Reduced level of the repair/transcription factor TFIIH in trichothiodystrophy

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Trichothiodystrophy (TTD) is a rare hereditary multisystem disorder associated with defects in nucleotide excision repair (NER) as a consequence of mutations in XPD, XPB or TTDA, three genes that are all related to TFIIH, the multiprotein complex involved in NER and transcription. Here we show that all the mutations found in TTD cases, irrespective of whether they are homozygotes, hemizygotes or compound heterozygotes, cause a substantial and specific reduction (by up to 70%) in the cellular concentration of TFIIH. Intriguingly, the degree of reduction in the level of TFIIH does not correlate with the severity of the pathological phenotype, suggesting that the severity of the clinical features in TTD cannot be related solely to the effects of mutations on the stability of TFIIH. We have also measured TFIIH levels in cells in which different mutations in the XPD gene are associated with clinical symptoms not of TTD but of the highly cancer-prone disorder xeroderma pigmentosum (XP). We have found mild reductions (up to 40%) in TFIIH content in some but not all of these cell strains. We conclude that the severity of the clinical features in TTD patients and the clinical outcome of differentially mutated XPD proteins is likely to depend both on the effects that each mutation has on the stability of TFIIH and on the transcriptional activity of the residual TFIIH complexes.

INTRODUCTION

Trichothiodystrophy (TTD) is a rare autosomal recessive multisystem disorder characterized by sulfur-deficient brittle hair, mental and physical retardation, ichthyosis, and, in many patients, cutaneous photosensitivity but no cancer. All sun-sensitive TTD cases appear to be defective in nucleotide excision repair (NER) as a consequence of alterations in one of three genes, namely XPB, XPD and TTDA (1–4). Intriguingly, in view of the very marked differences in the clinical phenotypes, defects in two of the genes altered in TTD (XPB and XPD) can also cause the cancer-prone disorder xeroderma pigmentosum (XP) or, in rare cases, the combined symptoms of XP and Cockayne syndrome (XP/CS). CS is another multisystem disorder characterized by postnatal growth failure, progressive neurological dysfunction, premature ageing and otherwise clinically heterogeneous features, which commonly include cutaneous photosensitivity but no cancer (5). A breakthrough in understanding the perplexing features of this complex triad of hereditary disorders and their puzzling genotype-phenotype relationships came from the discovery that the genes mutated in TTD are all related to TFIIH, a multiprotein complex involved in both initiation of transcription by RNA polymerase II and NER, XPB and XPD encode two subunits of TFIIH, whereas TTDA, whose identity remains unknown, is involved in the stabilization of the TFIIH complex (6,7).

The transcriptionally active form of TFIIH (holo-TFIIH) includes XPB, p62, p52, p44 and p34 (which are tightly associated in a subcomplex called core-TFIIH), XPD and three additional components, cdk7, cyclin H and MAT1, which constitute the CDK-activating kinase (CAK) subcomplex (8,9). XPB and XPD are ATP-dependent helicases with opposite polarity. The 3′→5′ helicase activity of XPB is essential for both transcription and repair, whereas the XPD 5′→3′ helicase activity is necessary for repair but dispensable for in vitro basal transcription (10–12). This probably accounts for the rarity of XP complementation group B (XP-B) families (4,13) compared with the relatively high frequency and variety of pathological phenotypes associated with XP-D defects. Mutations in the XPD gene have been found in many patients with TTD (14–17) or with XP (16,18–20) and in two XP/CS patients (18,21,22). To explain the paradox of mutations in the same gene resulting in distinct pathological phenotypes, it has been suggested that clinical features diagnostic for XP result from mutations in the XPD gene that affect only the NER function of TFIIH, while those typical of TTD and CS are due to a subtle impairment of

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its transcriptional role (23, 24). This notion has been supported by the XPD gene mutation spectrum in patients, indicating that the site of mutation determines the clinical phenotype (16, 17). Most of the mutations in TTD donors are localized at four sites (arg112his, arg658his, arg722trp, −1 frameshift at codon 730). In contrast, 80% of mutations in XP patients are localized at a single site, arg683his. Different changes were found in the two XP/CS cases. Since these phenotype–genotype studies also suggested possible gene dosage effects in TTD, we have taken advantage of our unique collection of fibroblast strains to analyze the steady-state level of TFIIH in patients representative of distinct clinical, cellular and molecular alterations. Fibroblast strains established from skin biopsies (i.e. an in vitro cell system that still maintains the cell contact inhibition and the cell density-dependent growth typical of the in vivo situation) comprise the only material that is available to investigate a significant number of patients.

