nature genetics

A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A

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DNA repair-deficient trichothiodystrophy (TTD) results from mutations in the XPD and XPB subunits of the DNA repair and transcription factor TFIIH. In a third form of DNA repair–deficient TTD, called group A, none of the nine subunits encoding TFIIH carried mutations; instead, the steady-state level of the entire complex was severely reduced[1]. A new, tenth TFIIH subunit (TFB5) was recently identified in yeast[2]. Here, we describe the identification of the human TFB5 ortholog and its association with human TFIIH. Microinjection of cDNA encoding TFB5 (*GTF2H5*, also called *TTDA*) corrected the DNA-repair defect of TTD-A cells, and we identified three functional inactivating mutations in this gene in three unrelated families with TTD-A. The *GTF2H5* gene product has a role in regulating the level of TFIIH. The identification of a new evolutionarily conserved subunit of TFIIH implicated in TTD-A provides insight into TFIIH function in transcription, DNA repair and human disease.

Hereditary mutations in the repair and transcription factor TFIIH are associated with three photo-hypersensitive syndromes: xeroderma pigmentosum, xeroderma pigmentosum combined with Cockayne syndrome (a neurodevelopmental disorder) and the Cockayne syndrome–like brittle-hair disease TTD[3,4] (**Table 1**). TFIIH has a central role in transcription of RNA polymerase I and II, nucleotide excision

Table 1 Clinical and cellular features

	General disease characteristics						Individuals in this study		
Gene (s)	XP[a] *XPA, XPC, XPE, ERCC2, ERCC3, ERCC4, ERCC5*	CS[b] *CKN1, ERCC6*	XP/CS[c] *ERCC2, ERCC3, ERCC5*	TTD[d] *ERCC2 (ERCC3)*	XP/TTD[e] *ERCC2*	COFS[f] *ERCC2, ERCC5, ERCC6*	TTD1BR[g] *GTF2H5*	TTD99RO *GTF2H5*	TTD13/14PV *GTF2H5*
Cutaneous symptoms									
Photosensitive skin	Mild–severe	Mild	Moderate–severe	Mild–moderate	Severe	Severe	Mild	Very mild	Mild
Skin cancer	Mild–severe	–	Mild–severe	–	Mild	–	–	–	–
Brittle low-sulfur hair	–	–	–	+	+/–	–	+	+	+
Other[h]	a,b	–	a,b	c,d	b,d	d	c–e	d	
CS-like symptoms[i]	–	++	+	+	–	+++	f,g,i,j	f–k	g,i,j
Cellular features									
GG-NER activity (UDS)[j]	0–70	100	0–40	10–45	0–30	<5	15–25	20	10
Recovery of transcription after UV (TC-NER)	Low–normal[k]	Low	Low	Low	Low	Low	Low	Low	Low
Overall UV sensitivity	1.5–10×	2.5–5×	4–10×	1.5–5×	2.5–10×	10×	2.1×	2.7×	3.5×
TFIIH level[j]	60–100	100	75	30–50	ND	ND	~30	~30	~30

[a]Xeroderma pigmentosum. [b]Cockayne syndrome. [c]Combined features of xeroderma pigmentosum and Cockayne syndrome. [d]Photosensitive TTD. [e]Combined features of xeroderma pigmentosum and TTD[9]. [f]Severe features of Cockayne syndrome[12]. [g]Ref. 13. [h]a, actinic keratosis; b, freckling; c, collodion baby; d, ichthyosis; e, eczema. [i]CS, Cockayne syndrome; f, cataracts; g, developmental delay; h, deafness; i, mental retardation; j, short stature; k, caries. [j]Percent of normal. [k]Low for *XPA, ERCC2, ERCC3, ERCC4* and *ERCC5*; normal for *XPC* and *XPE*.

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Published online 27 June 2004; doi:10.1038/ng1387

repair (NER) and transcription-coupled repair (TCR)[5]. NER removes a wide range of DNA helix–distorting injuries, including ultraviolet light (UV)-induced lesions[6]. TCR is a repair pathway that eliminates both classical NER lesions and oxidative DNA injuries from the transcribed strand[7,8]. Photosensitive features of xeroderma pigmentosum are caused by defective NER, whereas features of Cockayne syndrome and TTD are attributed to defective TCR and transcription function. Most individuals with these syndromes associated with TFIIH carry mutations of XPD or, in a few cases, of XPB, the two TFIIH helicase subunits[9–12]. A third, unidentified gene that causes photosensitive TTD[13,14], called *GTF2H5*, also shows association with TFIIH[1]. In **Table 1** we summarize the clinical and cellular features of all known cases of TTD-A, including two families recently diagnosed with TTD-A. Cells from individuals with TTD-A are only mildly UV-sensitive despite having low UV-induced DNA repair synthesis (UDS).

