Rescue of Progeria in Trichothiodystrophy by Homozygous Lethal Xpd Alleles

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Although compound heterozygosity, or the presence of two different mutant alleles of the same gene, is common in human recessive disease, its potential to impact disease outcome has not been well documented. This is most likely because of the inherent difficulty in distinguishing specific biallelic effects from differences in environment or genetic background. We addressed the potential of different recessive alleles to contribute to the enigmatic pleiotropy associated with XPD recessive disorders in compound heterozygous mouse models. Alterations in this essential helicase, with functions in both DNA repair and basal transcription, result in diverse pathologies ranging from elevated UV sensitivity and cancer predisposition to accelerated segmental progeria. We report a variety of biallelic effects on organismal phenotype attributable to combinations of recessive Xpd alleles, including the following: (i) the ability of homozygous lethal Xpd alleles to ameliorate a variety of disease symptoms when their essential basal transcription function is supplied by a different disease-causing allele, (ii) differential developmental and tissue-specific functions of distinct Xpd allele products, and (iii) interallelic complementation, a phenomenon rarely reported at clinically relevant loci in mammals. Our data suggest a re-evaluation of the contribution of "null" alleles to XPD disorders and highlight the potential of combinations of recessive alleles to affect both normal and pathological phenotypic plasticity in mammals.

Introduction

Interallelic complementation is defined as the ability of two differentially mutated alleles to function better together than either can on its own. Despite its near universality in lower organisms [1], its potential to contribute to clinical heterogeneity in human disease is seldom considered. Evidence of interallelic complementation at clinically relevant loci is limited to biochemical and cell-based studies of a handful of metabolic disorders with defects in enzymes including propionyl-CoA carboxylase [2], argininosuccinate lyase [3], galactose-1-phosphate uridylyltransferase [4], and methylmalonyl-CoA mutase [5].

Compound heterozygotes are individuals carrying two different mutant alleles of the same gene. In the absence of a dominant (wild-type [wt]) allele, genetic interactions between recessive alleles (referred to here as "biallelic" effects) could result in different phenotypic outcomes including interallelic complementation. Although amelioration of disease symptoms by interallelic complementation would create an ascertainment bias in the clinic, the lack of evidence concerning interallelic complementation or other biallelic effects in human disease is likely caused by the difficulty in distinguishing such effects from environment and genetic background.

XPD encodes one of the two helicase components of basal transcription/DNA repair factor IIH (TFIIH), a ten-subunit, multifunctional complex that is essential for multiple processes, including basal transcription initiation and DNA damage repair via the nucleotide excision repair (NER) pathway [6,7]. Alterations in XPD resulting in defective TFIIH function are associated with UV-sensitive, multisystem disorders including xeroderma pigmentosum (XP), XP combined with Cockayne syndrome (CS), and trichothiodystrophy (TTD) [8–10]. XP is marked by sun-induced pigmentation anomalies and a greater than 1,000-fold elevation in skin cancer risk. Severe cases can also present with growth retardation and primary neurodegeneration [11]. CS and
targeting construct is marked by a thick black line. Restriction sites: B, BamHI; C, ClaI; E, EcoRI; H, HindIII; Hp, HpaI; Sf, SfiI.

(B) Southern blot analysis of EcoRI-digested genomic DNA from wt, Xpd<sub>XPCS</sub>/wt and Xpd<sub>XPD</sub>/wt recombinant embryonic stem cell clones hybridised with the 3′ probe depicted in (A). The wt allele yields a 6.5-kilobase (kb) fragment, whereas both targeted Xpd<sub>XP</sub> and Xpd<sub>XPCS</sub> alleles yield a 5.1-kb fragment.

(C) Genotyping of wt and targeted alleles by PCR using primers F2, R1, and mR as indicated in (A) yields fragments of 399 bp and 468 bp, respectively.

(D) RT-PCR detection of mRNA expression originating from the targeted 1XP and 1XPCS alleles in embryonic stem cell clones using primers F1 (hybridising outside the targeting construct) and mR as indicated in (A) results in a 1,416-bp fragment.

(E) Northern blot analysis of total RNA isolated from testis of homozygous wt and Xpd<sub>XP</sub>/wt, heterozygous Xpd<sub>XPCS</sub>/Xpd<sub>XP</sub> and Xpd<sub>XPD</sub>/Xpd<sub>XP</sub> and compound heterozygous Xpd<sub>XPCS</sub>/Xpd<sub>XP</sub> mice as indicated. Hybridisation with a 1.4-kb mouse Xpd<sub>XPCS</sub>cDNA probe detects mRNAs of 4.3, 3.3, and 2.7 kb from wt, Xpd<sub>XPCS</sub> and Xpd<sub>XP</sub> alleles, respectively. An ethidium bromide (EtBr)-stained gel showing the amount of total RNA loaded is shown below.

