XPD mutations in trichothiodystrophy hamper collagen VI expression and reveal a role of TFIIH in transcription derepression

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Mutations in the XPD subunit of the transcription/DNA repair factor (TFIIH) give rise to trichothiodystrophy (TTD), a rare hereditary multisystem disorder with skin abnormalities. Here, we show that TTD primary dermal fibroblasts contain low amounts of collagen type VI alpha1 subunit (COL6A1), a fundamental component of soft connective tissues. We demonstrate that COL6A1 expression is downregulated by the sterol regulatory element-binding protein-1 (SREBP-1) whose removal from the promoter is a key step in COL6A1 transcription upregulation in response to cell confluence. We provide evidence for TFIIH being involved in transcription derepression, thus highlighting a new function of TFIIH in gene expression regulation. The lack of COL6A1 upregulation in TTD is caused by the inability of the mutated TFIIH complexes to remove SREBP-1 from COL6A1 promoter and to sustain the subsequent high rate of COL6A1 transcription. This defect might account for the pathologic features that TTD shares with hereditary disorders because of mutations in COL6A genes.

INTRODUCTION

Trichothiodystrophy (TTD; OMIM #601675) is a rare autosomal recessive multisystem disorder whose hallmark is sulfur-deficient brittle hair due to reduced levels of cysteine-rich matrix proteins. TTD patients also exhibit other features of varying clinical severity, which include ichthyotic skin, nail dysplasia, physical and mental retardation, decreased fertility, proneness to infections, signs of premature aging and, in about half of the patients, cutaneous photosensitivity (1,2). Cells from photosensitive TTD patients show an altered response to ultraviolet (UV) light caused by defects in nucleotide excision repair (NER), the sole mechanism that in human cells removes UV-induced DNA damage. The majority of these patients are mutated in XPD (TTD/XP-D), whereas rare cases were found mutated in either the XPB or TTDA gene [reviewed in (3,4)]. These genes encode subunits of the transcription factor IIH (TFIIH) that, besides participating in basal and activated transcription, is also engaged in NER (5). Mutations in the XPD gene have been found not only in TTD but also in several cases with the skin cancer-prone disorder xeroderma pigmentosum (XP; OMIM #278730) and in rare cases with the combined symptoms of XP and Cockayne syndrome (XP/CS). The finding that defects in a single gene are responsible for different disorders was rationalized by the discovery of the dual role of TFIIH in repair and transcription (6,7) leading to the hypothesis that XP is caused by mutations that interfere mainly with repair, whereas TTD clinical features are due to additional subtle impairments of transcription (8,9). This notion has been supported by the XPD gene mutation spectrum in patients, indicating that the mutations responsible for the distinct disorders are located in different sites of the gene (10). All the mutations responsible for TTD cause a decrease by up to 70% in the cellular content of TFIIH (11,12) and interfere with the basal transcription activity of TFIIH (13). In addition, emerging evidence in mouse model and in patient cells indicates that TTD clinical features are associated with impairments of the TFIIH regulatory role in transcription (14–16) and a reduced expression of several genes in terminally differentiating cells (17–21).

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In view of the skin defects typically present in TTD, we searched for possible alterations in the extracellular matrix (ECM), the structure synthesized mainly by dermal fibroblasts, that forms the bulk of the dermis and plays fundamental roles in normal tissue homeostasis, cell adhesion and cell migration. Collagen is by far the most abundant ECM component, it makes up 80% of the dry weight of the dermis and provides strength to the skin by structurally supporting the overlying layer of keratinocytes.

Here, we show that the amount of the alpha 1 subunit of collagen type VI (COL6A1), a ubiquitous component of connective tissues largely present in the dermis, is severely reduced in TTD primary skin fibroblasts upon confluence. The alteration occurs specifically in TTD and not in XP cells carrying XPD mutations and it is caused by a transcriptional defect that is recovered following the expression of wild-type XPD (wtXPD). Furthermore, we disclose the mechanism that in human dermal fibroblasts triggers COL6A1 transcription upregulation in response to cell density, an event that requires the TFIH-mediated removal of the sterol regulatory element-binding protein-1 (SREBP-1) from COL6A1 promoter. In addition, we reveal the basis of TFIH failure in modulating COL6A1 expression in TTD primary fibroblasts.

