Molecular insights into the recruitment of TFIIH to sites of DNA damage

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XPB and XPD subunits of TFIIH are central genome caretakers involved in nucleotide excision repair (NER), although their respective role within this DNA repair pathway remains difficult to delineate. To obtain insight into the function of XPB and XPD, we studied cell lines expressing XPB or XPD ATPase-deficient complexes. We show the involvement of XPB, but not XPD, in the accumulation of TFIIH to sites of DNA damage. Recruitment of TFIIH occurs independently of the helicase activity of XPB, but requires two recently identified motifs, a R-E-D residue loop and a Thumb-like domain. Furthermore, we show that these motifs are specifically involved in the DNA-induced stimulation of the ATPase activity of XPB. Together, our data demonstrate that the recruitment of TFIIH to sites of damage is an active process, under the control of the ATPase motifs of XPB and suggest that this subunit functions as an ATP-driven hook to stabilize the binding of the TFIIH to damaged DNA.

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Introduction

DNA and RNA helicases are a ubiquitous, yet diverse, group of enzymes present in viruses, prokaryotes and eukaryotes (Delagoutte and von Hippel, 2003). They convert chemical energy of nucleoside triphosphate hydrolysis to the mechanical energy necessary to transiently separate the strands of duplex nucleic acids (Tuteja and Tuteja, 2004). By this mean, they provide the single-stranded DNA or RNA intermediates necessary for replication, transcription, recombination or repair. Furthermore, it has been shown that helicases can also effectively displace bound proteins from DNA or RNA (von Hippel, 2004). There are several known human diseases caused by defective helicases (Ellis, 1997). Among these disorders, the cancer-prone *Xeroderma pigmentosum* (XP), alone or in combination with the Cockayne syndrome

(CS), and the *Trichothiodystrophy* (TTD) are noteworthy as they entail mutations in the XPB and XPD superfamily 2 helicases. Both of these helicases are part of the same TFIIH complex. TFIIH is composed of a seven-subunit core (XPB, XPD, p62, p52, p44, p34 and p8/TTD-A) associated with the CAK subcomplex (Cdk7, cyclin H, and MAT1) (Giglia-Mari *et al*, 2004; Ranish *et al*, 2004). TFIIH functions in both transcription initiations of mRNA and rRNA (Iben *et al*, 2002), as well as in nucleotide excision repair (NER) (Schaeffer *et al*, 1993).

XPB and XPD patients are photosensitive and display a 1000-fold increase in melanoma risk because of defects in the NER function of TFIIH (Lehmann, 2003). NER removes a broad spectrum of DNA lesions including UV-induced pyrimidine dimers and bulky, helix-distorting adducts caused by toxic chemicals such as the anticancer drug cisplatin (Sancar, 1996). In mammalian cells, the proteins necessary for the incision reaction include XPC-HR23b, TFIIH, XPA, RPA and the nucleases XPG and ERCC1-XPF (Araujo *et al*, 2000). The removal of lesions requires their recognition by the repair factor XPC-HR23b and the subsequent opening of the DNA duplex by TFIIH. The single-stranded structure is then stabilized by XPA and RPA, and the margins of the resulting DNA bubble are recognized by XPG and ERCC1-XPF, thereby generating 3' and 5' incisions relative to the damage, respectively (O'Donnovan *et al*, 1994; Sijbers *et al*, 1996).

As XPB and XPD helicases are both integral parts of TFIIH, their individual molecular roles in NER remain difficult to delineate. As XPB and XPD are helicases with opposite polarities, it was originally suggested that they could cooperate to open DNA on the 5' and 3' sides of a lesion, respectively (Schaeffer *et al*, 1994). Indeed, mutation of the ATPase activity of either XPB or XPD results in the inability to remove DNA lesions (Sung *et al*, 1988; Guzder *et al*, 1994). Refining these proposals, recent data bring into question the direct role of the helicase activity of XPB in NER and transcription, and suggest that only the ATPase activity is required (Lin *et al*, 2005; Coin *et al*, 2007; Richards *et al*, 2008). Supporting the prime role of the ATPase activity of XPB in TFIIH functions, we recently showed that this activity was regulated by the p52 subunit of TFIIH (Coin *et al*, 2007) and by the damage recognition factor XPC (Bernardes de Jesus *et al*, 2008). Contrary to XPB, the helicase activity of XPD, which is regulated by the p44 subunit of TFIIH (Coin *et al*, 1998), is required for efficient opening of the DNA around the damage, but is dispensable for transcription (Tirode *et al*, 1999; Coin *et al*, 2007).