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TTD, on the other hand, are segmental progeroid disorders characterised by progressive post-natal growth failure and primary demyelination resulting in severe neurodysfunction, but without a clear cancer predisposition [12–15]. Patients with TTD additionally display hallmark sulphur-deficient brittle hair and nails and scaling skin [13], resulting from a basal transcription defect in specific cell types [16,17]. A related disorder with the cancer predisposition of XP combined with the neurodevelopmental complications of CS (XPCS), although rare, has also been described [18].

Many XPD mutations are associated with an exclusive disease phenotype (e.g., XPD<sub>R722W</sub> with TTD and XPD<sub>G608W</sub> with XP) and are thus viewed as causative of the corresponding syndromes. Alleles not associated exclusively with one disorder are considered “likely null” alleles [19,20]. Some of these alleles fail to support viability in a haploid Schizosaccharomyces pombe yeast strain with a null mutation in the XPD homologue rad15 and are thus considered devoid of significant biological activity [19]. This classification of alleles as either causative or null currently defines what we refer to as a “monoallelic” paradigm of XPD disease. However, the identification in recent years of XP complementation group D patients with atypical disease presentation, including symptoms of both XP and TTD [8], casts doubt on the ability of such a monoallelic paradigm to explain clinical heterogeneity in compound heterozygotes.

Previously, we generated a TTD mouse model (XPD<sub>R722W</sub>) that phenocopies the human syndrome [15,21]. Here we report the generation of additional mutant Xpd alleles that fail to support viability on their own but nevertheless ameliorate TTD-associated premature segmental ageing, cutaneous features, cellular DNA repair capacity, and UV survival when present in a compound heterozygote state.

Results
Generation of Xpd Compound Heterozygotes

We generated an Xpd knock-in allele with a point mutation encoding a single amino acid change (XPD<sub>G502D</sub>) found in the XPCS patient XPCS2 (Figure 1A–1C). mRNA expression from the targeted allele could be detected in embryonic stem cells by RT-PCR (Figure 1D), although expression was reduced approximately 5-fold relative to wt mRNA transcript levels as...
determined by Northern blotting of RNA from the testis of heterozygous animals (Figure 1E). Because patient XPCS2 was a hemizygote with mutant XPD protein (XPD<sup>G602D</sup>) expressed from a single allele, the corresponding mutation was expected to be viable in the homozygous state. However, homozygous mutant mice were not observed, neither amongst live births nor embryonic day 13.5 (E13.5) or E3.5 embryos (Table 1). The corresponding hypomorphic, mutant allele was thus designated as homozygous lethal (XPCS<sup>−/−</sup>). Homozygous lethality of the XPCS allele is likely due to reduced levels of expression of this essential protein as a result of gene targeting (Figure 1A) rather than to the mutation itself. Xpd ablating (Xpd<sup>−/−</sup>) is similarly incompatible with life beyond the earliest stages of embryogenesis [22].

Consistent with this interpretation, a different targeted Xpd mutation encoding XPD<sup>ROSW</sup>, which is associated with XP in the homozygous state of humans, was similarly underexpressed and lethal in the homozygous state (designated as XP allele) (Figure 1A–1C; Table 1; unpublished data). Also, a different targeting approach leading to the use of the native 5′UTR and removal of the neo gene resulted in normalisation of Xpd<sup>XPCS</sup> mRNA levels and viable homozygous Xpd<sup>XPCS</sup>/XPCS (XPD<sup>G602W/G602W</sup>) animals [23].

"Null" Allele Can Alleviate Developmental Delay, Skin, and Hair Features of TTD

To test the potential of a homozygous lethal "null" allele to nevertheless contribute to organisinal phenotype, we combined an Xpd<sup>XPCS</sup> allele with a viable Xpd<sup>TTD</sup> allele by crossing the corresponding heterozygous animals. Similar to hemizygous TTD mice carrying one true Xpd knockout allele (Xpd<sup>TTDKO</sup>), compound heterozygous Xpd<sup>TTD/XPCS</sup> mice were born at the expected Mendelian frequencies. Expression from the Xpd<sup>XPCS</sup> allele was also reduced in the testis of compound heterozygous animals, whereas expression from the Xpd<sup>TTD</sup> allele was increased relative to wt by ~5-fold (Figure 1E). Because of a lack of available antibodies and the inability to distinguish amongst various mutant forms of XPD differing only by single amino acid substitutions, we were unable to ascertain the relative amount of XPD protein from the different alleles.

Despite reduced levels of mRNA expression, the homozygous lethal Xpd<sup>XPCS</sup> allele ameliorated multiple Xpd<sup>TTD</sup>-associated disease symptoms in compound heterozygous Xpd<sup>TTD/XPCS</sup> animals including the hallmark brittle hair and cutaneous features fully penetrant in homo- and hemizygous TTD mice (Figure 2A-2C). In marked contrast to Xpd<sup>TTD/TTD</sup> (and Xpd<sup>TTD/KO</sup>) mice, which display complete hair loss in the first hair cycle and partial hair loss in subsequent cycles throughout their lives [21], compound heterozygous Xpd<sup>TTD/XPCS</sup> mice displayed some hair loss only during the first hair cycle and only locally at the back (Figure 2A).