We previously found that treatment with highly purified TFIIH corrected the NER defect of cells from individuals with TTD-A, but none of the nine genes encoding TFIIH were mutated in these individuals[1]. Moreover, TFIIH isolated from cells of individuals with TTD-A had normal *in vitro* enzymatic activities and transiently restored the NER defect in these cells, suggesting that TFIIH was qualitatively not or only mildly defective. The total cellular TFIIH content of cells from individuals with TTD-A seemed to be very low[1].

Table 2 TFIIH subunits

Subunit	Mammalian	Yeast	Predicted protein size in kDa (human)	Properties	Percent identity between humans and yeast
1	XPB	RAD25/SSL2	89	Helicase, 5′–3′	49
2	XPD	RAD3	80	Helicase, 3′–5′	51
3	GTF2H1 (TFB1)	TFB1	62	–	21
4	GTF2H2	SSL1	44	Zn finger	34
5	GTF2H3 (TFB4)	TFB4	34	Ring finger	29
6	GTF2H4 (TFB2)	TFB2	52	–	34
7	MAT1 (TFB3)	TFB3	32	Ring finger	31
8	CDK7	KIN28	41	CTD kinase	41
9	CyclinH	CCL1	38	Cyclin motifs	23
10	TFB5	TFB5	8	Stabilization of the complex	28

Subunit 10 was identified by ref. 2.

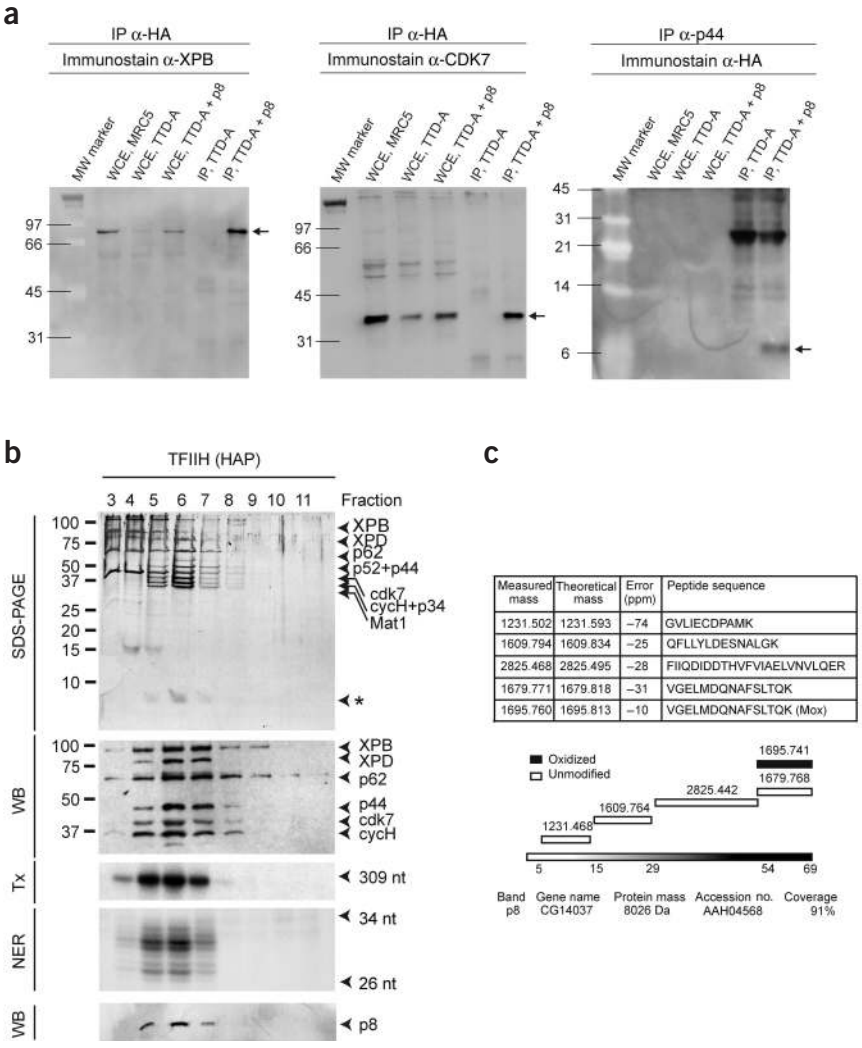
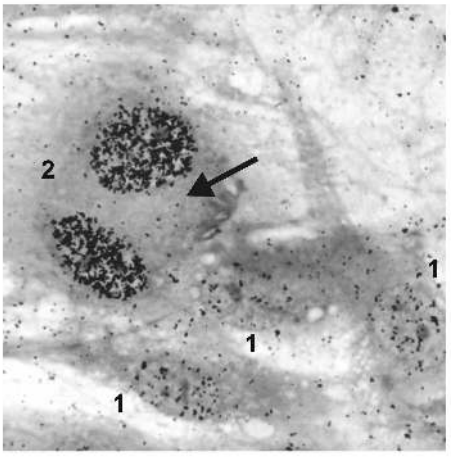