RESULTS

**COL6A1 is specifically reduced in high-cell density TTD/XP-D fibroblasts**

Skin alterations are typically present in TTD patients, the most common being dry, scaly ichthyotic skin (compare Fig. 1A and B with Fig. 1C) that is seen in almost all age groups and might be manifested already during the first weeks after birth. Other skin symptoms include collodion membrane at birth, erythroderma, eczema, freckles and telangiectasia (1,2). Because collagen is the most abundant component of the dermis and its synthesis by cultured fibroblasts depends on several conditions, including cell density (22), we analyzed COL6A1 protein levels in low- and high-cell density cultures of primary skin fibroblasts from normal and TTD/XP-D donors (Fig. 1D, phase contrast panels). Immunofluorescence assays using anti-COL6A1 antibodies showed no difference among low-cell density cultures, whereas in high-cell density, a weaker staining signal was commonly observed in TTD with respect to normal cells (Fig. 1D, COL6A1 panels). Immunoblot analysis in fibroblasts from three normal donors repeatedly revealed a 2–3 fold increased amount of COL6A1 in high-cell density when compared with low-cell density cultures (Fig. 1E, lanes 1–6). In contrast, no COL6A1 enhancement was detected in any of three TTD/XP-D patients (lanes 7–12). These findings were sustained by investigations on TTD family members (Fig. 1F). Confluent fibroblasts from patient parents (lanes 5–8) showed an accumulation of COL6A1 that was in the range detected for genetically unrelated healthy donors, whereas only slight variations were found between low- and high-cell density fibroblast cultures from the affected daughters (TTD2PV and TTD3PV, lanes 1–4). The analysis of COL6A1 content in high-cell density cultures was extended to a total of nine TTD cases who are homozygous, hemizygous or compound heterozygous for distinct mutated XPD alleles, including the changes arg112his and arg722trp that represent the most frequent alterations observed in TTD. All the analyzed patients show typical hair and skin alterations associated with different degrees of severity in physical and mental retardation, DNA repair defect and reduction in TFIH cellular levels (Table 1). In every case, mutations responsible for TTD resulted in reduced amounts of COL6A1 when compared with normal and XP fibroblasts, including XP strains homozygous and compound heterozygous for arg683 substitution, the alteration found in 80% of XP patients mutated in XPD (Fig. 1G). These results demonstrate that COL6A1 deficiency in confluent fibroblasts is marker specific for TTD/XP-D.

**wtXPD raises COL6A1 protein levels in high-cell density TTD fibroblasts**

To establish whether TTD/XP-D mutations are accountable for the COL6A1 defect, TTD dermal fibroblasts were transfected with the wtXPD-expressing plasmid (pXPD) co-expressing wtXPD and the reporter HaloTag-PDGFR-TM protein that allows the in vivo staining of wtXPD-expressing fibroblasts (Fig. 2A, left panel). Cells were then seeded at low- and high-cell density and analyzed 2 days later for restoration of TFIH activity in DNA repair, TFIH cellular amount and COL6A1 content. Following UV irradiation, HaloTag-positive cells (Fig. 2A, white arrow) showed increased DNA repair synthesis levels, with an average 8-fold increment in the number of autoradiographic grains over the nucleus (Fig. 2A, black arrow), demonstrating the capacity of wtXPD to restore the DNA repair function of TFIH in TTD [see also (12,23)]. In parallel, the cellular amount of TFIH that is typically reduced in TTD showed a substantial increase both in low- and high-cell density cultures, as indicated by the increased concentration of its cdk7 and p62 subunits (Fig. 2B). Concomitantly, we noticed the doubling of COL6A1 content in high-cell density TTD fibroblasts transfected with the pXPD plasmid, whereas no substantial change was observed in low-cell density cultures (Fig. 2B). These findings clearly indicate that the mutated XPD subunits present in TTD are responsible for the observed COL6A1 reduction in high-cell density fibroblast cultures.

**Altered COL6A1 promoter activity in TTD fibroblasts**

To establish the cause of the reduced COL6A1 protein levels in TTD, we first analyzed the expression of COL6A1 gene in low- and high-cell density cultures by quantitative real-time RT-PCR. Increased levels of COL6A1 mRNA were repeatedly observed in high-cell density when compared with low-cell density fibroblasts from normal and XP/XP-D donors (Fig. 3A). In contrast, no increase or even a decrease in COL6A1 transcripts occurred in confluent TTD cell strains representative of different combinations of mutated XPD alleles. The above data suggest that the reduced amount of COL6A1 observed in TTD cells might be due to a gene expression defect at the transcriptional level. This prompted us to investigate the regulatory domains of the COL6A1 promoter by dual luciferase assays in normal C3PV and in TTD fibroblasts. Luciferase activity was measured in cells...
TTD/XP-D confluent fibroblasts show a reduced amount of COL6A1. (A and B) Dry, scaly and ichthyotic skin typical of TTD in an 8-year-old patient. Scale bar, 4 mm. (C) Skin of a healthy 8-year-old child. Scale bar, 4 mm. (D) Indirect immunofluorescence staining with anti-COL6A1 antibodies in low- (upper panels) and high- (lower panels) cell density cultures from the normal individual C3PV and the TTD/XP-D patient TTD2PV. The same fields are shown in phase contrast. Scale bar, 40 μm. (E and F) COL6A1 immunoblot analysis in cell lysates of low- or high-cell density cultures of primary fibroblasts from three normal donors (C3PV, C377RM and C345RM) and three TTD/XP-D patients (TTD12PV, TTD23PV and TTD22PV) (E), two affected siblings (TTD2PV, TTD3PV) and their parents (F). γ tubulin is the loading control. Protein levels were normalized to the amount of γ tubulin, and the values reported in the diagram are the mean of three independent experiments. (G) COL6A1 immunoblot analysis on total extracts of confluent fibroblast cultures from the normal donor C3PV, seven TTD/XP-D patients (TTD2PV, TTD8PV, TTD11PV, TTD12PV, TTD20PV, TTD22PV and TTD24PV) and three XP patients mutated in either the XPD (XP16PV and XP17PV) or XPC (XP26PV) gene. The suffix PV in the strain code is omitted for the sake of brevity.
Table 1. Clinical, cellular and molecular features of the 11 XP-D patients analyzed in this study

