA, 1 mM DTT, 200 µM PMSF, protease inhibitors, and 0.1 µg/µl poly(dI-dC). Reaction components were combined in following order: recombinant proteins, the reaction buffer, and lastly ~30 fmole of labeled DNA probe. The binding reaction were performed at 30 °C for 40 min, and separated by PAGE (5% 19:1 acrylamide:bis, 0.25x TBE, 1 % glycerol, pre-run at 120 V for 30 min) and run at 120 V in a cold room for 3 h with constant buffer circulation. The gel was dried, then exposed to a phosphor-imager screen, and scanned by a Typhoon scanner. The scanned image was analyzed using TotalLab Quant software.

4.6. Stable hela cell lines

Hela cells were cultured in standard DMEM containing 10 % FBS. pcDNA3-DDB2-V5/His (NeoR) and derivatives were used to reverse-transfect Hela cells using Fugene-HD (Promega). 30 h post-transfection, cells were selected by 400 µg/mL G418 (Geneticin, Gibco) for 9 days, and were maintained in the presence of 100 µg/mL G418.

4.7. Microfilter UV irradiation

For co-IF of DDB2 and CPD, 4×10^4 HeLa cells were seeded onto 12 mm coverslips in a 24-well plate, precoated with 20 $\mu\text{g}/\text{mL}$ collagen and 1 $\mu\text{g}/\text{mL}$ fibronectin in PBS for 1 h at 37 °C. The coverslips were transferred with help of a hooked 30-G needle and a curved tweezer. 2~3 days post-seeding, cells were washed twice with PBS. 3 μM polycarbonate microfilter was cut into a 12 mm circle, pre-wet, and placed on top of 500 μL PBS in a 24-well plate. UVC irradiation was applied with a UVP Hybrilinker Oven (analytikjena) at 200 J/m^2 . Immediately prior to irradiation, PBS was gently withdrawn in full using a P1000 pipette to enable a close contact between cells and the membrane, and was applied back to the well immediately post-irradiation. Cells were fixed in 4 % formalin/PBS at room temperature for 10 min, washed twice with PBS, and permeabilized with 0.5 % Triton X-100/PBS on ice, followed by two washes with PBS. Only one coverslip was processed at a time.

4.8. Sequential staining procedure for co-IF of DDB2 and CPD

Cells were washed twice with PBS in the 24-well plate and blocked with 20 %FBS/PBS on a rotating nutator at 37 °C for 30 min. Then, cells were washed three time with 1 mL of PBS, and equilibrate once in 200 μL 5 %FBS/PBS. 1: 500 dilution of the rabbit anti-V5 primary antibody were diluted in 200 μL 5 %FBS/PBS, and incubated on a nutator at 37 °C for 30 min. After PBS washes, secondary antibody (donkey anti-Rabbit AlexFluor488) was applied to the samples similar as the primary antibody. Cells were subsequently washed four times with PBS. To preserve the DDB2 IF signal, cells were re-fixed in 4 % formalin/PBS at room temperature for 10 min, washed twice with PBS, and followed by a dehydration step in 70 % ethanol at room temperature for 10 min. After two PBS washes, cells were incubated in 2 M HCl at room temperature for 10 min. After three PBS washes, cells were re-blocked and were stained with the mouse anti-CPD antibody following the similar procedure as the first round of staining. Cells were counter-stained with 100 ng/mL of DAPI in PBS for 5 min prior to mounting with Prolong Gold medium. Images were captured by a Leica DM5000 B microscope with 63x oil objective. The images were processed in ImageJ with the Bio-Formats. The montages were generated using FigureJ.

4.9. Fractionation of chromatin-bound proteins upon UV irradiation

HeLa cells were growing in 10 cm dishes until confluency under standard condition. For dishes to be irradiated, cells were PBS-washed and aspirated in full, and immediately UVC-irradiated in a UVP Hybrilinker Oven (analytikjena) at 200 J/m^2 . Fresh culture medium was immediately replenished to allow cellular responses for 1 h in the tissue culture incubator. Irradiated and control cells were subsequently harvested by trypsinization, and resuspend in a 400 μL low salt NP40 lysis buffer (25 mM HEPES, 150 mM KCl, 1.5 mM MgCl_2 , 0.5 % NP40, 10 % glycerol, PH adjusted to 7.5 with KOH, with freshly added 1 mM DTT, 200 μM PMSF, and 1:1000 Protease Inhibitor Cocktail [Sigma, P8340]), and placed on a nutator for gentle mixing at 4 °C for 15 min, followed by a centrifugation step at 21,000 \times g for 20 min at 4 °C. After removal of the supernatant in full, the remaining chromatin pellets were completely resuspended by gentle pipetting using 400 μL DNaseI digestion buffer, containing 300 Kunitz units/mL of DNaseI (from bovine pancreas, Sigma DN25), 10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl_2 , 0.5 mM CaCl_2 , 10 % glycerol, and freshly added 1 mM DTT, 200 μM PMSF, and 1:1000 Protease Inhibitor Cocktail (Sigma, P8340). The digestion was performed on a nutator rocking at 37 °C for 30 min, followed by a centrifuge step of 21,000 \times g for 1 min at 4 °C. Soluble NP40 fractions and DNaseI-released fractions were quantitated using Bradford assays, and 50 μg of total proteins were separated by SDS-PAGE, and analyzed by western blotting. Total proteins were also visualized by Coomassie staining.

4.10. Structural visualization and superimposition

Molecular graphics and analyses were performed with UCSF Chimera (<http://www.rbvi.ucsf.edu/chimera/>). The RMSD value of superimposed structural model was calculated and rendered graphically using the structural comparison tool- MatchMaker.

4.11. Phylogenetic alignment

DDB2 homologs were identified by searching in the UniProt knowledgebase (<https://www.uniprot.org/>). The amino acid alignment was built using Geneious prime using Clustal Omega. To build the phylogenetic tree, pairwise distances were calculated based on the multiple sequence alignment using the Jukes-Cantor distance model. The tree was built using the UPGMA method.

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Footnotes

Declaration of Competing Interest

RRB directs the Veterinary Genetics Laboratory (University of California-Davis), where a commercial test for the DDB2-T338M mutation in horses is offered.

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