Figure 1 TFB5 is part of the TFIIH complex. (**a**) Immunoprecipitation (IP) assays. Lane 1, molecular weight (MW) marker; lanes 2–4: whole-cell extracts (WCE) from MRC5SV (MRC5; wild-type), TTD-A and TTD-A + p8 cells; lanes 5–6, immunoprecipitation from TTD-A and TTD-A + p8 cell extracts. Right panel, SDS-PAGE gel separation of 10 μg of proteins from whole-cell extracts and the immunoprecipitations with p44 of extracts from TTD-A and TTD-A + p8 cells. The blot was immunostained with antibody to HA. The arrow shows the immunostained band corresponding to p8-HA. Middle and left panels, SDS-PAGE gel separation of whole-cell extracts and the immunoprecipitations with antibody to HA of extracts from TTD-A and TTD-A + p8 cells. The blots were immunostained with antibodies to XPB and cdk7, respectively; the arrows show the corresponding immunostained proteins. (**b**) p8 is copurified with the other nine identified components of TFIIH (silver staining and western blotting; WB) and with TFIIH transcription (Tx) and repair (NER) activities. Lower panel, a rabbit polyclonal antibody raised against the C-terminal end of p8 was used to detect p8 in the hydroxyapatite (HAP) fractions containing TFIIH. (**c**) MALDI-MS identification of p8. Upper panel, experimentally determined values and the corresponding theoretical masses of p8 tryptic peptides are listed. Mass accuracy (ppm) and amino acid sequences are also shown for each peptide. Lower panel, schematic representation of MALDI peptide fingerprinting results. p8 was identified as CG14037 in the National Center for Biotechnology Information database.

Figure 2 Correction of the DNA-repair deficiency of TTD-A by *GTF2H5*. (**a**) Microinjection of an expression vector containing *GTF2H5* cDNA in TTD99RO (TTD-A) primary fibroblasts. Micrographs show the effect of p8 expression on DNA repair activity. Injected polynucleated primary fibroblasts (2), indicated by the arrow, had a high (wild-type) level of UDS, as apparent from the number of silver grains above their nuclei, relative to the uninjected surrounding fibroblasts from individuals with TTD-A (1), which had low residual UDS levels typical for TTD-A. UDS correction by p8 microinjection was also observed when injected into the other cells from individuals with TTD-A, TTD1BR and TTD13PV (data not shown). (**b**) Mutations found in the four cell lines analyzed derived from three unrelated individuals with TTD-A. The mutated base in the codon is shown in bold.



b

Cell line	Type of mutation	Amino acid position	Amino acid substitution
TTD99RO	CGA → TGA	56	Arg → Stop
TTD1BR	CGA → TGA CTG →CCG	56 21	Arg → Stop Leu → Pro
TTD13/14 PV	ATG →ACG	1	Met → Thr (no start)

The reduced steady-state levels of TFIIH are probably a result of complex fragility due to a mutation in an unknown gene[15].

A quantitative proteomic screening of promoter-associated RNA polymerase II preinitiation complexes from yeast nuclear extracts identified a new, very small protein of 72 amino acids (~8 kDa). This protein, called TFB5 (ref. 2), is a core component of yeast TFIIH. It was overlooked in previous analyses of TFIIH because of its exceptionally small size.