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Clinical severity</th>
<th>XPD alleles, amino acid changes</th>
<th>UDS, % of normal</th>
<th>TFIIF levels, % of normal</th>
<th>References</th>
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<tbody>
<tr>
<td>TTD2PV</td>
<td>Moderate</td>
<td>R112H/R112H</td>
<td>10</td>
<td>45</td>
<td>(12,17,20,32,39)</td>
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<tr>
<td>TTD3PV</td>
<td>Moderate</td>
<td>R112H/R112H</td>
<td>10</td>
<td>43</td>
<td>(17,20,32,39,40)</td>
</tr>
<tr>
<td>TTD8PV</td>
<td>Moderate</td>
<td>R112H/R112H</td>
<td>10</td>
<td>35</td>
<td>(12,32)</td>
</tr>
<tr>
<td>TTD11PV</td>
<td>Severe</td>
<td>R112H/V121_E159del</td>
<td>10</td>
<td>44</td>
<td>(12,32)</td>
</tr>
<tr>
<td>TTD12PV</td>
<td>Severe</td>
<td>R722W/C259Y</td>
<td>20</td>
<td>60</td>
<td>(12,32)</td>
</tr>
<tr>
<td>TTD20PV</td>
<td>Severe</td>
<td>R112H[L461V;V716_R730del]</td>
<td>10</td>
<td>48</td>
<td>This study</td>
</tr>
<tr>
<td>TTD22PV</td>
<td>Moderate</td>
<td>Q662X/E731F6X100 + E731GfsX50</td>
<td>25</td>
<td>60</td>
<td>(41)</td>
</tr>
<tr>
<td>TTD23PV</td>
<td>Severe</td>
<td>R112HUnexpressed</td>
<td>10</td>
<td>37</td>
<td>This study</td>
</tr>
<tr>
<td>TTD24PV</td>
<td>Moderate</td>
<td>R722W/E317D6X110 + normal</td>
<td>40</td>
<td>55</td>
<td>(41)</td>
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<tr>
<td>XP16PV</td>
<td>Mild</td>
<td>R683Q/R683Q</td>
<td>25</td>
<td>108</td>
<td>(10,12)</td>
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<tr>
<td>XP17PV</td>
<td>Mild</td>
<td>R683W/R616P</td>
<td>15</td>
<td>81</td>
<td>(12,42)</td>
</tr>
</tbody>
</table>

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. For details, see the quoted references.

UV-induced DNA repair synthesis (Unscheduled DNA Synthesis, UDS) after irradiation with 10 J/m² observed in patient cells is expressed as percentage of the value found in normal cells analyzed in parallel.

TFIIF levels in patient fibroblasts are expressed as percentages of the corresponding value in normal C3PV fibroblasts analyzed in parallel (12).

Sisters.

transfected with reporter plasmids containing the luciferase gene under the control of different fragments of COL6A1 promoter (Fig. 3B and Supplementary Material, Fig. S1, upper panel). In both normal and TTD cells, the COL6A1 promoter activity was undetectable with the shortest fragment (+1/+95). The other six genomic fragments (encompassing the COL6A1 DNA region from the position +95 up to −7995) were all able to transcribe the reporter gene. Remarkably, all the transcriptionally active COL6A1 reporter plasmids exhibited a substantially reduced firefly luciferase activity in TTD cells, ranging between 40 and 70% of normal. The transcription failure was specific for the COL6A1 promoter because TTD fibroblasts efficiently transcribed the reporter gene driven by the HSV-tk promoter (Supplementary Material, Fig. S1, lower panel). Overall our data suggest that the COL6A1 transcription deregulation in TTD occurs within the −304/+95 sequence surrounding the COL6A1 transcription start site.

In silico analysis of the human COL6A1 proximal promoter revealed a cluster of several putative DNA-binding sites for the transcription factor specificity protein 1 (Sp1) (Fig. 3C). Accordingly, upon addition of increasing amounts of nuclear extracts (NE) isolated from normal cells, in vitro DNase 1 footprint analysis of the proximal promoter unveiled the protection of four different regions (I–IV, Fig. 3D, lanes 2 and 3), which was suppressed by competitor oligonucleotides containing Sp1-binding sites (lanes 4–6). Deletion of most Sp1-binding sites (from −180 to −32) abolished COL6A1 expression both in normal and TTD fibroblasts (Fig. 3E). In silico analysis also identified one putative site for the transcription factor specificity protein 1 (Sp1) (Fig. 3C). Accordingly, upon addition of increasing amounts of nuclear extracts (NE) isolated from normal cells, in vitro DNase 1 footprint analysis of the proximal promoter unveiled the protection of four different regions (I–IV, Fig. 3D, lanes 2 and 3), which was suppressed by competitor oligonucleotides containing Sp1-binding sites (lanes 4–6). Deletion of most Sp1-binding sites (from −180 to −32) abolished COL6A1 expression both in normal and TTD fibroblasts (Fig. 3E). In silico analysis also identified one putative site for the SREBP-1 (in the region +79/+85, Fig. 3C), whose presence was confirmed by different experimental approaches (see below, Figs 4E and 6A). Strikingly, deletion of the SREBP-1-binding site (from +80 to +94) significantly increased the transcriptional activity of the −304/+95 COL6A1 fragment in normal (2-fold, P-value <0.001) and, at a lower degree, in TTD (1.3-fold, P-value <0.01) fibroblasts.