Scanning electron microscope analysis of Xpd<sup>TTD/XPCS</sup> hair revealed an almost normal appearance, with TTD-like features such as broken hairs found only at very low frequency (unpublished data). Amino acid analysis confirmed that cysteine levels in the hair of the Xpd<sup>TTD/XPCS</sup> mice were significantly higher than in Xpd<sup>TTD/TTD</sup> animals, but remained below the wt level (Figure 2C). TTD hemizygotes (Xpd<sup>TTD/KO</sup>) do not display significant differences in cutaneous features and longevity relative to homozygous Xpd<sup>TTD/TTD</sup> mice [21].

Other prominent TTD features in the epidermis, including acanthosis (thickening of the layer of the nucleated cells), hyperkeratosis (prominent thickening of the cornified layer), and pronounced granular layer and sebaceous gland hyperplasia (causing greasy appearance of the hair), were absent in the skin of Xpd<sup>TTD/XPCS</sup> mice, as established by blind microscopic examination of skin sections (Figure 2B). Furthermore, anaemia and developmental delay present in patients with TTD [24] and in Xpd<sup>TTD/TTD</sup> mice [15] were both partially rescued in compound heterozygous Xpd<sup>TTD/XPCS</sup> mice (Figure 2D and 2E).

Rescue of Progeroid Features in TTD Mice by Homozygous Lethal Xpd Alleles

Because patients with TTD, XPCS, and CS (but not XP) and the corresponding mouse models share similar accelerated progeroid symptoms [12,13,15,23], we next addressed ageing-related parameters in compound heterozygous mice (Figure 3). Whereas Xpd<sup>TTD/TTD</sup> animals show reduced bone mineral density as an indication of the early onset of osteoporosis before ~14 mo of age [15], tail vertebrae from compound heterozygous Xpd<sup>TTD/XPCS</sup> mice were comparable to wt even at 20 mo of age (Figure 3B and 3C). Furthermore, whereas Xpd<sup>TTD/TTD</sup> mice developed kyphosis earlier than wt animals (onset ~3 mo versus 12–20 mo), compound heterozygous Xpd<sup>TTD/XPCS</sup> mice did not (Figure 3B). Overall appearance and body weight curves revealed that TTD-associated age-related premature cachexia and lack of general fitness were fully rescued in compound heterozygous Xpd<sup>TTD/XPCS</sup> mice (Figure 3A and 3D). Finally, the life span of compound heterozygotes was extended relative to Xpd<sup>TTD/TTD</sup> mice (Table 2).

To determine whether the homozygous lethal Xpd<sup>XPCS</sup> allele was unique in its ability to ameliorate symptoms associated with the Xpd<sup>TTD</sup> allele, we generated compound

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**Table 1. Frequency of Xpd<sup>XP/XP</sup>, Xpd<sup>XP/PCS</sup>, and Compound Heterozygous Xpd<sup>XP/XP</sup> Embryos and Offspring**

| Genotype       | Age     | Analysed | Expected* (if Mendelian) | Found |
|----------------|---------|----------|--------------------------|-------|
| Xpd<sup>XP/PCS</sup> | E3.5    | 26       | 6.5                      | 0     |
|                | E13.5   | 26       | 6.5                      | 0     |
| Xpd<sup>XP/XP</sup> | Newborn | 129      | 32                       | 0     |
|                | Newborn | 29       | 7                        | 0     |
|                | Newborn | 144      | 36                       | 0     |
|                | Newborn | 33       | 8                        | 0     |

*Derived from Xpd<sup>XP/XP</sup>, Xpd<sup>XP/PCS</sup>, and Xpd<sup>XP/XP</sup> to Xpd<sup>XP/PCS</sup> intercrosses.

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heterozygous $Xpd^{TTD/XP}$ mice by crossing the corresponding heterozygous animals. Similar to the $Xpd^{XP}$ allele, the homozygous lethal $Xpd^{XP}$ allele rescued cutaneous symptoms including hair loss (except locally during the first round; unpublished data), reduced cysteine content (cysteine index 9.3 ± 0.9 standard deviation [87% of wt], $p = 0.01$ versus TTD), ageing-associated premature cachexia (males and females were 36.1 ± 6.4 g [93% of wt] and 39.2 ± 3.2 g [116% of wt], respectively), and reduced life span (Table 2). Taken together, these data indicate that two independent alleles, which on their own are unable to support viability (Table 1), were nonetheless able to ameliorate TTD-associated phenotypes in vivo (Table 2).