To further our understanding of the mechanistic details of XPB and XPD function, we analysed the behaviour of ATPase-deficient TFIIH complexes *in vivo*. We found that a TFIIH complex deficient in the ATPase of XPB was not recruited to sites of DNA damage, whereas a complex deficient in the ATPase of XPD did. More surprisingly, we discovered that the recruitment of TFIIH to these sites does not require the helicase activity of XPB but depends on two motifs, a R-E-D

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residue loop and a positively charged flexible Thumb (ThM) motif that were identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan *et al*, 2006). We analysed the molecular details of R-E-D and ThM impact on XPB activities and found that they were required to stimulate ATP hydrolysis in the presence of DNA. We propose a mechanism in which XPB functions as an ATP-dependent hook that uses the ATPase, R-E-D and ThM motifs to anchor TFIIH to the sites of DNA damage during DNA repair.

Results

The ATPase activity of XPB anchors TFIIH to the sites of DNA damage in vivo

To functionally discriminate between the ATPase activities of XPB and XPD, we produced recombinant TFIIH/XPD(K48R) and TFIIH/XPB(K346R) in baculovirus-infected cells (Tirode *et al*, 1999) and tested them in DNA repair and transcription assays. These complexes are mutated in the ATPase Walker A motif of XPD and XPB, respectively. When incubated in the presence of recombinant TBP, TFIIA, TFIIB, TFIIE and TFIIF transcription factors in addition to purified RNA polymerase II and a linearized DNA template containing the adenovirus major late promoter (Tirode *et al*, 1999), TFIIH/XPD(K48R) supported transcription, contrary to TFIIH/XPB(K346R), which was totally inactive (Figure 1A, upper panel). To test the repair capacity of the different TFIIH complexes, we used a reconstituted dual incision assay composed of the recombinant XPC-HR23b, XPA, RPA, XPG, ERCC1-XPF factors and a closed-circular plasmid (Pt-DNA) containing a single 1,3-intra-strand d(GpTpG) cisplatin-DNA crosslink as a template (Araujo *et al*, 2000). None of the mutated complexes was able to excise the damaged oligonucleotide (Figure 1A, lower panel). In a permanganate footprinting assay that measures the opening of the DNA around the lesion (Tapias *et al*, 2004), addition of TFIIH(WT) induced an increased sensitivity of nucleotides at positions T+5, T+6, T-4, T-5, and, to a lesser extent, T-7 and T-10 (Figure 1B, lane 2), indicative of DNA opening. In contrast, neither TFIIH/XPB(K346R) nor TFIIH/XPD(K48R) were able to open damaged DNA (compare lanes 3–4 with lane 2).

To analyse the behaviour of ATP-deficient TFIIH complexes *in vivo*, we used a stably transfected Chinese hamster ovary (CHO)-UV5 cell line expressing an HA-tagged version of the human XPD(K48R) protein (Winkler *et al*, 2000). Using the CHO-27-1 cells mutated in the hamster homologue of XPB (Ma *et al*, 1994), we also generated a stably transfected cell line expressing a C-terminally GFP-tagged version of the human XPB WT or K346R protein. The functionality of an XPB-GFP fusion construct was established earlier (Hoogstraten *et al*, 2002). We used immunofluorescent labeling after local UV irradiation of stably transfected cells (Volker *et al*, 2001) to assess the nuclear distribution pattern of XPB and XPD. Immunostaining with antibodies against cyclobutane pyrimidine dimers (CPDs) showed that UV damages were located in discrete local spots in the nucleus (Figure 1C, panels a, e, i, m). Both, human wild-type XPB and XPD proteins colocalized with CPD spots, indicating that TFIIH was efficiently recruited to the damaged sites in these cells (panels a–d and i–l). Surprisingly, although signals of XPD(K48R) colocalized with CPD spots in CHO-UV5 cells (panels m–p), signals of XPB(K346R) showed an homoge-

nous distribution pattern through the nucleus (panels e–h), indicating that TFIIH/XPB(K346R) complex was not recruited to the damaged sites (Figure 1D). These data suggest that the accumulation of TFIIH to sites of DNA damage takes place in the absence of an active XPD protein but requires functional XPB.