Altogether, the above results indicate that the Sp1-binding sites are essential to sustain the transcriptional activity of COL6A1 promoter, whereas the SREBP-1-binding site might exert a negative regulatory function.

COL6A1 transcription dynamic is restored in TTD cells upon wtXPD expression

To gain further insights into COL6A1 transcription regulation, we analyzed the dynamics of RNA polymerase II (RNAPol II), Sp1 and SREBP-1 on COL6A1 promoter in low- and high-cell density cultures of normal and TTD fibroblasts, following chromatin immunoprecipitation (ChIP). Co-immunoprecipitated chromatin fragments were evaluated by quantitative real-time PCR using primers encompassing the COL6A1 promoter domains −304/−186 or +1/+92 or the exon II region +898/+992 (Fig. 4A). In normal C3PV cells at low-cell density, RNAPol II as well as Sp1 and SREBP-1 are present on COL6A1 promoter (Fig. 4B–E, compare amplicons I and II with amplicon III). In these conditions, the phosphorylation of RNAPol II at serine 5 [Fig. 4C, serine 5 phosphorylated-carboxi-terminal domain (SSP-CTD)] and at serine 2 [Supplementary Material, Fig. S2, serine 2 phosphorylated CTD (S2P-CTD)] clearly indicates the engagement of the RNA synthesis process (24). Concomitant with COL6A1 transcription upregulation (Fig. 4A), a higher recruitment of RNAPol II and Sp1 on COL6A1 promoter was observed in high-cell density when compared with low-cell density C3PV cultures (Fig. 4B–D). Conversely, the occupancy of SREBP-1 on COL6A1 promoter was reduced by 50% (Fig. 4E), suggesting a repressor-SREBP-1-based transcriptional regulation mechanism. Interestingly, in TTD fibroblasts the high-cell density was not associated with either increased RNAPol II and Sp1 occupancy (Fig. 4F–H) or SREBP-1 removal from COL6A1 promoter (Fig. 4I). Overexpression of wtXPD fully restored the recruitment of RNAPol II and Sp1 as well as the concomitant removal of SREBP-1 (Fig. 4J–M). Altogether, these results suggest the involvement of SREBP-1 in COL6A1 transcription deregulation observed in TTD cells.
SREBP-1 removal is a key event in COL6A1 transcription upregulation

The role of SREBP-1 in COL6A1 gene expression regulation was further investigated in normal fibroblasts, following treatment with short interfering RNA (siRNA) molecules. When compared with the control siRNA, the silencing of SREBP-1 led to an increased recruitment of RNApol II and Sp1 on COL6A1 promoter (Fig. 5A). This resulted in a significant increment of COL6A1 level (Fig. 5B) that agrees with the increased transcriptional activity of COL6A1 promoter lacking its SREBP-1-binding site (Fig. 3E). Interestingly, Sp1 silencing impeded the recruitment of RNApol II (Fig. 5A), underlining the role of Sp1 in the formation of the transcription complex in the TATA-less COL6A1 promoter.