The results show that alterations in any of the gene products that result in the clinical phenotype of TTD specifically reduce the cellular content of the TFIIH complex. Extension of our investigations to XP-D patients with clinical symptoms of XP or XP/CS indicates that the clinical outcome of an XPD mutation is the result of the effects that a mutated XPD subunit has not only on the stability of TFIIH but also on the multiple functional roles of the residual TFIIH complexes.

RESULTS

The level of TFIIH was analyzed by western blots of cell lysates from 19 patient strains (Table 1), using antibodies against the p62 and p44 subunits of the core TFIIH as well as the cdk7 subunit of CAF1. In parallel, we measured the content of actin, a cellular matrix protein as a control, and of the α subunit of TFIIIE, a basal transcription factor that is involved in recruiting TFIIH to the promoter (9) and regulating its enzymatic activities (25).

All the genetic alterations observed in the repair-defective TTD patients are associated with reduced levels of TFIIH

In the strains TTD1BR and TTD6VI (i.e. the sole TTD representatives of the TTD-A and XP-B groups, respectively) the amount of TFIIExz was nearly normal, whereas the content of the TFIIH subunits cdk7, p44 and p62 was strikingly lower than in normal fibroblasts. TFIIH content ranged between 32% and 55% of normal (Fig. 1A and C). The concentration of the TFIIH subunits, but not of TFIIExz, was also drastically reduced in two TTD cases (TTD6PV and TTD8PV) mutated in the XPD gene (2) (Fig. 1B and D). Thus, mutations in any of the three genes (XPD, XPB and TTDA) responsible for the photosensitive form of TTD result in a reduction in the steady-state level of TFIIH subunits, whereas TFIIExz is unaffected. In contrast, the cellular amounts of the different TFIIH subunits in the parents of the patients TTD6PV and TTD8PV were comparable to those detected in C3PV normal cells (Fig. 1B). Furthermore, the levels of cdk7, p44 and p62 observed in four TTD parents were similar to those detected in four healthy unrelated individuals (Fig. 1E), and no statistically significant differences were found between the two donor groups in the mean levels of TFIIH subunits. These findings demonstrate that the occurrence of one normal XPD allele is sufficient to ensure normal TFIIH levels.

The expression of the XPD wild-type protein in XPD-mutated TTD cells is sufficient to restore normal levels of the other TFIIH subunits

To demonstrate that the presence of a mutated XPD protein in TTD patients is responsible for the observed reduction in the other TFIIH subunits, we transfected TTD8PV fibroblasts with a construct containing the wild-type XPD cDNA cloned in frame with GFP and we isolated stably transformed clones. Sequence analysis of the XPD cDNA amplified from a TTD8PV[XPD–GFP]+ clone showed at position 413 the G present in the wild-type XPD sequence, as well as the homozygous A mutation in the genome of TTD8PV cells (Fig. 2A). Immunoblots on parallel samples demonstrated that the expression of the recombinant wild-type XPD–GFP chimeric protein results in a specific and substantial increase (from 30% to 70% of normal) in the steady-state levels of the cdk7, p44 and p62 subunits of TFIIH (Fig. 2B and C). The amount of TFIIH components was also analyzed directly in vivo by immunofluorescence. TTD8PV cells revealed a specific reduction in the intensity of the signal of the XPD and XPB proteins similar to that observed for the other TFIIH subunits (30–40% of normal). These alterations were corrected by transfection with wild-type XPD, as demonstrated by the recovery to normal level of the five TFIIH subunits in >70% of the TTD8PV[XPD–GFP]+ stably transformed fibroblasts (Fig. 3). This was paralleled in vivo by a drastic increase in the average repair activity (from 6.8 to 47.1 grains/nucleus) and by the restoration of normal repair capability in >70% of the TTD8PV[XPD–GFP]+ cells (Fig. 2D). These observations also suggest that the lack of complete restoration to normal levels of TFIIH subunits, as detected by western blots (Fig. 2B and C), can probably be attributed to loss of the transgenic XPD cDNA or its expression in a small proportion of the cells in the transfected clone. The overall results of in vitro and in vivo approaches clearly indicate that the expression of normal XPD protein is able to raise the level of the other TFIIH subunits and to restore TFIIH functionality.