Database screening identified several orthologs of TFB5 (**Supplementary Fig. 1** online), including a presumed human homolog, which consists of 71 amino acids and has a predicted molecular weight of 8 kDa (referred to as p8). TFB5 was highly conserved, with a sequence identity of 28% and 56% similarity between human and yeast (**Table 2**). The strong evolutionary conservation of each TFIIH subunit (**Table 2**), in combination with the overall structural homology of the complex[16,17] and the fact that the recently identified *Chlamydomonas reinhardtii* TFB5 ortholog (REX1S) is involved in NER[18], prompted us to investigate whether p8 (the human TFB5 ortholog) is also part of mammalian TFIIH and whether it is the factor mutated in TTD-A.

We cloned the cDNA encoding p8 from primary fibroblasts. We expressed a hemagglutinin (HA)-tagged version of p8 in fibroblasts from individuals with TTD-A and immunoprecipitated it with antibody to HA (**Fig. 1a**). Both the XPB core TFIIH and the associated CAK component (CDK7) coprecipitated with p8-HA, in contrast to replication-repair factor RPA1 (data not shown). Conversely, antibody to p44 (another core TFIIH component) coprecipitated p8-HA. We conclude that, as in yeast, this small polypeptide is associated with TFIIH in mammalian cells.

We further analyzed the association of p8 with TFIIH using purified TFIIH from HeLa cells[19]. Silver staining showed that a protein band of ~8 kDa copurified with the other TFIIH subunits and with the transcription and DNA repair activities (**Fig. 1b**). MALDI peptide mass fingerprint analysis on tryptic digests of this ~8-kDa band showed that all identified peptides were part of the human ortholog of TFB5 (**Fig. 1c**). Finally, immunoblotting using a polyclonal antibody raised against this 8-kDa polypeptide showed that it copurified with TFIIH (**Fig. 1b**). These experiments unambiguously identify this polypeptide (p8) as the human ortholog of TFB5 and as a new, tenth component of TFIIH.

We microinjected the cDNA into polynucleated fibroblasts from individuals with TTD-A and determined the DNA repair capacity of the injected cells by measuring UDS. Injected cells had greater UDS

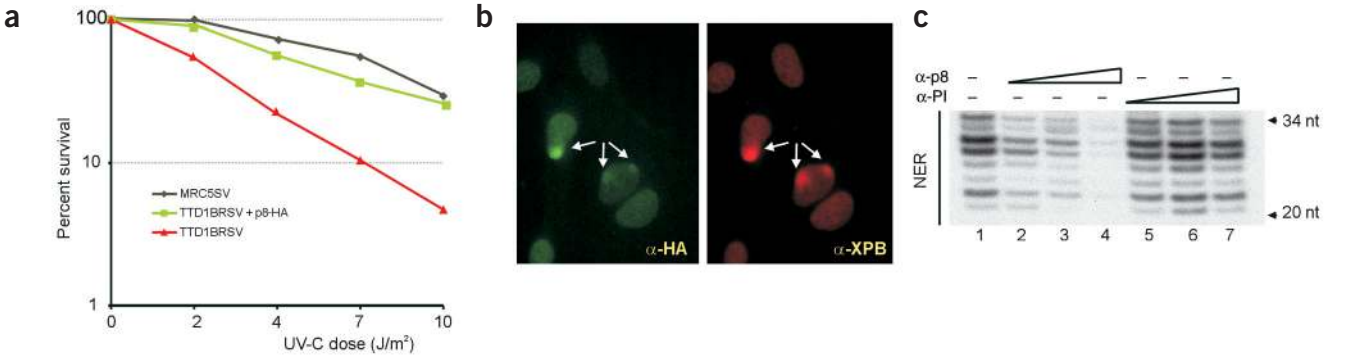


Figure 3 NER characteristics of TFB5. (**a**) UV survival using wild-type MRC5SV cells (black diamonds), TTD1BRSV cells (red triangles) and TTD1BRSV cells expressing TFB5-HA (green squares). The percentage of surviving cells is plotted against the UV-C dose. (**b**) Locally UV-irradiated (40 J m^{-2}) TTD1BRSV cells expressing TFB5-HA were stained with antibodies to HA (left panel) and XPB (right panel). Locally damaged areas are indicated by the arrows. (**c**) TFIIH hydroxyapatite fraction 6 (1 μl) was preincubated with no antibody (lane 1), with increasing amounts of antibody to p8 (1, 5 and 10 μg; lanes 2–4, respectively) or with antibody control (α-PI; 1, 5 and 10 μg; lanes 5–7, respectively) before being supplemented with all other NER factors (XPC-R23b, XPA, RPA, XPG and ERCC1-XPF) in addition to damaged DNA to measure dual incision.

