Mislocalization of XPF-ERCC1 Nuclease Contributes to Reduced DNA Repair in XP-F Patients

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Abstract

Xeroderma pigmentosum (XP) is caused by defects in the nucleotide excision repair (NER) pathway. NER removes helix-distorting DNA lesions, such as UV-induced photodimers, from the genome. Patients suffering from XP exhibit exquisite sun sensitivity, high incidence of skin cancer, and in some cases neurodegeneration. The severity of XP varies tremendously depending upon which NER gene is mutated and how severely the mutation affects DNA repair capacity. XPF-ERCC1 is a structure-specific endonuclease essential for incising the damaged strand of DNA in NER. Missense mutations in XPF can result not only in XP, but also XPF-ERCC1 (XFE) progeroid syndrome, a disease of accelerated aging. In an attempt to determine how mutations in XP can lead to such diverse symptoms, the effects of a progeria-causing mutation (XPFR153P) were compared to an XP–causing mutation (XPFR799W) in vitro and in vivo. Recombinant XP harboring either mutation was purified in a complex with ERCC1 and tested for its ability to incise a stem-loop structure in vitro. Both mutant complexes nicked the substrate indicating that neither mutation obviates catalytic activity of the nuclease. Surprisingly, microinjection of XPFR153P–ERCC1 into the nucleus of XPF–deficient human cells restored nucleotide excision repair of UV–induced DNA damage. Intriguingly, in all XP mutant cell lines examined, XPF-ERCC1 was detected in the cytoplasm of a fraction of cells. This demonstrates that at least part of the DNA repair defect and symptoms associated with mutations in XP are due to mislocalization of XPF-ERCC1 into the cytoplasm of cells, likely due to protein misfolding. Analysis of these patient cells therefore reveals a novel mechanism to potentially regulate a cell’s capacity for DNA repair: by manipulating nuclear localization of XPF-ERCC1.

Introduction

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by photosensitivity and a greater than a 1000-fold increased risk of skin cancer in sun-exposed areas of the skin [1]. The severity of the disease is determined largely by which gene is mutated and to what extent the mutation affects NER.

NER removes helix-distorting lesions in DNA, for example cyclobutane pyrimidine dimers (CPDs) and pyrimidine pyrimidone photoproducts (6–4PPs) caused by the ultraviolet (UV) component of sunlight [4]. There are two ways by which DNA damage is recognized in NER. Lesions anywhere in the genome can be recognized by the complex XPC-RAD23B [5,6]. For some lesions, this is facilitated by a second complex XPF/DDB2-DDB1 [7]. Alternatively, lesions that occur in the coding strand of DNA, within transcribed regions, can trigger NER if they stall progression of RNA polymerase II [8,9]. This requires CSA, CSB and XAB2 [10–12]. Once the damage is recognized, the

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**Author Summary**

XP-FERCC1 is a nuclease that plays a critical role in DNA repair. Mutations in XP-F are linked to xeroderma pigmentosum, characterized by sun sensitivity, high incidence of skin cancer, and neurodegeneration, or XFE progeroid syndrome, a disease of accelerated aging. Herein we report the unexpected finding that mutations in XP-F cause mislocalization of XP-FERCC1 to the cytoplasm. Recombinant mutant XP-FERCC1 derived from XP- and XFE-causing alleles are catalytically active and if delivered to the nucleus of cells restore DNA repair. This demonstrates that protein mislocalization contributes to defective DNA repair and disease arising as a consequence of mutations in XP-F. It also illustrates a novel mechanism of regulating a cell's capacity for DNA repair: by manipulating nuclear localization of XP-FERCC1 to enhance or inhibit repair and to prevent cancer or tumor resistance to chemotherapy, respectively.