Mutations in the XPD gene associated with distinct pathological phenotypes differentially affect the level of TFIIH in the cell

We extended our analysis to a total of nine TTD patients carrying different mutated XPD alleles (Fig. 4) and different severity of symptoms in terms of severity of mental and growth retardation, proneness to infections and age at death (Table 1). In every case mutations responsible for TTD resulted in reductions in the amount of TFIIH (Fig. 4: lower panel). This indicates that all the mutations found in TTD patients, irrespective of whether they are homozygotes, hemizygotes or compound heterozygotes, interfere with the stability of TFIIH. The reduction in the level of TFIIH subunits is particularly striking (by 55% for p44 and ~65% for cdk7 and p62) in the four cases with the arg112his substitution, the change most frequently found in TTD (17). It should be noted...
that the three patients homozygous for the arg112his change (TTD11PV, TTD2PV and TTD8PV) are less severely affected at the clinical level than is the patient TTD11PV, who is compound heterozygote for the arg112his change. This suggests that the mutation present in the second allele of TTD11PV, resulting in the deletion of exon 6, is more detrimental to the functional role of TFIIH than is the arg112his change. Other mutations in TTD, which have a less marked destabilizing effect on TFIIH, are nevertheless associated with severe clinical features. This is clearly the case for the XPD alleles resulting in either arg673gly or loss of the last 17 amino acids, which are the only expressed alleles in TTD6PV and TTD1BI patients, respectively (14, 17). These findings indicate that the degree of reduction in the TFIIH level does not correlate with the severity of the mental and physical impairment.

To verify whether mutations in XPD associated with distinct pathological phenotypes differentially interfere with the level of TFIIH in the cell, six XP cases and two XP/CS cases were also investigated (Fig. 4; middle and upper panels). Normal amounts of TFIIH were present in XP patients homozygous for the substitution of arg683, the alteration found in 80% of XP-D patients (16), whereas a reduction by up to 45% in the TFIIH subunits was observed in the XP compound heterozygotes in which the second allele is a null (16). A slight reduction (25%) was observed in XP/CS cases, as previously reported (32). Therefore, the XPD mutations associated with the XP and XP/CS phenotypes may also affect proper protein–protein interactions within the TFIIH complex. This interference gives rise to a slight reduction in the steady-state level of TFIIH only in functionally hemizygous patients in which one allele is either lethal (in the case of patients XP1NE, XPLABE and XP17PV) or unexpressed (in the case of XPCS2). None of the mutations found in XP patients is sufficient to cause detectable transcription-related symptoms, even when associated with a 40% reduction in the amount of TFIIH.

### DISCUSSION

Our analysis shows that mutations in any of the three genes (XP-D, XPB and TTD4) responsible for the photosensitive form of TTD cause a decrease by up to 70% in the cellular concentration of the repair/transcription factor TFIIH. A general implication of our findings is that a limited availability of TFIIH interferes with NER but is compatible with life. This implies that the lowered TFIIH level in TTD cells does not seriously impede transcription of most genes, but it must impair transcription only under certain conditions or in specific cell compartments affecting, for example, only a limited set of genes that critically demand optimal TFIIH function (33). This notion

### Table 1. Clinical and DNA repair data of the patients analyzed in this study

<table>
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<th>Clinical phenotype</th>
<th>Genetic defect</th>
<th>Patient code</th>
<th>Status of the mutated alleles</th>
<th>Clinical severity</th>
<th>UDS</th>
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*TTD, trichothiodystrophy; XP, xeroderma pigmentosum; CS, Cockayne syndrome. TTD patients show sulfur-deficient brittle hair, ichthyosis, physical and mental retardation, unusual face, and photosensitivity, but no cancer. XP patients show hypersensitivity to sun exposure, pigmentary alterations and premalignant lesions in sun-exposed areas of the skin, a high incidence of skin cancer, and, in several cases, neurological abnormalities of varying severity due to primary neuronal degeneration. In XP/CS patients, the cutaneous alterations of XP are combined with some of the major clinical symptoms of CS, namely pigmentary retinal degeneration, primary demyelination, calcification of the basal ganglia and cachetic dwarfism.

1. Comp. heteroz., compound heterozygote; Fn. Hemizygote, functional hemizygote because only one allele is expressed. XPD mutated alleles are shown in Figure 4.

2. In TTD, clinical severity refers to the degree of physical and mental impairment, whereas in XP, it refers to the type and severity of skin lesions. Neurological abnormalities of varying severity are present in all but two cases (XP16PV and XPLABE). XP3NE and XP17PV developed skin cancer. For clinical details, see the quoted references.