The repressor role of SREBP-1 on COL6A1 transcription was further investigated in a dual luciferase assay using the (−304/+95) COL6A1 promoter fragment. Transfection of C3PV normal cells with increasing concentrations of SREBP-1 decoy oligonucleotides (that sequester SREBP-1 from its endogenous DNA targets) resulted in a progressive increment of the luciferase activity by up to three times when compared with basal levels (Fig. 5C, compare lanes 5 and 9 with lane 1). In TTD23PV cells, transfection of either increasing concentrations of SREBP-1 decoy oligonucleotides or pXPD resulted in a 2-fold increased luciferase activity, thus approaching the basal level observed in normal C3PV cells (Fig. 5C, compare lanes 2, 6, 10 and lane 4 with lane 1). These findings demonstrate that both SREBP-1 removal and wild-type TFIIH play crucial roles in COL6A1 transcription regulation. As a control, we used scrambled oligonucleotides that did not significantly modify the luciferase expression in both normal and TTD cells (Fig. 5C, compare lanes 13, 17 with lane 1 and lanes 14, 18 with lane 2). Interestingly, co-transfection of TTD cells with both wtXPD (that restores the cellular concentration and functional activities of TFIIH Fig. 2B) and increasing amounts of SREBP-1 decoy oligonucleotides resulted in luciferase expression levels similar to those observed in normal cells transfected with SREBP-1.
Figure 3. The transcriptional activity of COL6A1 promoter is reduced in TTD/XP-D primary fibroblasts. (A) Real-time quantification of COL6A1 mRNA levels in high- and low-cell density cultures from normal (C3PV, C345RM and C377RM), TTD (TTD12PV, TTD22PV and TTD23PV) and XP (XP16PV and XP17PV) donors. COL6A1 expression was normalized to the expression of the GAPDH housekeeping gene. The reported values represent the means of at least three independent experiments (∗P < 0.01; **P < 0.005; ***P < 0.001; ns, not statistically significant; Student’s t-test). (B) COL6A1 promoter activity in C3PV and TTD23PV fibroblasts 48 h after co-transfection with pCOL6A1 promoter-luc2 plasmids and the pGL4.74-hRluc/tk plasmid. In the pCOL6A1 promoter-luc2 plasmids, the firefly luciferase reporter gene is placed under the control of different fragments of COL6A1 promoter, all sharing a common 3’end located at position +95 (in the untranslated region of exon 1) and extending up to −7995 bp. The pGL4.74-hRluc/tk plasmid contains the renilla luciferase gene under the control of HSV-tk promoter. The reported firefly luciferase activities were normalized to the renilla luciferase activity, and the values represent the means of at least four independent experiments performed in triplicate (∗P < 0.01; **P < 0.005; ***P < 0.001; ns, not statistically significant; Student’s t-test). (C) putative transcription factor-binding sites on the −304/+105 region of the human COL6A1 gene, as predicted by MatInspector (38) with a filter for ubiquitous and connective tissue and a score of matrix and core similarity >75%. The reported sequence corresponds to the nucleotide region 47401359–47401767 of COL6A1 sequence (GenBank NC_000021.8) and includes the promoter region (lowercase), the 5’UTR (uppercase) and the first codon (bold). +1 indicates the transcription initiation site (NM_001848.2). (D) DNase 1 footprinting analysis of COL6A1 proximal promoter. The −304/+95 COL6A1 promoter fragment was labeled at the 5’end and incubated with increasing amounts of nuclear extract from normal cells (NE). Increasing amounts of unlabeled oligonucleotide containing Sp1 response elements (Sp1-RE) were used as competitors. Four cis-regions (designated I-IV) specifically protected by Sp1 were localized in the proximal promoter. The arrow indicates the transcription start site. Lane 1 shows the digestion pattern of the DNA fragment in the absence of NE. (E) COL6A1 promoter activity in C3PV and TTD23PV fibroblasts processed as in (B). The pCOL6A1 promoter-luc2 plasmids are deleted in either the SREBP-1 (+80/+94) or Sp1 (+180–+32) potential binding sites. On the top, the −304/+95 COL6A1 promoter fragment is indicated in which the gray box represents multiple (>20) Sp1-binding sites, the black box indicates the unique potential SREBP-1-binding site and +1 indicates the transcription start site (NM_001848.2). ∗P < 0.01; ***P < 0.001.
Figure 4. The dynamic of RNApol II, Sp1 and SREBP-1 occupancy on COL6A1 promoter is altered in TTD/XP-D fibroblasts, and the alterations are rescued by wtXPD expression. (A) Schematic organization of the genomic fragment included between nucleotides −304 and +2521 of COL6A1 gene (GenBank accession number NG_008674.1). Introns are shrunk to a minimal length. The arrow indicates the transcription start site. The gray box indicates multiple (>20) Sp1-binding sites, whereas the black box indicates the unique potential SREBP-1-binding site. Horizontal bars show the position of the fragments amplified by quantitative ChIP assays (amplicon location: I −304/−186, II +1/+92 and III +898/+992). (B–I) RNApol II, Sp1 and SREBP-1 occupancy on the promoter and proximal exons of COL6A1 gene, in low- (gray boxes) and high- (black boxes) cell density cultures of normal (C3PV, B–E) and XPD mutated (TTD23PV, F–I) fibroblasts. ChIP assays on RNApol II were performed with distinct antibodies that recognize either the hypophosphorylated (RNApol IIA) or phosphorylated CTD at ser5 (S5P-CTD). The values are the mean of at least three independent experiments (**P < 0.005; ***P < 0.001; ns, not statistically significant; Student’s t-test). (J–M) RNApol II, Sp1 and SREBP-1 occupancy on the promoter and proximal exons of COL6A1 gene in high-cell density TTD23PV cells 48 h after transfection with the pXPD plasmid (gray boxes) or the empty vector pCtr (white boxes). ChIP assays were performed as described in (B–I).
Figure 5. SREBP-1 is a transcriptional repressor of COL6A1 in human primary fibroblasts. (A) RNApol II A, Sp1 and SREBP-1 occupancy on COL6A1 transcription start site (amplicon II in Fig. 4A) as detected by ChIP assays in C3PV fibroblasts approaching the high-cell density, 72 h after transfection with either SREBP-1, Sp1 or control siRNA. The values are the mean of at least three independent experiments. (B) Western blot (WB) analysis of COL6A1, Sp1 and SREBP-1 in cell lysates from samples analyzed in A. Protein levels were normalized to the amount of γ tubulin, and the values reported in the diagram are the mean of three independent experiments. (C) COL6A1 promoter activity in normal C3PV (yellow bars) and TTD23PV fibroblasts (gray bars) co-transfected with ~304/+95 COL6A1-promoter-luc2 and pGL4.74-hRluc/tk plasmids and increasing concentrations of SREBP-1 decoy or scrambled control oligonucleotides. Paralleled TTD23PV samples were co-transfected also with empty pCtr (white bars) or pXPD (black bars) plasmid. The reported firefly luciferase activities were normalized to the renilla luciferase activity, and the values are the mean of four independent experiments. (D) Indirect immunofluorescence staining with anti-COL6A1 antibodies in high-cell density TTD fibroblasts after transfection with either the empty vector pCtr or the pXPD plasmid and SREBP-1 or control siRNA. The intensity profile of COL6A1 immunofluorescence was obtained with the MetaMorph image analysis software. The same fields are shown in phase contrast. Scale bars, 40 μm.
decoy oligonucleotides (compare lanes 4, 8 and 12 with lanes 1, 5 and 9). These results demonstrate the capability of normal XPD subunits to recover TFIIH quantitative/conformational alterations that impair SREBP-1 removal and hamper COL6A1 transcriptional burst in TTD. Further support to this hypothesis was provided by the analysis of the endogenous COL61 promoter. TTD23PV fibroblasts cultured at low- and high-cell density were transfected with an empty pCtr (used as control) or the pXPD plasmid. Twelve hours after transfection, cells were treated with either SREBP-1 or control siRNA (lanes 9–12). Altogether, our results indicate that a TFIIH-binding affinity toward the COL6A1 promoter fragment (DNA) with purified SREBP-1a (lanes 2–12) and, when indicated, rTFIIH, lanes 3, 4, 11 and 12), NE from normal cells (NE, lanes 6 and 7), (NEΔTFIIH, lanes 9–12) and 300 mM ATP (lanes 4, 7, 10 and 12). After extensive washes (300 mM KCl), the binding of SREBP-1 or Sp1 was visualized by immunoblot. Lane 1 shows the non-specific SREBP-1 binding to magnetic beads in the absence of the COL6A1 promoter fragment (DNA). The intensity of SREBP-1 and Sp1 protein levels are reported in the diagram below. (A) Immunoblot analysis of cdk7, Sp1 and SREBP-1 proteins in immunoprecipitations (IP) performed with control IgG or antibodies raised against cdk7, SREBP-1 or Sp1 in NE of normal C3PV fibroblasts. (B) Immunoblot analysis of cdk7, Sp1 and SREBP-1 expression, as observed in TTD23PV fibroblasts cultured at low- and high-cell density cultures of normal C3PV fibroblasts, untreated (white boxes) or treated with control (gray boxes) or cdk7 (black boxes) siRNA. Each value was normalized to the value of the corresponding control (white boxes) and represents the mean of at least three independent experiments.