New motifs in XPB required for the activity of TFIIH in NER

By introducing mutations in some of the seven canonical helicase motifs of XPB, we demonstrated recently that its helicase activity was not required for TFIIH repair function (Coin *et al*, 2007). Recently, three additional motifs were identified in a homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan *et al*, 2006). To determine whether these newly identified motifs have a function in the activities of the human TFIIH complex in transcription and repair, we designed four mutants (E253A, E253A/R283A, E473A and Δ 516–526) depicted in Figure 2. We introduced an E253A substitution located at the end of the first β -strand that was combined, when indicated, with an R283A mutation located at the beginning of the α -helix of a putative damage recognition domain (DRD). We also designed an E473A substitution in the R-E-D residue loop to change the local negative charge of the motif, and we deleted the positively charged ThM domain from amino acid 516 to 526 (Δ 516–526) (Figure 2).

To investigate the importance of the DRD, R-E-D and ThM motifs of XPB in the repair function of TFIIH, we first performed a host-cell reactivation assay (Carreau *et al*, 1995). A UV damaged reporter construct, carrying a luciferase gene (pLuc) was transiently transfected into CHO-27-1 cells, together with vectors coding for β -galactosidase and for human XPB proteins. Transfection of either XPB(E253A) or XPB(E253A/R283A) restored luciferase expression that reached the level observed with XPB(WT) (Figure 3A, lanes 1–5). In marked contrast, XPB(E473A) and XPB(Δ 516–526) were not able to restore luciferase expression (lanes 7–8), a defect also observed with XPB(K346R) (lane 6). The various XPB were expressed at a similar level with the exception of XPB(Δ 516–526) whose expression was slightly reduced compared with the wild type (Figure 3A).

Next, we carried out a UV-survival assay and for that purpose we established CHO-27-1 cells stably expressing the new XPB-GFP mutant proteins. Immunoprecipitations using a rabbit polyclonal antibody, recognizing the hamster homologue of the core TFIIH subunit p62, demonstrated that the various XPB were efficiently incorporated into the hamster TFIIH complex (Figure 3B). The stably transfected CHO-27-1 cells were UV irradiated at different doses (3, 6 and 9 J/m²) and their survival was measured. Expression of XPB(WT), XPB(E253A) and XPB(E253A/R283A) induced a substantial rescue of the UV survival of the CHO-27-1 cells compared with nontransfected control (Figure 3C). On the other hand, the UV-survival curve of XPB(E473A) and XPB(Δ 516–526) transfected cells fell into the range of both the nontransfected parental CHO-27-1 cells and those transfected with the NER-deficient XPB(K346R) control. These data indicate that the R-E-D and ThM domains of XPB are crucial for the repair activity of TFIIH, while the putative DRD is dispensable.

Figure 1 The ATPase activity of XPB is required to anchor TFIIF to damaged chromatin. **(A)** A measure of 25, 50 and 100 ng of TFIIF(WT), TFIIF/XPB(K48R) or TFIIF/XPB(K346R) was tested either in a reconstituted transcription assay (Tx, upper panel) or in a dual incision assay (NER, lower panel) as described (Coin *et al*, 2004). The sizes of the incision and transcription products are indicated. **(B)** TFIIF(WT), TFIIF/XPB(K48R) or TFIIF/XPB(K346R) (100 ng) were incubated with a radio-labelled linear DNA fragment from the Pt-DNA plasmid and 40 ng of XPC-HR23b, 25 ng of XPA, 50 ng of RPA and 150 ng of XPG in a KMnO_4 footprinting assay. Lane 1; Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate KMnO_4 sensitive sites. Adducted strand residues to the 3' and 5' of T0 are denoted by positive and negative integers (+ N, -N), respectively. **(C)** Stably transfected CHO-27-1 expressing a GFP-tagged version of the human WT or K346R XPB proteins (upper panel) and stably transfected CHO-UV5 cells expressing an HA-tagged version of the human WT or K48R XPD proteins (bottom panel) (Winkler *et al*, 2000) were UV irradiated at 100 J/m^2 through the $3 \mu\text{m}$ pore filter and fixed 30 min later. Immunofluorescent labelling was performed using a rabbit polyclonal anti-GFP (panels b and f), a rat monoclonal anti-HA (panels j and n) or a mouse monoclonal anti-CPD (panels a, e, i, m). Nuclei were counterstained with DAPI (panels c, g, k, o), and slides were merged (panels d, h, l, p). **(D)** Quantitative analysis of the recruitment of TFIIF to sites of DNA damage in transfected cells. Values represent averages $\pm$ s.d. ($n = 100$ sites of DNA damage) from three independent experiments.