3. UV-induced DNA repair synthesis (UDS) after irradiation with 10 J/m² observed in patient cells is expressed as percentage of that in normal cells analyzed in parallel. In TTD and XP cells, the magnitude of the UDS defect correlates with the degree of sensitivity to the killing effects of UV. In contrast, XP/CS cells are extremely sensitive to UV killing because the high values of residual UDS reflect abortive DNA synthesis at sites distant from the damage (31).
is supported by several observations on cells from TTD patients mutated in the XPD gene. (i) We have not found any alteration in cellular transcription, measured by [3H]uridine uptake, in TTD fibroblasts (data not shown), but a marked reduction in T-cell proliferation in response to mitogens was detected in TTD lymphocytes from the same patients (30). (ii) Alterations in T cells and dendritic cells (DC) suggestive of a subtle transcriptional defect of a set of genes involved in DC maturation and function have been reported in a TTD child with severe immunodeficiency (34). (iii) Reduced levels of synthesis of globin have recently been described in several TTD patients (35). These observations are paralleled by the finding of reduced transcription of the skin-specific gene in late stages of terminally differentiating cells in the TTD mouse expressing the arg722trp mutated XPD protein (33). Therefore, emerging evidence supports the hypothesis that the reduced amount of TFIIH may become limiting in terminally differentiated tissues in TTD patients. This deficiency is likely to have its strongest effect on the synthesis of highly expressed genes, thus accounting for the typical TTD clinical symptoms, namely, sulfur-deficient brittle hair, nail dysplasia and ichthyosis.

Our extensive analysis demonstrates not only that a reduced steady-state level of TFIIH is a common feature in all TTD patients mutated in XPD, XPD and TTD4 genes, but also that the severity of the clinical features within the group of TTD patients cannot be related solely to the effects of mutations on TFIIH stability. Substantial reductions in TFIIH levels have been detected in patients showing relatively moderate psychomotor retardation and no increased proneness to infections (as in the case of the patients TTD1PV, TTD2PV and TTD8PV, altered in the XPD gene, as well as in TTD6V1, defective in the XPD gene). Conversely, less marked reductions by 35–45% of normal levels were found in the patients TTD1RO, TTD6PV, TTD1BI, TTD7PV and TTD12PV, who had drastically compromised pathological phenotypes in terms of severity of mental and growth retardation, proneness to infections, and age at death (see references in 1). As already mentioned, thermostability of TFIIH has been demonstrated in vivo and in vitro in TTD1RO (28). The lack of any evidence hinting at any sort of fever-dependent reversible deterioration of clinical symptoms in the patients TTD6PV, TTD1BI, TTD7PV and TTD12PV leads us to propose that the severity of the clinical features in TTD is likely to be determined by the combination of the effects that each mutation has on the stability of TFIIH and on the transcriptional activity of the residual TFIIH complexes.

**Basis of TFIIH instability**

XPD and XPB are subunits of TFIIH, and it is not unprecedented that a defective or absent protein might reduce the stability of the entire complex by compromising the
stability of interacting proteins (36–38). We can postulate that XPD and XPD mutations may render unstable either the transcript or the protein, or interfere with correct folding or proper associations of the protein with the other components of TFIIH. Uncomplexed proteins are then rapidly degraded. Alternatively, the mutated component may induce slight conformational changes in the architecture of the entire TFIIH complex that in turn may favour its degradation. Several observations indicate that a mutated TFIIH subunit may affect the stability of the entire complex. It has been shown that mutations in XPB or in p44 that modify the XPB–p44 interaction affect the composition of TFIIH by decreasing the amount of XPD and CAK subunits associated with the core and/or weakening the anchoring of CAK to the core TFIIH (39,40). Mutations in XPB and p52 may prevent the XPB anchoring within the core TFIIH (41), and mutations in p44 may prevent incorporation of the p62 subunit within the core TFIIH (42). We have shown that the expression of the XPD wild-type protein in XPD-mutated TTD cells drastically increases the level of the other TFIIH subunits and that this increase is paralleled by the restoration of normal repair activity. These findings, together with the normal TFIIH level observed in TTD parents, indicate that a normal XPD protein provides stability to the TFIIH complex, probably by restoring proper protein–protein interactions, and ensures its correct functioning in NER.