Molecular Mechanisms of Biallelic Effects

We next turned to UV-based cellular assays including unscheduled DNA synthesis after UV irradiation (UV-UDS), recovery of RNA synthesis after UV irradiation (UV-RRS), and UV survival, which report on the NER subpathways (global genome NER and transcription-coupled NER) and total NER, respectively. In none of these assays was the response to UV improved in compound heterozygotes relative to TTD homozygotes (Figure 4A–4C). However, unlike the in vivo TTD phenotypes described above, in which $Xpd^{TTD/TTD}$ and $Xpd^{TTD/KO}$ animals were indistinguishable, $Xpd^{TTD}$ dosage effects were observed in UV survival, UV-UDS, and UV-RRS, indicating that cellular parameters as measured in fibroblasts here do not always correlate with the phenotype at the level of the intact organism. $Xpd^{TTD/KO}$ hemizygous cells were thus used as the baseline on which to compare the activity of compound heterozygous cells. Relative to $Xpd^{TTD/KO}$ hemizygote cells, UV survival was improved by the homozygous lethal $Xpd^{XP}$ allele in $Xpd^{TTD/XP}$ compound heterozygous cells and to a lesser degree by the $Xpd^{XP}$ allele (Figure 4A). Because of
embryonic and cellular lethality, we were unable to test UV survival associated exclusively with the \textit{Xpd} \textit{XPCS} and \textit{Xpd} \textit{XP} alleles. However, homozygous \textit{XPDXP} (\textit{XPDG602D}) and hemizygous \textit{XPDXPCS} (\textit{XPDG602D}) human cells are known to be highly sensitive to UV [19,25], as are cells from a homozygous viable \textit{Xpd} \textit{XPCS} mouse model (Figure 4A, dotted line) [23]. Thus, the survival of \textit{Xpd} \textit{TTD/XP} cells likely represents a level of UV resistance that neither mutant allele can impart on its own (Table 2).

Figure 3. Rescue of TTD-Associated Segmental Progeroid Features in Compound Heterozygous \textit{Xpd} \textit{TTD/XP} Mice

(A) Photographs of 20-mo-old \textit{wt}, compound heterozygous \textit{Xpd} \textit{TTD/XP}, and homozygous \textit{Xpd} \textit{TTD/TTD} mice. Note the extreme cachexia (lack of subcutaneous fat) in the \textit{Xpd} \textit{TTD/TTD} mouse and the absence of this phenotype in \textit{wt} and \textit{Xpd} \textit{TTD/XP} mice.

(B) Radiographs of 20-mo-old male \textit{wt}, \textit{Xpd} \textit{TTD/XP}, and \textit{Xpd} \textit{TTD/TTD} mice. Ageing \textit{Xpd} \textit{TTD/TTD} mice develop kyphosis (curvature of the spinal column) and reduction of bone mineral density as shown in the 6–8 segment of the tail vertebrae counted from the pelvis (see close-up at right). Note the absence of these features in the \textit{Xpd} \textit{TTD/XP} mouse.

(C) Quantification of relative bone mineral density of tail vertebrae from 20-mo-old male \textit{wt} (\textit{n} = 4), \textit{Xpd} \textit{TTD/XP} (\textit{n} = 4), and \textit{Xpd} \textit{TTD/TTD} (\textit{n} = 3) mice. The \textit{p}-values indicate the significance of the difference relative to \textit{Xpd} \textit{TTD/TTD}. Error bars indicate SEM.

(D) Body weight curves as a function of time. Note that the age-dependent cachexia observed in \textit{Xpd} \textit{TTD/TTD} mice was rescued in both male and female \textit{Xpd} \textit{TTD/XP} mice. Significant differences between \textit{wt} and \textit{Xpd} \textit{TTD/TTD} but not between \textit{wt} and \textit{Xpd} \textit{TTD/XP} mice were observed at 9 and 18 mo of age as indicated by asterisks. Error bars indicate SEM.

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Next we asked whether the \textit{Xpd} \textit{XPCS} and \textit{Xpd} \textit{XP} alleles, despite decreased mRNA levels, ameliorated TTD symptoms by increasing overall TFI IH levels in compound heterozygous \textit{Xpd} \textit{TTD/XP} cells. Previously, using comparative immunohistochemistry, we and others have shown an up to 70% reduction of TFI IH complexes from patients with TTD compared with \textit{wt} controls due to reduced stability [16,17]. Despite overexpression of mRNA from the \textit{Xpd} \textit{TTD} allele relative to the \textit{wt} allele (Figure 1E), TFI IH protein levels were reduced by 50% in primary mouse reaction reconstituted with different mutant TFI IH complexes (Figure 4D). Taken together, these data are consistent with interallelic complementation of UV sensitivity in cells but underscore the lack of any correlation between UV-related repair characteristics and TTD progeroid phenotypes in animal models.
the ability of the encoded protein (XPDG602D) to restore the quantitative effect on these phenotypes in vivo.

of TFIIH levels, suggesting a qualitative rather than a progeroid symptoms (Figure 3) were not due to normalisation heterozygote cells (Figure 4A) and likely the rescue of TTD 4F). Thus, the improved UV survival observed in compound ;

Transcription-coupled NER UV-RRS

TTD hair phenotype to normal. Notably, data). Thus, the range of expression levels from these two

animals had wt hair, correlating with normal expression levels from the viable XpdXPCS allele (Table 2 and unpublished data).