TFIIH activity is required for SREBP-1 removal from COL6A1 promoter

To elucidate whether TFIIH plays a direct role in SREBP-1 displacement from COL6A1 promoter, we designed an in vitro assay in which the −304/+95 COL6A1 promoter template was immobilized on magnetic beads and subsequently incubated with SREBP-1 in the presence or absence of highly purified recombinant TFIIH (rTFIIH). Under these experimental conditions, the capability of SREBP-1 to bind COL6A1 promoter was very weak (Fig. 6A, lanes 2, 5 and 8). Addition of NE isolated from normal cells strongly enhanced the binding of SREBP-1 to COL6A1 promoter (compare lanes 5 and 6), suggesting the involvement of additional partner(s) in SREBP-1 binding to COL6A1. Interestingly, when ATP was added to promote the formation of the transcription initiation complex, a 50% release of SREBP-1 was detected in the presence of NE (compare lanes 7 and 6). To determine whether ATP hydrolysis by TFIIH is implicated in SREBP-1 removal, the assay was performed using NE depleted of endogenous TFIIH (NEΔTFIIH). We observed that NEΔTFIIH was still able to promote the binding of SREBP-1 to the DNA fragment (compare lanes 8 and 9), indicating that the SREBP-1-binding affinity for COL6A1 promoter does not depend on the presence of TFIIH. Addition of either ATP alone or rTFIIH did not substantially disturb the SREBP-1/COL6A1 promoter interaction (lanes 10 and 11, respectively). However, the addition of both rTFIIH and ATP to the NEΔTFIIH clearly promoted the removal of more than 70% of SREBP-1 (lane 12). Under all these experimental conditions, Sp1 did not show relevant changes in its capability of binding COL6A1 promoter fragment (lanes 9–12). Altogether, our results indicate that a TFIIH-dependent ATP hydrolysis is necessary to promote the in vitro release of SREBP-1 from COL6A1 promoter. In vivo, the SREBP-1 protein directly interacts with the TFIIH complex, as shown by NE co-immunoprecipitation experiments with antibodies against cdk7 or SREBP-1 (Fig. 6B,
lanes 3 and 4, respectively). No binding of Sp1 was observed with either cdk7 or SREBP-1. Furthermore, treatment of normal primary fibroblasts with cdk7 siRNA did not affect Sp1 occupancy, but led to SREBP-1 accumulation and fibroblasts the removal of SREBP-1 from (Fig. 6C). Overall, these findings demonstrate that in human fibroblasts the removal of SREBP-1 from COL6A1 promoter in response to high-cell density and the COL6A1 transcription upregulation are both dependent on TFIIH activity.

DISCUSSION

Thus far, investigations on the mechanistic defects leading to XP, CS or TTD have been beneficial in understanding the function of TFIIH in both transcription and DNA repair and might help to define therapeutic approaches. In the present study, we provide evidence of alterations in the ECM of TTD primary skin fibroblasts with mutations in XPD (i.e. the gene encoding a subunit of TFIIH), by showing that collagen VI, one of the most abundant collagens of the dermis and all connective tissues, is significantly reduced in confluent fibroblasts. The COL6A1 protein reduction occurs in TTD/XP-D fibroblasts representative of different types and combinations of mutated XPD alleles. In contrast, a normal COL6A1 pattern was observed in cells from XP patients, even in those mutated in XPD. Thus, a reduced amount of COL6A1 in confluent fibroblasts appears to be a novel molecular marker of the most frequent form of TTD.