A different situation has to be envisaged in TTD-A. No causative mutation has been identified in any of the TFIIH subunits or in any of the known NER genes in TTD-A cells, which nevertheless have a 3–4-fold reduced amount of TFIIH. It has been proposed that TTD-A cells might lack a factor that stabilizes TFIIH or protects it against degradation (6). Therefore, a 3–4-fold reduced amount of an otherwise normal TFIIH complex consistently reduces NER efficiency (to 25% of normal) and confers subtle defects in transcription resulting in a TTD phenotype with a physical and mental impairment of moderate severity. If the sole defect in TTD-A cells is indeed a reduced level of TFIIH with normal composition, we are led to the proposal that a drastic reduction in TFIIH content (to ≤35% of normal levels) is sufficient to confer the clinical features of TTD. Our analysis shows that less severe reductions may be necessary but are not sufficient to generate the TTD phenotype.

**XPD mutations and clinical outcome**

Although some of our TTD cell strains (6 out of the 11 analyzed) had drastic reductions in TFIIH content, in others we observed a less severe reduction (by 35–45% of normal levels), comparable to that in compound heterozygous XP patients [XP1NE, XPLABE and XP17PV reported in the present study and XP7BE and XP17BE described elsewhere (32)]. Therefore, we propose that the clinical outcome of XPD mutations is the combined result of the reduction in TFIIH content and the effects of the specific mutations on the interactions of TFIIH with other components of the transcription machinery that may partially compromise transcription activity. Emerging evidence indicates that the involvement of TFIIH in transcription is multifaceted, ranging from initiation and promoter escape in basal transcription to regulation of gene expression [see (43) and references therein]. It has been demonstrated that the transcriptional activator FUSE-binding protein (FBP), a regulator of MYC expression, binds specifically to TFIIH. In XP-B and XP-D cells, this interaction was either abolished or attenuated, resulting in impaired regulation of MYC expression (44). A specific involvement of TFIIH in activation of estrogen receptor α (ERα) and retinoic acid receptor γ (RARγ), two members of the nuclear receptor superfamily of transcription factors, has been demonstrated (45,46). Furthermore, it has recently been shown that hormonal responses operate through TFIIH and that some mutations in XPD prevent an optimal phosphorylation of nuclear receptors by cdk7, with, as a consequence, a drop in the expression of genes sensitive to hormonal action. Transactivation was restored upon overexpression of the wild-type XPD (47). Together, these studies underscore the importance of TFIIH as a potential convergence point of diverse regulatory signals, which ultimately control gene expression. Mutations altering TFIIH conformation may therefore affect to different degrees its stereospecific interactions with transcription-specific regulators (activators and repressors). While XP-type mutations could interfere with a
specific class of regulators involved in carcinogenesis, TTD-type mutations may affect the interaction of TFIIH with transcription factors involved in other regulated pathways, such as differentiation, development and neurogenesis. The possibility that XPD mutations, whilst preserving basal transcription, differentially affect activated transcription mediated by the interaction of TFIIH with different transcription regulators may help to explain the lack of cancer-proneness in TTD, despite the reduced efficiency of NER, and the many different pathological phenotypes that can result from mutations in the XPD gene. These include not only XP, TTD and XP/CS but also other pathological phenotypes ranging from cerebro-oculo-facio-skeletal syndrome (48) to combined XP/TTD features (49).

In conclusion, we have shown that reduced steady-state levels of TFIIH are a common feature of all TTD patients in which photosensitivity is due to distinct genetic and molecular alterations. However, the severity of the clinical features in TTD patients and the clinical outcome of differentially mutated XPD proteins are likely to depend on the effects that each mutation has on the architecture of the residual TFIIH complexes and, consequently, on its structural and functional roles in transcription. In this context, the variety of clinical features associated with XP-D defects provides a unique tool to dissect the complex interplay between repair and transcription and the phenotypic consequences of mutations that affect the stability and/or the activity in repair and transcription of the TFIIH complex.

**MATERIALS AND METHODS**

**Cells and culture conditions**

Primary fibroblast cultures established from biopsies were routinely grown in DMEM medium (Euroclone, Wetherby, UK) supplemented with 10% fetal bovine serum (Euroclone) and subcultured by trypsinization. Clinical and DNA repair data, and related literature references of the analyzed patients, are reported in Table 1. In parallel, fibroblasts from four healthy individuals (C3PV, 377, 380 and 383) and from the parents of the patients TTD6PV and TTD8PV were analyzed. The study was approved by the appropriate institutional review board, and

![Figure 3](https://academic.oup.com/hmg/article-abstract/11/23/2919/641241)}
Appropriate informed consent was obtained from human subjects.