R-E-D and ThM motifs are needed for an optimal ATPase activity of XPB

To decipher the molecular details of the repair defect generated by the E473A and $\Delta 516$ –526 mutations, we produced recombinant TFIIF complexes in baculovirus-infected cells containing the corresponding mutated XPB subunits (Tirode *et al*, 1999). Western blot analysis of the recombinant TFIIF/XPB(E253A), XPB(E253A/R283A), XPB(E473A) and XPB($\Delta 516$ –526) complexes revealed a similar subunit composition compared with the TFIIF(WT) complex (data not shown). When tested in either a dual incision or in a transcription assays, TFIIF/XPB(E253A) and XPB(E253A/R283A) were as active as TFIIF(WT) in excising damaged DNA (Figure 4A, upper panel, compare lanes 5–8 with lanes 3–4) or synthesizing RNA (lower panel). In contrast, TFIIF/XPB(E473A) and XPB($\Delta 516$ –526) were inactive in repairing damaged DNA and in synthesizing RNA (Figure 4A, compare lanes 11–12 and 13–14 with lanes 3–4), similarly to TFIIF/XPB(K346R) (lanes 9–10).

In a permanganate footprint assay, TFIIF/XPB(E473A) and XPB($\Delta 516$ –526) were unable to open the damaged DNA (Figure 4B, lanes 5–6), compared with TFIIF(WT) or

XPB(E253A/R283A) (lanes 2–3). Altogether, the above data drew our attention to the critical role of both the R-E-D and ThM motifs of XPB in damaged DNA opening.

R-E-D and ThM motifs are needed for the anchoring of TFIIF to the sites of DNA damage

We next measured the recruitment of the TFIIF complexes carrying mutations in the newly identified motifs to sites of DNA damage *in vivo*. XPB(E253A/R283A) colocalized with CPD spots (Figure 5, panels e–h), indicating that the corresponding TFIIF complexes translocated to the sites of DNA photolesions. XPB(E473A) and XPB($\Delta 516$ –526), however, displayed a homogeneous distribution pattern through the nucleus (panels i–l and m–p), which parallels the pattern observed in Figure 1C with XPB(K346R). This homogenous distribution contrasted with the local accumulation to the damaged sites of a TFIIF/XPB(T469A) complex (panels q–t) containing a mutation that has been shown to impede the helicase activity of XPB but not the NER function of TFIIF (Coin *et al*, 2007). We conclude from these data that the R-E-D and ThM motifs are required, together with the Walker A

Figure 2 XPB conserved sequences, motifs and structural architecture. (A) Upper panel shows the location of the human XPB structural domains: the four classical helicase motifs (I, Ia, II and III) of the first helicase module (HD1) are indicated in blue. The three helicase motifs (IV, V and VI) of the second helicase module (HD2) are indicated in green. The putative damage recognition (DRD), R-E-D and Thumb (ThM) domains identified in an homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* (Fan *et al*, 2006) are indicated, respectively, in light blue, red and purple. The mutations E253A, R283A, K346R, E473A and $\Delta 516-526$ are annotated. Lower panel shows the sequence conservation of the DRD and R-E-D motifs between human (h) and *Archaeoglobus fulgidus* (Af) XPB proteins. The β -strands (S1-4) and the α -helix (H1) are indicated. The conserved helicase motif III indicated by a blue square is located close to the R-E-D motif indicated by a red opened square. The three hairpin loops potentially involved in DNA binding are indicated ($\Pi 1$, $\Pi 2$, $\Pi 3$). Residues mutated in this study are marked in red. (B) View of the ribbon representation of AfXPB. The HD1 is indicated in blue, HD2 in green. The putative DRD, R-E-D and ThM domains are indicated in light blue, red and purple, respectively. The positions of the new mutations are indicated.

motif, for the recruitment of the TFIIF complex to sites of DNA damage.

R-E-D and ThM motifs stimulate the DNA-dependent TFIIF ATPase activity

Data above show common biochemical and biological defects for TFIIF complexes mutated either in the ATPase, R-E-D or ThM motifs of XPB and suggest a link between the newly

identified motifs of XPB and its ATPase activity. To assess the contributions of these motifs on the hydrolysis of ATP by XPB, we performed ATPase assay. Our data show that the TFIIF/XPB(E473A) and TFIIF/XPB($\Delta 516-526$) displayed about 40% of residual ATPase activity compared with TFIIF(WT) (Figure 6A, compare lanes 1-3 with 10-12 and 13-15). TFIIF/XPB(E253A) or TFIIF/XPB(E253A/R283A) exhibited the same activity as TFIIF(WT) (lanes 1-6 and data