In this study, we demonstrate that in normal primary human fibroblasts, COL6A1 expression is downregulated by the transcription factor SREBP-1. Indeed, transfection with either siSREBP-1 RNA or SREBP-1 oligonucleotide competitor (Fig. 5A–C) as well as deletion of SREBP-1 DNA-binding site (Fig. 3E) considerably enhance COL6A1 promoter activity.

The overall results of our investigations prompt us to propose the following scenario. Sp1 binds to the TATA-less COL6A1 promoter (Figs 3D, 4D and 6A) to allow the formation of an active transcription preinitiation complex, as demonstrated by the finding that COL6A1 expression is compromised by the deletion of Sp1-binding sites and Sp1 silencing (Figs 3E and 5A). The presence of SREBP-1 on its DNA-binding site downregulates COL6A1 expression. The COL6A1 transcriptional upregulation in high-cell density normal fibroblasts requires the displacement of SREBP-1. Our results demonstrate that SREBP-1 removal from COL6A1 promoter is mediated by TFIIH in an ATP-dependent manner (Fig. 6A and C). ATP hydrolysis allows the promoter opening by the TFIIH helicase (25,26) and the phosphorylation of RNApol II by the TFIIH kinase, which stimulates promoter escape. These reactions are likely combined with an additional event involving the cdk7/SREBP-1 interaction (Fig. 6B) that could result in SREBP-1 post-transcriptional modifications. So far, several studies have shown the crucial role of TFIIH in regulating basal and activated transcription by phosphorylating DNA-binding activators, including nuclear receptors (13–15,27). Our work provides evidence for an additional role of TFIIH in removing a chromatin-bound transcriptional repressor.

Little is known about molecular details of how fibroblasts sense density changes and activate downstream signaling events. Because high-cell density has been shown to regulate the expression of specific target genes by triggering a cascade of phosphorylation-mediated pathways (28,29), we are tempted to speculate that some components of the transcriptional machinery acting on COL6A1 promoter might be modified by signaling events activated by cell—cell contacts. This, in turn, may expose SREBP-1 to post-translational modifications that facilitate the TFIIH-dependent removal of SREBP-1 from COL6A1 promoter by altering, for instance, its DNA-binding affinity. The signaling cascade activated by the high-cell density might also directly influence the local kinase and/or ATPase activity of the TFIIH complex, thus triggering SREBP-1 removal and, ultimately, a more efficient COL6A1 transcription.

In TTD fibroblasts, we observed a COL6A1 transcriptional impairment caused by the persistent occupancy of SREBP-1 on COL6A1 promoter. This defect was recovered, following SREBP-1 silencing or transfection with either SREBP-1 decoy oligonucleotides (Fig. 5D and C) or wtXPD (Figs 4J–M and 5C–D), further demonstrating the tight connection between SREBP-1 and TFIIH in COL6A1 expression upregulation. However, to reach the optimal COL6A1 expression levels observed in normal cells transfected with SREBP-1 decoy oligonucleotides, a wild-type TFIIH complex is required (Fig. 5C). Thus, our study discloses two distinct failures of TFIIH in TTD primary fibroblasts: inability to displace the repressor from COL6A1 promoter and incapacity to sustain an increased COL6A1 transcriptional demand. The finding that reduced amounts of TFIIH may become limiting in TTD cells is in agreement with previous observations in terminally differentiated tissues from patients (19,20) and TTD mouse model (16).

The reduced amount of COL6A1 in TTD fibroblasts might provide an explanation for those pathologic features observed within TTD patients, which are present in collagen VI-related disorders. Indeed, changes in the expression and localization of collagen VI microfibrils are associated with common musculoskeletal diseases, such as osteoarthritis, and mutations in the COL6A1 genes are responsible for Bethlem myopathy (BM, OMIM #158810) and Ullrich congenital muscular dystrophy (UCMD, OMIM #254090). Depending on the type of mutation, BM and UCMD patients show different age onsets and different degrees of clinical severity (30,31). Besides the most striking clinical manifestation of muscle weakness, these disorders exhibit joint contractures, skin defects, distal hyper-extensibility and kyphosis, also present in TTD. It is worthwhile mentioning that COL6 forms a microfibrillar extracellular network that interacts with basement membranes, fibrillar collagens and other ECM components and with receptors at the cell surface. Therefore, it is not unexpected that limiting amounts of structurally normal collagen VI molecules might interfere with all the network. The finding that COL6A1 haploinsufficiency causes BM (30) further supports the notion that a reduction in an otherwise normal COL6A1 may be a pathogenic mechanism. In addition, to deepen our understanding on the etiology of some TTD clinical features and to provide a new diagnostic tool, our study discloses the mechanism of COL6A1 regulation in primary fibroblasts.
MATERIALS AND METHODS

Cell culture and transfection

The XP-D cell strains used in this study are listed in Table 1. In parallel, we used fibroblasts from the healthy donors C3PV, C345RM, C377RM and B119, the parents of TTD2PV and TTD3PV patients (32) and the XP-C patient XP26PV (33). Human primary fibroblasts in vitro established from skin biopsies were routinely cultured in HAM F10 medium (Cambrex) supplemented with 2% fetal calf serum (Euroclone). Cells were seeded in a 1:3 ratio and collected 4 days later, when the cultures reached the 70% (low) and 100% (high) cell density, respectively. For transfection experiments, 10⁶ fibroblasts were resuspended in 100 µl Human Dermal Fibroblast Nucleofector solution (VPD1001-Amamax) mixed with 3 µg DNA, electroporated accordingly to Nucleofector P-22 or U-23 programs, seeded and lysed 48 h later.