**Transfection of primary fibroblasts, selection and characterization of stably transfected clones**

Eight 100 mm dishes were seeded with $8 \times 10^5$ TTD8PV fibroblasts/dish. After 24 h, the cultures were transfected according to manufacturer’s protocol with 40 μl Cytofectene transfection reagent (BioRad Laboratories, Hercules, CA) and 7 μg pXPD-EGFP construct carrying the wild-type XPD cDNA cloned in frame to the 5’ end of the GFP (green fluorescent protein) gene in the pEGFP-N1 expression vector harboring the dominant selectable neo marker gene (50). Transfected cells were cultured for 5 days and then selected in medium supplemented with 100 μg/ml G418-sulfate.

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**Figure 4.** Relationship between mutations in the XPD protein found in TTD (lower panel), XP (middle panel) and XP/CS patients (upper panel) and steady-state levels of the TFIIH subunits cdk7, p44 and p62. The XPD protein is shown with the helicase domains (black boxes). Amino acid changes resulting from the mutations found in the patients are shown boxed, and numbers 1 and 2 after the patient code denote the different alleles. The changes responsible for the pathological phenotype, those resulting in deletions likely to affect cellular viability and mutations described as lethal <sup>16</sup> are indicated by solid, dashed and dotted arrows, respectively. The mutations found in both TTD and XP patients (the leu461val substitution and the 716–730 deletion, which have been always found associated in a single haplotype, and the arg616pro change) are lethal and do not contribute to the phenotype, which is determined by the other less severely affected allele <sup>16</sup>. Levels of TFIIEα (white columns), cdk7 (dotted columns), p44 (dashed columns) and p62 (black columns) in the patients are expressed as percentages of the corresponding values in normal C3PV cells. The reported values are the means of at least two independent experiments, with SE values always <10%.
(Invitrogen). Five weeks later, seven G418 resistant clones, designated TTD8PV[XPD–GFP]⁺, were trypsinized within cloning rings and transferred to 35 mm dishes. Five of them were expanded and tested for their repair capability following ultraviolet (UV) irradiation. Compared with TTD8PV fibroblasts, two TTD8PV[XPD–GFP]⁺ clones showed a drastic increase in the ability to perform UV-induced DNA repair synthesis as well as in the amount of TFIIH subunits, as detected by immunofluorescence. One clone was further characterized for the steady-state level of TFIIH subunits on western blot. In parallel, the expression of the transfected XPD cDNA was analyzed by RT–PCR followed by sequencing, as described previously (17).

UV-induced DNA repair synthesis (UDS)

The response to UV irradiation was analyzed by measuring UDS following irradiation with an UV dose of 20 J/m², as routinely performed in our laboratory (1–3).

Western blot (WB) analysis

Samples of 8 × 10⁵ fibroblasts were resuspended in 100 μl of lysis buffer [62.5 mM Tris–HCl pH 6.8, 4 M urea, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol and 0.006% bromophenol blue], sonicated three times for 30 s on ice, incubated at 65°C for 15 min and stored at −20°C. Different samples of lysates were separated on 12% polyacrylamide–SDS gels and transferred onto nitrocellulose membranes (Protran, Schleicher and Schuell, Dassel, Germany). The membranes were incubated for 1 h at room temperature in blocking buffer (PBS/Tween 0.05% containing 5% skim milk) and hybridized overnight at 4°C with primary antibodies against cdk7, p44, p62 or actin (Sigma, Saint Louis, MO) diluted in blocking buffer as follows: anti-cdk7 5000 a/C2, anti-p44 4000 a/C2, anti-XPD 2000 a/C2, anti-XPB 1000 a/C2, anti-XPUIEx 5000 a/C2. Slides were washed three times for 10 min in PBS/Tween 0.05% and incubated with rhodamine-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 50 ×. The XPD hybridization signal was amplified by incubation with biotin–SP-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories) followed by streptavidin–Texas red conjugate (Invitrogen, Carlsbad, CA). Nuclei were stained with Hoechst 33258 and slides were mounted with Mowiol 4-88 (Calbiochem, San Diego, California). Epifluorescent images were obtained using a Leitz Orthoplan microscope equipped with a digital camera (Olympus Camedia 2000) and digitally processed using the Adobe Photoshop 5.0 software.

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