Figure 3 Analysis of the function of the new motifs in NER *in vivo*. (A) CHO-27-1 cells were transfected with UV-irradiated pLuc plasmid expressing the luciferase gene (lanes 2–8) in combination with vector expressing either XPB(WT) (lane 3), XPB(E253A) (lane 4), XPB(E253A/R283A) (lane 5), XPB(K346R) (lane 6), XPB(E473A) (lane 7) or XPB($\Delta 516-526$) (lane 8). The luciferase activity in cell lysates (48 h post-transfection), normalized with the internal β -galactosidase standard, assesses repair complementation. Results are expressed as relative luciferase activity. Values represent averages $\pm$ s.d. from three independent experiments. A measure of 50 μ g of total extract were resolved by SDS-PAGE and western blotted (WB) with a mouse anti-human XPB antibody (Coin *et al*, 2004). Note that XPB($\Delta 516-526$) migrates slightly lower than the others because of the deletion. (B) TFIIH from 100 μ g of extracts prepared from untransfected CHO-27-1 (lane 1) or CHO-27-1 stably expressing XPB (lanes 2–6) was immunoprecipitated with a polyclonal antibody against the p62 subunit of TFIIH and resolved by SDS-PAGE, followed by Western blotting with a mouse anti-human XPB and a mouse anti-p62 antibody. LC; light chain of the antibody. (C) Quantitative UV-survival analysis of transfected CHO-27-1 cell lines. Results are expressed as a ratio between the cells that survived after irradiation and the cells that survived without treatment. Values represent averages $\pm$ s.d. from three independent experiments.

not shown). TFIIH/XPB(K346R) showed only 20% residual ATPase activity (corresponding to that of XPD (Coin *et al*, 2006)), compared with TFIIH(WT) (compare lanes 1–3 with 7–9).

XPB and XPD are DNA-dependent ATPases (Roy *et al*, 1994). The ATPase activity of TFIIH(WT), low in the absence of DNA, is stimulated by double-stranded DNA (Figure 6B, compare lanes 1–2 with 7–8). In contrast,

TFIIH/XPB(E473A) showed almost no DNA-induced ATPase stimulation (Figure 6B, compare lanes 5–6 with 11–12). More importantly, in the absence of DNA, TFIIH/XPB(WT) and XPB(E473A) displayed similar specific ATPase activities (Figure 6B, compare lanes 1–2 and 5–6) that were slightly higher than the TFIIH/XPB(K346R) activity (compare lanes 1–2, 5–6 and 3–4). Similar observations were obtained with TFIIH/XPB($\Delta 516-526$) (Supplementary data 1). Altogether,

Figure 4 Mutations in R-E-D and ThM motifs impair the ATPase activity of XPB. **(A)** A measure of 25 and 75 ng of TFIIH(WT), TFIIH/XPB(E253A), XPB(E253A/R283A), XPB(K346R), XPB(E473A) or XPB(Δ516–526) was tested in a dual incision assay (NER, upper panel) or in a reconstituted transcription assay (Tx, lower panel) as described (Coin *et al*, 2004). Lane 1 contains highly purified Hela TFIIH (Giglia-Mari *et al*, 2004). Lane 2 contains all the factors except TFIIH. The sizes of the incision or transcription products are indicated. The transcription and repair signals were quantified using Genetool (Syngene). **(B)** A measure of 100 ng of the various TFIIH complexes were tested in a KMnO₄ footprint assay (see Figure 1B). Lane 1; Pt-DNA with BSA only. Residues are numbered with the central thymine of the crosslinked GTG sequence designated T0. Arrows indicate KMnO₄ sensitive sites. Adducted strand residues to the 3' and 5' of T0 are denoted by positive and negative integers (+N, -N).

these data indicate that the R-E-D and ThM motifs do not affect the basal intrinsic ATPase activity of XPB but are required for the stimulation of this activity by DNA.

Discussion

To efficiently protect the genome, cells need to detect all types of DNA structural alterations embedded in billions of normal base pairs. The identification of the various proteins that execute NER was done through extensive studies of human cells deficient in this repair pathway (Maillard *et al*, 2007). Both *in vivo* and *in vitro* experiments identified XPC as the first factor that binds the damaged DNA (Sugasawa *et al*, 1998; Volker *et al*, 2001; Riedl *et al*, 2003). TFIIH is recruited to the lesion immediately after XPC (Yokoi *et al*, 2000; Riedl *et al*, 2003), presumably through direct protein–protein interaction (Bernardes de Jesus *et al*, 2008). The role of TFIIH is devoted to the opening of the DNA around the damaged site, but the individual function of its helicase subunits in this step remains difficult to delineate.