Immunoblot and immunofluorescence analysis

Analyses were performed according to standard protocols (34) using primary antibodies raised against COL6A1 (sc-20649) and Sp1 (sc-59, Santa Cruz Biotechnology); SREBP-1 (2A4, Abcam); cdk7, p62 and XPB (16); γ tubulin and actin (Sigma-Aldrich).

Quantitative RT-PCR

RNA extraction was performed using the RNaseasy Kit (Qiagen) according to the manufacturer’s instructions. In a single RT reaction, 1 µg RNA was reverse transcribed using the SuperScript III kit (Life Technologies). The cDNAs were diluted 1:10 in water, and 8 µl were used as template in real-time PCR reactions containing 10 pmol each forward and reverse primers (Supplementary Material, Table S1) using the LightCycler 480 (Roche).

Plasmids

The cloning of COL6A1 promoter and the generation of pCOL6A1-promoter-luc2 constructs as well as the pXPD and pCtr plasmids are described in the Supplementary Material, Methods.

Luciferase assays

Cells were transfected with 3 µg COL6A1-promoter-luc2 derivatives (renilla luciferase reporter) and 150 ng pGL4.74-hRluc/tk (renilla luciferase reporter) and increasing amounts of decoy or scrambled control oligonucleotides (Supplementary Material, Table S2). Human primary fibroblasts were transfected with the pXPD or the control pCtr vector, seeded at low and high-cell density on glass slides and analyzed 48 h later for UDS, as described in (35), and for the presence of the reporter HaloTag-PDGFR-TM protein by in vivo staining with the tetramethyl-rhodamine HaloTag-ligand (HaloTag-TMR), according to the manufacturer’s instruction (Promega). HaloTag-positive cells were visualized by fluorescence microscopy with XF204 filter. UDS was measured by counting the number of grains on the nucleus of at least 50 HaloTag-positive and -negative cells.

RNA interference

1.5 × 10⁶ fibroblasts were transfected with 160 pmol control (AllStar negative control, Qiagen) Sp1, SREBP-1 or cdk7 (Supplementary Material, Table S3) siRNA using the HiPerfect Transfection Reagent (Qiagen) and incubated for 72 h at 37 °C. Transfected cells were then processed for immunoblot or ChIP analysis.

Protein co-immunoprecipitation and ChIP

Co-immunoprecipitations were performed using the Nuclear Complex Co-IP Kit (Active Motif), and the NE were incubated with control IgG or antibodies raised against SREBP-1, Sp1 or cdk7. ChIP experiments were performed as described (36). Immunoprecipitated DNA was quantified by real-time PCR and analyzed using the LightCycler 480 (Roche) and the appropriate primers (Supplementary Material, Table S1). Primary antibodies were purchased as follows: anti-RNApol II (SW16 for RNApol IIA, H14 for S5P-CTD and H5 for S2P-CTD, Covance), anti-SREBP-1 (2A4, Abcam), anti-p89 (S-19, sc-293) and anti-Sp1 (PEP2, sc-59 Santa Cruz).

Protein purification

Wild-type TFIH (rTFIIH) complexes were purified from baculovirus-infected insect cells, as described (37). SREBP-1a, 1c and CTD were expressed in Escherichia coli and purified using the GST tag.

DNase 1 footprinting

The COL6A1 promoter fragment (from nucleotides −304 to +105) was amplified using a forward primer previously radiolabeled by the T4 Polynucleotide kinase (Biolabs). The probe (20,000 cpm) was incubated with NE isolated from normal cells, as previously described (16).

Protein-binding studies on immobilized DNA

The COL6A1 promoter fragment (from nucleotides −304 to +105) was amplified using a forward biotinylated primer. Reaction mixtures, each containing 500 fmol of immobilized DNA were incubated with control IgG or antibodies raised against SREBP-1, Sp1 or cdk7. Co-immunoprecipitations were performed using the Nuclear Complex Co-IP Kit (Active Motif), and the NE were incubated with control IgG or antibodies raised against SREBP-1, Sp1 or cdk7.

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DNA, 5 μl streptavidin magnetic beads (Dynabeads, Life Technologies) and purified SREBP-1a, were supplied with rTFIIH and/or NE depleted or not of endogenous TFIIH and incubated for 45 min at 25°C in 20 mM Hepes, 100 mM KCl, 0.05% NP40, 0.2 mM EDTA, 12.5 mM MgCl2, 15% Glycerol, 2 mM DTT and protease inhibitors. Magnetic beads were collected on a magnetic particle concentrator and washed 4× in binding buffer containing 300 mM KCl. Washed beads were further analyzed for bound proteins by immunoblot.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement: None declared.

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