Transcription-coupled NER UV-RRS

Global genome NER UV-UDS

DNA repair

UV-damage repair

Table 2. Pleiotropic Xpd Biallelic Effects in Mice and Cells

| Presumed TFIIH Function | Phenotype | Genotype |
|-------------------------|-----------|----------|
|                         | TTD/TTD   | TTD/KO   | XPCS/XPCS<sup>a</sup> | KO/KO and<sup>e</sup> XPCS/XPCS | TTD/TTD/XPCS (Allelic Relation)<sup>b</sup> |
| General transcription defect | Embryonic lethality | Absent | Absent | Absent | Present | Absent |
| Transcription defect of hair/skin/blood | Fragile hair with reduced cytostane content | Present | Present | Absent | n.a | Intermediate |
| | Acanthosis, hyperkeratosis, sebaceous gland hyperplasia | Present | Present | Absent | n.a | Absent |
| | Anaemia | Present | n.d. | n.d. | Absent | Absent |
| UV-damage repair | Cellular UV hypersensitivity | Mild | Moderate<sup>a</sup> | Severe | n.a | Mild (interallelic comp) |
| | UV-RRS | ~30% | ~15% | <5% | n.a | ~25% (TTD dominant)<sup>a</sup> |
| | UV-UDS | ~50% | ~25% | ~30% | n.a | ~25%<sup>f</sup> |
| | Developmental delay | Moderate | Moderate | Mild | n.a | Mild |
| | Cachexia | Severe | Severe | Mild | n.a | Absent (interallelic comp)<sup>a</sup> |
| | Premature loss of bone mineral density | Severe | n.d. | n.d. | n.a | Absent (interallelic comp)<sup>b</sup> |
| | Life span<sup>c</sup> | Reduced | Reduced | Reduced | n.a | Reduced |
| | Life span in Xpa<sup>c</sup> background<sup>d</sup> | ~3 wk | n.d. | ~3 wk | n.a | ~6 mo |

<sup>a</sup>For a complete description of Xpd<sup>XPCS</sup>/XPCS homozygous mice, please see [23].
<sup>b</sup>Allelic relationships in Xpd compound heterozygotes are colour-coded as follows: blue, XPCS or XPCS allele dominant over TTD allele; green, TTD allele dominant over XPCS or XPCS allele; red, interallelic complementation (comp).
<sup>c</sup>Dominant is defined as closer to wt function in comparison with the other mutant allele present in the compound heterozygote, with respect to a given phenotype/function.
<sup>d</sup>XPD protein expression level–dependent.
<sup>e</sup>Also demonstrated in Xpd<sup>TTD/XPCS</sup> compound heterozygous animals.
<sup>f</sup>Because in XPCS cells the 30% UV-UDS is not associated with actual lesion removal, whereas in TTD it is [23], the allelic relationship between the alleles with respect to UV-UDS is unknown.

<sup>c</sup>In the 129Oa/C57BL6 mixed genetic background used in a previous study [15], the average life span of Xpd<sup>TTD/TTD</sup> mice was approximately 12 mo. Nevertheless, three out of six Xpd<sup>TTD/TTD</sup> mice included in this study lived until 19–20 mo, although their overall appearance was poor at the time they were killed (Figure 3A). Xpd<sup>TTD/XPCS</sup> mice (n = 6) reached the age of 19–20 mo and were in a good condition at the time they were killed (Figure 3A). Two Xpd<sup>TTD/XPCS</sup> animals not killed died at 24 and 26 mo of age, which is in the wt range. Similar condition and life span extension were observed in Xpd<sup>TTD/XPCS</sup> mice (n = 6) killed at the age of 19–20 mo for analysis. Xpd<sup>PCS/PCS</sup> mice had a mean life span of ~18 mo that was significantly shorter than controls (~21 mo, log rank p = 0.001; [23]).

<sup>d</sup>In the absence of XPA, another essential NER protein, DNA repair capacity is further reduced, resulting in accelerated TTD- and XPCS-like features and a reduced life span of ~3 wk in Xpd<sup>XPCS/XPCS</sup>/Xpa<sup>−/−</sup> and Xpd<sup>TTD/TTD/Xpa<sup>−/−</sup></sup> mice. Xpd<sup>TTD/XPCS</sup>/Xpa<sup>−/−</sup> animals survived significantly longer (van de Ven HWM, Andressoo JO, Jong W, Holcomb VB, de Zeeuw CI, et al., unpublished data).

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XP<sup>dTTD/TTD</sup> fibroblasts (Figure 4E and 4F), thereby mimicking the situation in human patients with TTD. In accordance with the gene dosage, a further reduction of up to 70% of the wt level was observed in hemizygous XP<sup>dTTD/XPCS</sup> cells. Consistent with low mRNA expression levels, neither the Xpd<sup>XPCS</sup> nor the Xpd<sup>TTD</sup> allele was able to restore TFIIH abundance to wt levels in XP<sup>dTTD</sup> compound heterozygote cells (Figure 4E and 4F). Thus, the improved UV survival observed in compound heterozygote cells (Figure 4A) and likely the rescue of TTD progeroid symptoms (Figure 3) were not due to normalisation of TFIIH levels, suggesting a qualitative rather than a quantitative effect on these phenotypes in vivo.