(elevated grain number) than uninjected neighboring cells (**Fig. 2a**). *GTF2H5* cDNA corrected the repair defect of cells from individuals with TTD-A to a level comparable to that observed in wild-type cells assayed in parallel, suggesting that *GTF2H5* is mutated in TTD-A.

To verify that *GTF2H5* is implicated in TTD-A, we analyzed genomic DNA of three unrelated individuals with TTD-A (**Table 1**). Each of these individuals had a different inactivating mutation in *GTF2H5* (**Fig. 2b**). Individual TTD99RO carries a homozygous C→T transition at codon 56, converting CGA (arginine) into a TGA stop codon and truncating the protein by 23%. Siblings TTD13PV and TTD14PV carry (homozygously) a mutation in the start codon, converting ATG to ACG. This mutation will result in either a complete loss of protein synthesis or the production of an N-terminally truncated polypeptide (lacking the first and most conserved 15 amino acids; 21%) when a downstream AUG at codon 16 is used. Because only one allele was detected, both mutations can be considered functionally homozygous. Finally, individual TTD1BR seemed to be a compound heterozygote, with one allele identical to that of individual TTD99RO and the other allele carrying a T→C transition at codon 21, converting the conserved leucine residue to a proline. Genomic mutations were also identified in isolated mRNA, as shown by RT-PCR, indicating that these alleles are expressed (data not shown). Taken together, our data unambigously identified *GTF2H5* as the gene causing the NER defect in TTD-A.

To further explore the role of TFB5 in NER, we generated TTD1BR-SV cells stably expressing HA-tagged TFB5. Exposure of these cells to UV-C light (**Fig. 3a**) showed that the UV sensitivity of the cells was comparable to that of NER-proficient cells assayed in parallel.

Using an assay in which UV damage is locally inflicted in cell nuclei by irradiation through a filter that contains pores (5 μm), we previously showed that TFIIH components transiently accumulate at these sites[20–22]. We applied the same procedure to cells expressing TFB5-HA to test the participation of TFB5 in the NER reaction *in vivo*. At local damaged sites, marked by the accumulation of endogenous XPB, TFB5-HA also accumulated (**Fig. 3b**). This indicates that TFB5 participates in the NER reaction, like other NER factors[21–23].

We further tested the involvement of TFB5 in NER in a reconstituted *in vitro* incision-excision repair assay, using recombinant NER factors, purified TFIIH and a plasmid containing a cisplatin adduct. Addition of increasing amounts of purified polyclonal antibodies to TFB5 incubated with a fixed amount of purified TFIIH inhibited the repair reaction, whereas treatment with preimmune serum had no effect (**Fig. 3c**). These results indicate that TFB5 participates in NER as a part of TFIIH.

The NER defect and TTD-specific features in TTD-A seem to be linked to a reduced steady-state level of the total amount of TFIIH[1]. Cells that express TFB5-HA have a concomitantly higher XPB level (**Fig. 4a**), suggesting that TFB5 has a stabilizing function and protects TFIIH from degradation. Similar observations were made in cells stained with antibodies directed against other TFIIH components, namely p62, p44 and CDK7 (data not shown), indicating that the intranuclear levels of the entire TFIIH complex were increased. In contrast, levels of non-TFIIH NER factors (ERCC1) were not increased (**Fig. 4b**). Additionally, in immunoblot experiments (**Fig. 1a**), elevated levels of XPB and CDK7 were observed after expression of TFB5 expression. These experiments suggest that TFIIH concentration depends on the presence of TFB5. We transiently overexpressed TFB5-HA in MRC5 cells (wild-type SV40-immortalized). Even in these TFB5-proficient cells, transient overexpression of TFB5 seemed to increase the cellular TFIIH content (**Fig. 4c**), suggesting that expression of TFB5 regulates TFIIH concentration *in vivo*.