Earlier studies from our laboratory have shown that mutations in the helicase motifs III (T469A) or VI (Q638A), which impaired the helicase activity of the XPB subunit, did not inhibit the NER activity of TFIIH (Coin *et al*, 2007), thus raising the question of the role of XPB in NER. Here, we showed that TFIIH containing mutation in the motif III of XPB is recruited to the DNA repair sites after UV irradiation. However, a mutation in the helicase motif Ia, which abolishes the ATPase activity of XPB, thwarts the accumulation of TFIIH to these sites. This implies that the recruitment of TFIIH to sites of damage is an active process that requires

ATP hydrolysis. In contrast, the ATPase activity of XPD, the second helicase of TFIIH, is not required to recruit TFIIH to the damage sites, although it is needed for DNA repair.

In addition to the aforementioned ATPase motif, we found that two additional motifs, the R-E-D and ThM motifs, are implicated in the recruitment of TFIIH to sites of DNA damage. These two domains, highly conserved in human XPB, were identified in an homologue of XPB from the thermophilic organism *Archaeoglobus fulgidus* and were suggested to be involved in TFIIH functions (Fan *et al*, 2006). Mutations in the R-E-D and ThM motifs mimicked the biochemical and biological defects obtained with a mutation in the ATPase motif. This suggests that the ATPase, R-E-D and ThM motifs work together to ensure a correct recruitment of TFIIH to the damaged sites before the opening and dual incision steps take place during NER. How the R-E-D and ThM motifs participate to the anchoring of TFIIH? The ThM domain has not been found in other helicases, including XPD (Bienstock *et al*, 2002; Fan *et al*, 2008; Liu *et al*, 2008; Wolski *et al*, 2008), but a similar helical protrusion has been observed in DNA polymerases (Doublié *et al*, 1998) and in *Sulfolobus solfataricus* SWI2/SNF2 ATPase Rad54 (Durr *et al*, 2005), in which it is expected to grip double-stranded DNA from the minor groove. The structure of XPB suggests that the energy furnished by the ATP hydrolysis is used to induce a flip of 170° of the HD2 domain after the binding of XPB to DNA (Fan *et al*, 2006) (Figure 7). The R-E-D (present in HD1) and the ThM (present in HD2) are then in close vicinity and are used to stabilize TFIIH on the DNA by introducing a wedge (the E473 residue) in the double-stranded DNA, gripped by the ThM motif. To obtain experimental evidence

Figure 5 Recruitment of TFIIH to local sites of DNA damage. (Left panel) CHO-27-1 cells were stably transfected with pEGFP plasmids expressing various forms of GFP-tagged XPB proteins. These cells were irradiated with UV light (100 J/m^2) through the $3\text{-}\mu\text{m}$ pore filter and fixed 30 min later. Immunofluorescent labelling was performed using either a mouse monoclonal anti-CPD (panels A, E, I, M, Q) or a rabbit polyclonal anti-GFP (panels B, F, J, N, R). Nuclei were counterstained with DAPI (panels C, G, K, O, S), and slides were merged (panels D, H, L, P, T). (Right panel) Quantitative analysis of the recruitment of TFIIH to sites of DNA damage in transfected cells. Values represent averages $\pm$ s.d. ($n = 100$ sites of DNA damage) from three independent experiments.

for this model, we compared the ATPase activities of the WT and mutated complexes with or without DNA. Indeed, like most SF1 and SF2 members, DNA stimulates the ATPase activity of TFIIH (Roy *et al*, 1994). In the presence of DNA, mutations in the R-E-D and ThM motifs induces 60% inhibition of the ATPase activity compare with TFIIH(WT). In the absence of DNA, the three ATPase activities are strictly identical and are slightly higher than the ATPase activity of the TFIIH complex mutated in the ATP-binding site of XPB. These data further support the model of the conformation change proposed above, as it demonstrates that R-E-D and ThM are used to stabilize the binding of XPB to DNA. Furthermore, the fact that these mutations inhibit both, TFIIH transcription and repair activities, suggests a common mode of recruitment of TFIIH to the promoters and to the damage sites.

The recruitment of TFIIH through the action of the ATPase activity of XPB may also induce a reorganization of the protein–DNA complexes in transcription and repair that will allow new protein–protein or protein–DNA contacts. Indeed, using photocrosslink experiments, we have shown that

addition of ATP in NER induced a re-positioning of XPC on the damaged DNA, which dependent on TFIIH (Tapias *et al*, 2004). After the recruitment of TFIIH to the damaged DNA through the energy furnished by the ATPase activity of XPB, the DNA would be opened by XPD, which has a processive and robust helicase activity stimulated by the p44 subunit of the core TFIIH (Coin *et al*, 1998). Here, a mutation in the ATPase activity of XPD still allowed TFIIH to bind the damaged sites *in vivo* but was unable to open the DNA around the lesion. Altogether, our data brings a new conceptual view of the roles of XPB and XPD in NER by revealing their different molecular functions within this genome caretaking event.