In contrast, the level of XPCS mRNA expression did affect the ability of the encoded protein (XPG<sup>GG322D</sup>) to restore the TTD hair phenotype to normal. Notably, XP<sup>dTTD/XPCS</sup> animals had a partial TTD hair phenotype, correlating with low levels of Xpd<sup>XPCS</sup> expression, whereas XP<sup>dTTD/XPCS</sup> animals had wt hair, correlating with normal expression levels from the viable Xpd<sup>XPCS</sup> allele (Table 2 and unpublished data). Thus, the range of expression levels from these two mutant alleles affected their ability to complement some phenotypes (hair). An overview of the functional relationships between Xpd alleles, phenotypes, and the presumed underlying TFIIH function in mice and cells is presented in Table 2.

Discussion

Dissection of Biallelic Effects from other Determinants of Phenotype

Although phenotypic consequences, referred to here as biallelic effects, resulting from two different mutant alleles in compound heterozygote patients have been postulated, such effects have historically been difficult to distinguish from the influence of environment and genetic background. We used a genetically defined mammalian model system under controlled environmental conditions to reveal phenotypic effects attributable specifically to combinations of differentially mutated Xpd alleles.

The observed biallelic effects were of three general types. In the first, the allele associated in a homozygous state with a phenotype closer to wt singularly determined the
A phenotypic outcome, a phenomenon widely known in human recessive disease. Because these Xpd alleles functioned at or near wt levels with respect to a particular function, we call these effects “dominant”. Such alleles can also be referred to as “separation of function” alleles, because they allow dissection of the roles of multifunctional proteins in specific phenotypes.

Secondly, highlighting the potential relevance of current findings to all diploid organisms including humans was the observation that in one compound heterozygous animal, the Xpd allelic relationship could shift from A_dominant/A_recessive to A_recessive/A_dominant with respect to different phenotypes in a time-dependent and tissue-specific manner (see below and Table 2).

In the third type of biallelic effect, known as interallelic complementation, two mutant alleles produced a phenotype closer to wt than either could alone in a homo- or hemizygous state. As summarised in Table 2, examples of all types of biallelic effects were observed in a variety of Xpd-associated phenotypes, ranging from brittle hair to segmental progeria.

Figure 4. TFIH Functions and Mechanisms of XPD-Associated Disease Pleiotropy

(A) Cellular survival after UV irradiation. Rescue of hemizygous XpdTTD/KO survival by XpdXPCS and XpdXP alleles is illustrated by arrows marked A and B, respectively. UV survival of homozygous XpdXPCS/XPCS cells (asterisk) from the normally expressed viable allele (XpdXPCS) is depicted by a dotted line. Survival curves represent an average of four independent experiments; 1–2 cell lines per genotype were included in each experiment. Error bars indicate SEM between experiments.

(B) UV-UDS, a measure of global genome repair. Number of experiments: n = 15 (XpdTTD/TTD), n = 6 (XpdTTD/KO), n = 4 (XpdTTD/XPCS), n = 2 (XpdTTD/XP); 1–2 cell lines per genotype were included in each experiment. The asterisk indicates significant difference with XpdTTD/TTD; crosses indicate significant differences with XpdTTD/KO.

(C) UV-RRS, a measure of transcription-coupled repair of UV-induced lesions. Number of experiments: n = 7 (XpdTTD/TTD), n = 2 (XpdTTD/KO), n = 4 (XpdTTD/XPCS), n = 2 (XpdTTD/XP); 1–2 cell lines per genotype were included in each experiment.

(D) Incision/excision activity of combinations of altered TFIH complexes in a reconstituted NER reaction. Equal amounts of single or mixed populations of recombinant TFIHs (containing XPD, XPB, p62, p52, His-p44, Flag-p34, cdk7, cyclin H, Mat1, and p8) were mixed with recombinant XPG, XPF/ERCC1, XPC/hHR23B, RPA, and a radiolabelled synthetic NER substrate. The excision products (26–34 nucleotides in length) were visualised at nucleotide resolution on a denaturing polyacrylamide gel as indicated. Note the weak activity corresponding to each single and combined TFIH complex (lanes 3–8) relative to the wt (lane 1) and negative controls (lane 2).

(E) Xpd dose-dependent reduction of TFIH in homozygous XpdTTD/TTD, hemizygous XpdTTD/KO, and compound heterozygous XpdTTD/XPCS and XpdTTD/XP cells by comparative immunofluorescence of the p62 subunit of TFIH. Roman numerals represent different microscopic slides and Arabic numerals different cell lines labelled as follows: (I) wt cells (1) labelled with 2-μm beads, XpdTTD/TTD cells (2) with 0.79-μm beads, and XpdTTD/KO cells (3) with no beads; (II) wt cells (1) labelled with 0.79-μm beads and XpdTTD/KO cells (4) with 0.79-μm beads, and XpdTTD/XP cells (5) with no beads.