One of the most obvious cellular phenotypes of cultured cells from individuals with TTD-A is the severely reduced level (~30%) of TFIIH (**Table 1**). The fact that TFB5 transiently increases the level of TFIIH, even in wild-type cells (**Fig. 4c**), indicates that this 8-kDa protein is involved in regulating steady-state levels of TFIIH. Because mRNA levels of TFIIH components are not reduced in cells from individuals with TTD-A[1], control of the amounts of TFIIH by TFB5 must be post-transcriptional. Regulation at the translational or post-translational level is suggestive of a chaperone-like function for TFB5 in complex assembly or maintenance. An altered tertiary structure of TFIIH, by the absence of TFB5, might impede both the longevity of this complex and its repair and transcription activity.

Although parallels of TFIIH composition, structure and function (**Table 2**) can be drawn between humans and yeast, differences in the relative contribution of TFB5 to transcription efficiency are also observed. In yeast, the absence of TFB5 seemed to affect the efficiency of transcription and NER, whereas in humans, mutations of TFB5 do not seriously affect transcription[24]. Furthermore, in humans the absence of TFB5 seemed to predominantly affect TFIIH stability and steady-state levels, whereas in yeast, absence of TFB5 did not reduce

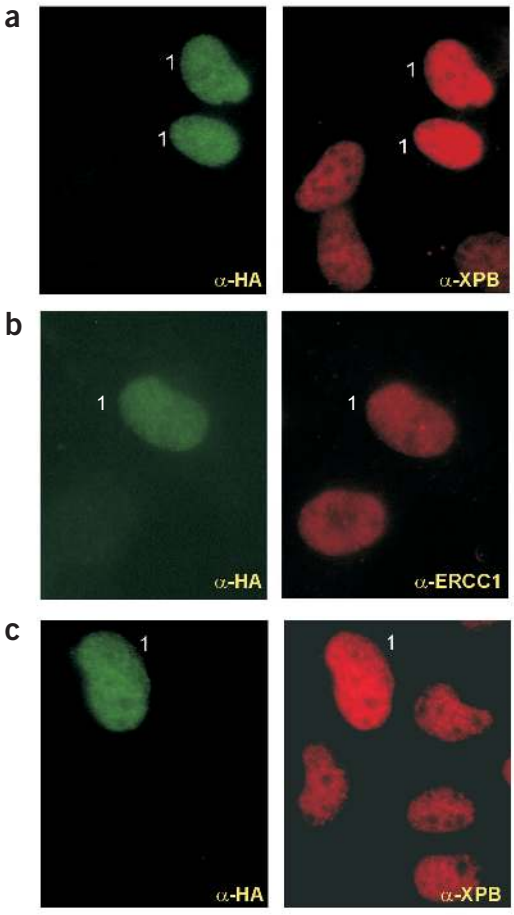


Figure 4 Recovery of reduced concentrations of TFIIH in fibroblasts from individuals with TTD-A expressing TFB5-HA. (**a**) Mass population of TTD1BRSV cells expressing TFB5-HA. Cells expressing TFB5-HA (left panel) had an increased level of XPB (right panel). (**b**) Mass population of TTD1BRSV cells expressing TFB5-HA. ERCC1 levels (right panel) were not influenced by expression of TFB5-HA (left panel). (**c**) MRC5SV cells expressing TFB5-HA (left panel) showed an increased level of XPB (right panel) 1, cells expressing TFB5-HA.

TFIIH levels, as judged by levels of TFB1 and Kin28 (J.A.R., unpublished data). Future experiments should identify the mechanistic details of TFB5 function in humans and yeast.

The severe effect of *GTF2H5* mutations on NER function suggests that NER requires higher concentrations of TFIIH than does transcription. Live cell studies showed that TFIIH participates substantially longer in NER than in transcription[22], providing a possible explanation for the increased need for sufficient amounts of TFIIH in NER. Additionally, an altered structure of TFIIH caused by mutant TFB5 may primarily affect NER function.

In conclusion, the identification of TFB5 as a new, evolutionarily conserved, tenth subunit of TFIIH provides essential information to understand the molecular mechanism of TFIIH function in DNA repair, transcription and human disease. These findings unambiguously identify *GTF2H5* as the gene causing the NER defect in TTD-A and provide an opportunity to begin to dissect the molecular details underlying the disease.