Materials and methods

Cell lines

CHO-27-1 is a CHO mutant cell line belonging to the third rodent complementation group (the hamster ERCC3 gene is the homologue of the human XPB gene) (Hall *et al*, 2005). CHO-UV5 belongs to the second rodent complementation group (the hamster ERCC2 is the homologue of the human XPD gene) (Winkler *et al*, 2000).

Figure 6 Mutations in the R-E-D motif impair DNA-dependent TFIIH ATPase activity. (A) 50, 100, and 150 ng of TFIIH(WT), TFIIH/XPB(E253A), XPB(K346R), XPB(E473A) or XPB(Δ516–526) were tested in an ATPase assay in the presence of 200 ng of double-strand circular DNA (Coin *et al*, 2007). The average percentage $\pm$ s.d. of phosphate released (Pi/(ATP + Pi)) from three independent experiments is represented in the graph. (B) 50 and 150 ng of TFIIH(WT), TFIIH/XPB(K346R) or TFIIH/XPB(E473A) were tested in an ATPase assay without (lanes 1–6) or with (lanes 7–12) 200 ng of double-strand circular DNA. The average percentage $\pm$ s.d. of phosphate released (Pi/(ATP + Pi)) from three independent experiments is represented in the graph.

Construction of the plasmids

Baculovirus allowing the expression of mutated XPB were constructed in the FLAG tag pSK278 vector (BD Biosciences). XPB was inserted at the BamHI/EcoRI site, in fusion with the FLAG tag at its 5' side. The mutants were obtained by site-directed mutagenesis (Quickchange, Stratagene). The resulting vectors were recombined with baculovirus DNA (BaculoGold DNA, PharMingen) in *Spodoptera frugiperda* 9 (Sf9) cells. *In vivo* experiments were carried out with the pEGFP-N1 plasmid (Clontech) containing the XPB cDNA inserted in frame with the green fluorescent protein tag (Hoogstraeten *et al*, 2002).

Stable cell lines

CHO-27-1 cells (10^6) were transfected with 2 μ g of pEGFP-N1/XPB plasmid in 10 cm Petri dishes using lipofectamine (Invitrogen). Forty hours after transfection, the fluorescent cells were sorted on the FACS DIVa (BD; Becton, Dickinson and Company). The cells with the highest level of fluorescence (about 5% of total cells) were maintained in the selective medium with G418 (Geneticin, 800 μ g/ml), expanded and analysed for XPB expression.

Damaged DNA substrates

Covalently closed circular Pt-DNA containing a single 1,3-intra-strand d(GpTpG) cisplatin-DNA crosslink was prepared as described (Frit *et al*, 2002).

Dual incision assay

Dual incision assay was carried out in 25 μ l of Repair buffer (45 mM Hepes-KOH (pH 7.8), 5 mM MgCl₂, 1 mM DTT, 0.3 mM EDTA, 10% glycerol, 2.5 μ g BSA, 50 mM KCl) supplemented with 2 mM ATP. Each reaction contained 5 ng of XPG, 15 ng of XPF/ERCC1, 10 ng of

XPC-HR23b, 50 ng of RPA and 25 ng of XPA. After pre-incubation 10 min at 30°C, 30 ng of Pt-DNA was added and reaction was continued for 90 min at 30°C. The excised fragment was detected on a 14% urea-acrylamide after annealing with 9 ng of the complementary oligonucleotide and addition of four radiolabelled dCMP α -P³² (3000 μ Ci/mmol) residues by Sequenase V2.1 (USB).

KMnO₄ footprint assay

The damaged strand probe was obtained on AgeI/AseI digestion of the Pt-DNA and radiolabelling at the 3' end in a Klenow reaction, the Pt adduct is located at 156 bp from the labelled end. The resulting fragment was purified by the 'crush and soak' method after migration in a 5% nondenaturing PAGE. Reactions (75 μ l) were carried out in 20 μ l of Repair buffer (+2 mM ATP) containing the labelled cisplatinated probe (40 fmol) and 40 ng of XPC-HR23b, 25 ng of XPA, 50 ng of RPA and 150 ng of XPG. After incubation at 30°C for 15 min, 3 μ l of 120 mM KMnO₄ was added, and oxidation was allowed to proceed for 3 min at room temperature before reduction by adding 6 μ l of 14.6 M β -mercaptoethanol for 5 min on ice. After organic extraction and ethanol precipitation, dried pellets were resuspended in 100 μ l of a solution containing 1 M piperidine, 1 mM EDTA and 1 mM EGTA and incubated at 90°C for 25 min. Samples were next ethanol precipitated, and final pellets were recovered in 10 μ l of loading buffer and analysed in 8% urea PAGE.