(F) Quantification of immunofluorescent signal from at least 50 nuclei per cell line and 2–6 experiments per genotype. Bars representing cells analysed on the same microscopic slide are depicted side by side, with wt set at 100%. The p-value indicates minimum significant difference between wt and the indicated cell lines analysed on the same microscopic slide within one experiment.

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TFIIH in Transcription and Repair: Mechanisms of XPD Disease Pleiotropy

We observed differences in the ability of XpdTTD versus homozygous lethal XpdXPCS and XpdXP alleles to function in two transcription-related phenotypes separated in the organism by both time and space: embryonic lethality and terminal differentiation of enucleating skin and blood cells. The preblastocyst-stage homozygous lethality shared by the XpdKO, XpdXPCS, and XpdXP alleles most likely reflects a defect in basal transcription that is incompatible with life. In XpdTTD/XPCS and XpdTTD/XP compound heterozygous mice, embryonic lethality was fully rescued by the XpdTTD allele. Because embryonic lethality was also fully rescued in XpdTTDKO hemizygous mice, the XpdTTD allele can be considered as wt and thus dominant to each of the homozygous lethal alleles (XpdKO, XpdXPCS, and XpdXP) with respect to this particular phenotype (Table 2).

TTD-specific cutaneous and anaemic features, on the other hand, are thought to result from a specific kind of transcriptional insufficiency caused by depletion of unstable TFIIH during the terminal differentiation of skin, hair-shaft, and blood cells [16,24]. In compound heterozygous mice, both homozygous lethal XpdXPCS and XpdXP alleles were able to alleviate XpdTTD-specific cutaneous and anaemic features and can thus be defined as dominant over the XpdTTD allele with respect to these phenotypes. We conclude that the defects leading to embryonic lethality and aberrant terminal differentiation of the skin, hair, and blood represent two qualitatively and/or quantitatively different transcriptional deficiencies. During early embryonic development, XpdTTD is dominant over the XpdXPCS and XpdXP alleles, whereas later in the ontogenesis of skin, hair-shaft, and blood cells, the situation is reversed.

In its role in the repair of UV photolesions, the XpdXPCS allele imparted a clear UV survival benefit over a single XpdTTD allele or two XpdXPCS alleles independent of expression levels, which is consistent with interallelic complementation. However, the observation that no other cellular or biochemical UV-related parameters were improved in XpdTTD/XPCS argues against complementation of this repair activity in the rescue of TTD progeroid symptoms in vivo.

Interallelic Complementation and XPD Function

What does interallelic complementation tell us about the mechanism of XPD function? Interallelic complementation is most often observed in multi-allelic proteins with multiple functional domains. Unfortunately, the structure–function relationship between disease-causing mutations and XPD functional domains, including detailed structural information on XPD or even its stoichiometry within TFIIH, remains unknown. However, based on the ability of cell extracts that are defective in two different TFIIH components (XPD and XPB) to complement NER activity in vitro [26], it is likely that TFIIH (or its components) can either multimerise or exchange at least during the NER reaction. Furthermore, XPD is known to be a "loosely bound" subunit of TFIIH [27]. We thus envisage the molecular mechanism of interallelic complementation to involve the exchange of XPD molecules within the TFIIH complex or turnover of TFIIH complexes containing different XPD molecules at the site of DNA damage during the course of the global genome as well as transcription-coupled repair of either UV-induced or endogenous DNA damage.

Monoallelic hypothesis: one allele determines phenotype

![Diagram showing genotypes and phenotypes](image)

**Figure 5. Genotype–Phenotype Relationships in XPD Disorders**

According to the current monoallelic hypothesis, phenotype is determined solely by the causative allele product. If a second, different allele is present, it is considered a functional null. There is a lack of any correlation between the site of the XPD mutation and the resulting disorder. We propose a biallelic hypothesis for compound heterozygotes in which both alleles can contribute to the phenotype. Examples of compound heterozygous patients in which a second, presumed null allele is likely to contribute to disease outcome are provided above in comparison to corresponding homo- or hemizygous patients with the same causative allele. Numbers in the schematic of the protein indicate the helicase domains.

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A Biallelic Paradigm for XPD Disorders

Recently, proteins originating from presumed null alleles were biochemically characterised as inactive in basal transcription [27], providing an explanation as to why these alleles failed to rescue lethality in haploid S. pombe with a null mutation in the XPD homologue rad15 [19]. Our data suggest that certain presumed null alleles, although unable on their own to support basal transcription, may in fact have a substantial impact on disease outcome in compound heterozygous humans, as they do in mouse models.