METHODS

Cell lines, culture conditions and transfections. We cultured primary human fibroblasts TTD99RO, TTD1BR, TTD13PV, TTD14PV (all TTD-A) and C5RO (wild-type, NER-proficient) under standard conditions at 37 °C, 5% CO_2 and 3% oxygen in Ham's F-10 medium supplemented with 12% fetal calf serum and antibiotics (penicillin and streptomycin). We cultured SV-40 transformed cell lines TTD1BR-SV (TTD-A) and MRC5-SV (wild-type) at 37 °C, 5% CO_2 in a 1:1 mixture of Ham's F-10 medium and Dulbecco's modified Eagle medium (Gibco) supplemented with 8% fetal calf serum and antibiotics. We cloned C-terminally HA-tagged TFB5 into the expression vector pCDNA3 (Invitrogen) and transfected this construct into TTD1BRSV fibroblasts using FUGENE 6 transfection reagent (Roche). We subjected transfectants to triple UV-C irradiation of 8 J m^{-2} with 2-d intervals to enrich the population of cells expression TFB5-HA. One week after the last irradiation, stable transfectants were single cell–sorted (FACSVantage, Becton Dickinson). We tested colonies derived from single cells by immunofluorescence with antibody to HA after expressing the fusion gene. We tested the repair capacity of a selected clone stably expressing TFB5-HA (UV survival).

Immunoprecipitation and immunoblot analysis. We prepared whole-cell extracts from MRC5SV, TTD1BRSV and TTD1BRSV+TFB5HA fibroblasts by isolating cells from six petri dishes (13-cm) per cell line. We collected the cell pellets and washed them once in phosphate-buffered saline (PBS) before lysing them by douncing (20 strokes using a 12.61-mm dounce homogenizer, Bellco Glass) in 2 ml of buffer A (50 mM Tris (pH 7.9), 150 mM NaCl, 20% glycerol, 0.1% Nonidet-P40 and 5 mM β-mercaptoethanol) supplemented with antiproteases. We incubated cellular extracts from MRC5SV, TTD1BRSV and TTD1BRSV+TFB5HA fibroblasts overnight at 4 °C in buffer A with antibodies to HA (12CA5) and p44 (1H5) cross-linked to protein A–sepharose beads (Amersham Biosciences). Before immunoprecipitations, we washed cross-linked beads three times in buffer A. After incubation with the extracts, we washed the beads extensively with buffer A and then analyzed them by SDS-PAGE and immunoblotted them using antibodies to XPB (1B3), cdk7 (2F8) and HA (3F10).

Mass spectrometry. We separated TFIIH subunits on a 15% polyacrylamide SDS-PAGE gel and stained them with silver[25]. The band migrating below the 10-kDa marker was excised and digested in the gel with trypsin[26]. We concentrated the peptide extracts by speed-vac, purified them by chromatography on a C18 reverse-phase 'ZipTip' pipette tip (Millipore) and finally eluted them in 2 μl of 50% acetonitrile. We mixed 0.5 μl of peptides either with an equal volume of saturated alpha-cyano-4 hydroxycinnamic acid (dissolved in 50% acetonitrile) or with the same volume of saturated 2,5-dihydroxybenzoic acid (dissolved in 20% acetonitrile). Both matrices were purchased from LaserBio Labs. We carried out mass measurements on a Bruker Reflex IV MALDI-TOF spectrometer in the positive-ion reflector mode. The mass acquisition range was 800–3,000 Da with a low mass gate set at 700 Da. Internal calibration was done using autolytic trypsin peptides (MH^+ with m/z = 842.51, 2211.11 and 2807.47). Monoisotopic peptide masses were assigned manually using the Bruker X-TOF software. We carried out database searches (National Center for Biotechnology Information, proteins of human origin) with the Profound program using the following parameters: molecular weight of 0–10 kDa, trypsin digestion (one missed cleavage tolerated), cysteines modified by carbamidomethylation, methionine oxidation and mass tolerance of 75 ppm. Four of five p8 peptides were detected in the MALDI spectrum using both alpha-cyano-4 hydroxycinnamic (data not shown) and 2,5-dihydroxybenzoic acids as matrix.