ATPase assay

Protein fractions were incubated for 2 h at 30°C in the presence of 1 μ Ci [γ -³²P]ATP (7000 Ci/mmol, ICN Pharmaceuticals) in a 20 μ l reaction volume in 20 mM Tris-HCl pH 7.9, 4 mM MgCl₂, 1 mM DTT, 50 μ g/ml BSA and when indicated 200 ng of double-strand DNA (pcDNA3+). Reactions were stopped by addition of EDTA

Figure 7 Proposed structure-based mechanism for binding of XPB to DNA. In this model, adapted from Fan *et al* (2006), XPB is in an opened conformation in the absence of DNA. When XPB binds to DNA, the rotation (170°) of the second helicase domain (HD2) together with the ThM domain, facilitated by HD1-mediated ATP hydrolysis, forms the closed and stable XPB–DNA complex.

(50 mM) and SDS (1% (w/w)). The reactions were then diluted five-fold, spotted onto polyethylenimine (PEI) TLC plates (Merck), run in 0.5 M LiCl/1 M formic acid and autoradiographed.

Local UV irradiation and immunofluorescence

The cells were rinsed with PBS and covered with an isopore polycarbonate filter with pores of $3\mu\text{m}$ diameter (Millipore, Bedford, MA). Cells were then exposed to UV irradiation with a Philips TUV lamp (predominantly 254 nm) at a dose of 100 J/m^2 (Volker *et al*, 2001). Subsequently, the filter was removed, the medium was added back to the cells, and they were returned to culture conditions for 30 min. Then, cells were fixed in 2% formaldehyde for 15 min at room temperature and permeabilized with PBS/0.5% Triton X-100 for 5 min. After washing with PBS-Tween (0.05%), the slides were incubated for 1 h with the indicated antibodies. After extensive washing with PBS-Tween, they were incubated for 1 h with Cy3-conjugated donkey anti-rabbit IgG, goat anti-mouse Alexa 488 IgG or goat anti-rat Alexa 488 IgG (Jackson Laboratories) diluted 1:400 in PBS-Tween/0.5% Foetal Calf Serum. The slides were counterstained for DNA with DAPI prepared in Vectashield mounting medium (Vector lab). All images were collected using a Leica Confocal TCS 4D microscope equipped with both UV laser and an Argon/Krypton laser, and standard filters to allow collection of the data at 488 and 568 nm. The software TCSTK was used for three-colour reconstructions, and figures were generated using the PLCHTK software.

Host-cell reactivation assay

The pGL3 vector expressing *Photinus pyralis* (firefly) luciferase was purchased from Promega and the pCH110 vector expressing the β -galactosidase from Invitrogen. The pGL3 vector was UV irradiated (254 nm, 1000 J/m^2) at a concentration of 1 mg/ml in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. CHO-27-1 cells were transfected in a six-well plate at a confluence of 95% using Lipofectamine Plus (Invitrogen). Each transfection mixture contained 500 ng of pGL3 (UV +/–), 100 ng of pCH110 (nonirradiated) and 10 ng of the various pcDNAXPB plasmids. After 4 h of incubation, the transfection

reagents were replaced by medium. Cells were lysed after 24 h to measure luciferase activity on a microtiter plate luminometer (Dynex). All results (mean values of at least five measurements) were normalized by calculating the ratios between luciferase and galactosidase activities.

UV-survival assay

Cells (10^3) were plated per 6 cm petri dishes, cultured overnight and UV irradiated at 254 nm at various doses ($0.5\text{ J/m}^2/\text{s}$). After 14 days, the cells are stained by trypan blue and counted.

Antibodies

Mouse monoclonal antibodies towards TFIIH subunits were used as described (Coin *et al*, 2007). Primary antibodies (the final dilutions are indicated in parentheses) used in fluorescent labelling were purified rabbit anti-GFP (Torrey Pines Biolabs, Inc) (1:1000), rat monoclonal anti-HA 3F10 (Roche) (1:1000) and mouse IgG monoclonal anti-CPD (TDM2) (1:2000) (MBL international corp.).

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Conflict of interest

The authors declare that they have no conflict of interest.

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