Clinical evidence in support of this hypothesis comes from a number of XP complementation group D patients that do...
not fit within the framework of the current monoallelic paradigm of XPD disorders (Figure 5). In contrast to two hemizygous XPD<sup>XPCS</sup> patients carrying the XPD<sup>G47R</sup> or XPD<sup>R683W</sup>-encoding alleles who died of the disease before 2 y of age, two compound heterozygous XPD<sup>XPCS</sup> patients carrying the same XPD<sup>G47R</sup> or XPD<sup>R683W</sup>-encoding alleles in addition to the presumed null XPD<sup>L461V<del del1="1">-del716</del>+</sup> allele both had considerably milder disease symptoms and survived more than ten times longer (A. Lehmann, personal communication) (Figure 5). Compound heterozygosity is also associated with the recently reported combination XP and TTD (XPTTD) syndrome [8]. Similar to the Xpd<sup>TTD</sup><sup>xps</sup>C160<sup>-</sup> and Xpd<sup>TTD</sup><sup>xps</sup>C160<sup>+</sup> mice described here, both patients with XPTTD described so far had had intermediate hair cystine values. Furthermore, XPTTD patient XP38BR carried a “causative” TTD mutation in one allele and a novel point mutation encoding XPD<sup>L463P</sup> in the other. Although the XPD<sup>L463P</sup>-encoding allele fails to complement viability in the haploid S. pombe rad15 deletion strain and is thus interpretable as a null allele [8], we nonetheless suggest that the combined XPTTD phenotype in this patient involves phenotypic contributions from both alleles. Taken together, these data suggest a shift to a biallelic paradigm for compound heterozygous patients in XP complementation group D.

Potential of Combined Recessive Alleles to Affect Phenotypic Diversity in Mammals

In humans, the clinical relevance of biallelic effects such as interallelic complementation remains unknown. Although interallelic complementation between two endogenous mutant alleles has been described in cells from a compound heterozygous patient with methylmalonic acidemia, no observable effects on disease outcome were noted in the patient [28]. Thus, to the best of our knowledge, the amelioration of progeroid features observed here is the first in vivo demonstration in compound heterozygous animals of interallelic complementation relevant to a human disease. Keeping in mind that the ~1,200 alleles known to exist for the CTRF gene implicated in the common autosomal recessive disorder cystic fibrosis alone [29] can theoretically result in ~700,000 different allelic combinations, the potential number of allelic combinations of different recessive mutations and single nucleotide polymorphisms genome-wide is currently incalculable. We suggest biallelic effects as a previously underestimated yet important variable in considering genotype-phenotype relationships from autosomal recessive disease to normal phenotypic diversity in mammals. Extension of the above concept implies that recessive mutations can enter evolutionary selection in F1 provided that the second allele carries a different recessive alteration. Finally, our data highlight the potential of clinically relevant alleles previously designated as null, with little or no detectable expression or activity, to nonetheless contribute to phenotype.

Materials and Methods

Derivation and analysis of mutant mice. Generation of Xpd<sup>TTD</sup> (XPD<sup>G47R</sup> and XPD<sup>R683W</sup>) mice has been described previously [21,22]. A detailed description of the generation of targeting constructs for Xpd<sup>TTD</sup> and Xpd<sup>TTD</sup> mice carrying mutations encoding the G602D and R683W alterations is provided upon request. Chimeric mice and mouse embryonic fibroblasts were generated according to standard procedures. Haematoxylin and eosin staining was performed according to standard procedures. Amino acid analysis was conducted as described in [21]. Blood values were analysed using Animal Blood Counter Vet (ABX Diagnostix, Montpellier, France). Radiographs were taken, and relative bone mineral density was calculated as described in [15]. Mice used in this study were in a 129Ola/C57Bl6 mixed background unless noted differently. All experiments involving mice were judged and approved by the national committee for genetic identification of organisms and the animal ethical committee, and were conducted according to national and international guidelines.

UV sensitivity, UV-UDS, UV-RRS, and TFIHF incision/excision activity. UV survival, UV-UDS, and UV-RRS assays were performed as described previously [21,30]. For UV-RRS, average values from the representative experiment containing two wt, three Xpd<sup>TTD</sup><sup>-</sup>, two Xpd<sup>TTD</sup><sup>xps</sup>, and one Xpd<sup>TTD</sup><sup>xps</sup> cell line are presented. The ~48% UV-UDS value presented in this study for Xpd<sup>TTD</sup><sup>xps</sup> cells differs from our previously published data of 25% UV-UDS [21], possibly because of the high variability intrinsic to the assay or routine variations in the cell culture conditions. For the incision/excision activity assay, recombinant TFIHF was prepared and assayed as described previously [27].

Comparative immunofluorescence. Latex bead labelling and comparative immunofluorescence analysis of the p62 subunit of the TFIHF was performed as described previously [16,17] using primary mouse embryonic fibroblasts at passages 2–5. Two or more cell lines per genotype (except for the Xpd<sup>TTD</sup><sup>xps</sup> cell line, in which only one cell line was used in repeated experiments) were used, and experiments were repeated 2-6 times per genotype.

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Author contributions. JOA, JJ, JHJH, GTJvdH, and JRM conceived and designed the experiments, JOA, JJ, JdW, FC, DH, MdW, WT, JH, WfL, and JRM performed the experiments. JOA, JJ, JdW, FC, DH, MdW, JH, HBT, WfL, JME, JHJH, and JRM analyzed the data. JdW and GTJvdH contributed reagents/materials/analysis tools. JOA, JHJH, and JRM wrote the paper.

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