Microinjection and DNA repair assays. We microinjected *GTF2H5* cDNA constructs (with and without a HA tag) into fibroblasts from individuals with TTD-A (TTD99RO, TTD1BR) as described[27]. Three days before microinjection, we fused cells using inactivated Sendai virus and seeded them onto coverslips. To allow the expression of the injected gene, we exposed cells to 15 J m^{-2} UV-C 24 h after injection and incubated them for 2 h in culture medium supplemented with ^{3}H-thymidine. After removing excess free ^{3}H-thymidine, we fixed cells. We determined DNA repair capacity (UDS) as the amount of autoradiographic grains above the nuclei, which is usually severely reduced in NER-deficient cells (for details see ref. 1). We determined the UV sensitivity of TTD1BRSV (TTD-A), TTD1BRSV+TFB5-HA (TTD-A corrected) and NER-proficient MRC5SV (wild-type) human fibroblasts by incorporation of ^{3}H-thymidine, as described previously[28,29].

Mutation analysis of *TFB5*. We extracted total RNA (10 μg) from wild-type fibroblasts (C5RO) and primary fibroblasts from individuals with TTD-A (TTD99RO; TTD1BR; TTD13PV and TTD14PV) using RNeasy Mini columns (Qiagen) and used it to prepare cDNA by reverse transcription. Reverse transcription was carried out using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). We amplified *GTF2H5* cDNA using puReTaq Ready-To-Go PCR Beads (Amersham) and 10 pmol of primers (Tm: 60°). We amplified the coding region of genomic *GTF2H5* using two sets of primers covering the coding exons 2 and 3. Primers sequences are available on request. We amplified genomic DNA (100–500 ng) using puReTaq Ready-To-Go PCR beads (Amersham) and 10 pmol of each primer set. Amplification products were cloned into T-easy vector (Promega). For each cell line, we sequenced 18 independent clones using BaseClear lab services.

Immunofluorescence. Two days after micro injection or transfection, we washed cells twice in PBS, fixed them in 2% paraformaldehyde, permeabilized them two times for 10 min, rinsing in PBS containing 0.1% Triton X-100 (PBS-T) and then washed them with PBS plus 0.15% glycine and 0.5% bovine serum albumin (PBS+). We diluted antibodies in PBS+ and incubated cells with antibodies for 2 h at room temperature in a moist chamber. Dilutions were 1,000× for antibodies to XPB (IB3) and to HA (3F10, Roche). We then washed coverslips (five times for 5 min each) in PBS-T and incubated them with the secondary antibody: goat antibody to mouse conjugated with Cy3 (The Jackson Laboratory) and goat antibody to rat conjugated with Alexa 488 (Molecular Probes), respectively, each diluted 1,000× in PBS+. After the same washing procedure, we mounted coverslips in Vectashield mounting medium (Vector Laboratories) containing 1.5 μg μl^{-1} of DAPI. We produced epifluorescent and phase-contrast images on a Leitz Aristoplan microscope equipped with epifluorescence optics and a PLANAPO 63×/1.40 oil immersion lens.

Dual incision NER assay. We carried out the dual incision assay as described[30] in 25 μl of dual incision buffer supplemented with 2 mM ATP. Each reaction contained 5 ng of XPG, 15 ng of ERCC1, 10 ng of XPC-HR23B, 50 ng of RPA, 25 ng of XPA and TFIIH as indicated. After 10 min of preincubation at 30 °C, we added 30 ng of Cys-platinum damaged DNA and continued the reactions for 90 min at 30° C. We detected the excised fragment on 14% urea-acrylamide after annealing with 9 ng of the complementary oligonucleotide and adding four radioactively labeled dCMPα-P^{32} (3,000 μCi $mmol^{-1}$) residues by Sequenase V2.1 (USB). Before adding it to the repair reactions, we preincubated TFIIH with either polyclonal antibodies to p8 or preimmune serum. The rabbit polyclonal antibody was produced by coupling the peptide VGELMDQNAFSLTQK (57–71) to ovalbumin and injecting it in rabbits.

Local ultraviolet irradiation. We irradiated cultured cells with UV (254 nm) at 40 J m^{-2} through an isopore polycarbonate filter (Millipore) containing 5-μm pores as described[20,21]. After removing the filter, we fixed cells with paraformaldheyde and further processed them for immunocytochemistry as described above.

URL. The Profound program is available at http://prowl.rockefeller.edu/.

GenBank accession numbers. The complete human cDNA sequence is available under BC060317, AK055106 or AJ634743.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

This work was supported by grants from European Molecular Biology Organization, the Dutch Basic Research Organization, the Dutch Cancer Society, European Union and the National Institute of Health.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 13 February; accepted 3 May 2004
Published online at http://www.nature.com/naturegenetics/

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