**Results**

**Characterization of R153P-XPF-ERCC1 activity in vitro**

We first asked if mutations in XP-F that cause mild or severe disease differentially affect the biochemical properties of XP-FERCC1. To answer this, we compared the biochemical properties of XP-FERCC1 from two patients, XP12RO [a patient with mild XP, homozygous for a mutation causing an R799W substitution in XP-F [33]] and XP51RO [a patient with XFE progeroid syndrome, homozygous for a mutation causing an R153P substitution in XP-F [32]] to that of wild type XP-FERCC1. Recombinant XPFWT-ERCC1, XPF R153P-ERCC1 and XPF R799W-ERCC1 were purified from baculovirus-infected Sf9 insect cells using a His6 tag on ERCC1. We previously reported [27] that our purified preparations of XPFWT-ERCC1 elute from a gel-filtration column in three fractions: (1) a minor fraction in the void volume (~45 ml) containing aggregated, inactive protein; (2) active heterodimeric XPFWT-ERCC1 at ~65 ml, which corresponds to a molecular weight of ~200 kD, as expected, and (3) monomeric ERCC1, which peaks at ~78 ml, which corresponds to ~50 kD. Recombinant XPFWT-ERCC1 eluted as expected (Figure 1A). Both mutant protein complexes eluted with similar profiles that differed substantially from that of XPFWT-ERCC1. The majority of the mutant complexes eluted at ~45 ml, rather than at 65 ml, indicating that they were aggregated. The peak at 78 ml, corresponding to free ERCC1 was identical for both mutant and WT XPFWT-ERCC1 preps. These results suggest that the mutations in XP-F that cause both mild and severe disease lead to protein misfolding that does not interfere with ERCC1 binding, but does lead to protein aggregation.

We were able to purify a small amount of XPF R153P-ERCC1 and XPF R799W-ERCC1 from the fractions eluting at 65 ml, indicating that at least some of the mutant proteins are likely to be properly folded. SDS-PAGE analysis of the complexes after an additional purification step over a heparin column revealed dramatically reduced yields of the complexes of XPF R153P-ERCC1 and XPF R799W-ERCC1 compared to XPFWT-ERCC1 (Figure 1B). Similarly, the amount of XP-F protein detectable by immunoblot in whole cell extracts of human fibroblasts harboring the XPF R153P and XPF R799W mutations (XP51RO and XP42RO, respectively) was reduced compared to normal cells (C5RO) (Figure 1C).

The catalytic activity of the purified heterodimers was investigated by measuring their ability to incise a 32P-end-labeled stem–loop DNA substrate at the single-strand:double-strand DNA junction in the presence of 0.4 mM MnCl2 or 2 mM MgCl2 at a 2-fold molar excess of protein over substrate (Figure 1D). With XPFWT-ERCC1, >80% of the stem-loop substrate was cleaved. Both XPF R153P-ERCC1 and XPF R799W-ERCC1 also incised the DNA substrate, demonstrating that both mutant complexes retain catalytic activity (Figure 1D, lanes 5 & 7). Incision by both mutant complexes was reduced compared to the WT complex. This may simply reflect the fact that preparations of mutant heteroduplexes were less concentrated than XPFWT-ERCC1 (Figure 1B), inevitably leading to differences in the buffering conditions between incision reactions.

We previously observed that mutant XPFWT-ERCC1 complexes tend to be more active in the presence of Mn2+ than Mg2+ since this metal has less stringent requirements for the proper alignment of the active site residues [27]. Consistent with this, incision by XPF R153P-ERCC1 and XPF R799W-ERCC1 was increased ~2-fold in the presence of Mn2+ compared to Mg2+, whereas the cation had no effect on incision by XPFWT-ERCC1 (Figure 1D). These data support the conclusion that even monomeric XPF R153P and XPF R799W are to some extent misfolded. Notably, there was not a
ERCC1, XPF R153P-ERCC1 and XPF R799W-ERCC1, respectively, after PAGE analysis of purified protein complexes. Lane 1, 3 and 5 (D): XPF-ERCC1, column; heterodimeric XPF-ERCC1 elutes at 0.4 mM MnCl₂ (lanes 2, 4 and 6) or 2 mM MgCl₂ (lanes 3, 5 and 7).

Lanes 2 and 4 (A) show the proteins present in the fractions eluting at 45 ml in the gel filtration column step of XPFR153P-ERCC1 and XPFR799W-ERCC1 (200 fmol) on a 5'-32P-labeled stem-loop DNA substrate (100 fmol) in the presence of either XPFR153P or XPFR799W mutant cells. The star indicates the migration of a cross-reactive XPF signal. XPFR153P-ERCC1 and XPFR799W-ERCC1 (200 fmol) on a 5'-32P-labeled stem-loop DNA substrate (100 fmol) in the presence of either XPFR153P or XPFR799W mutant cells.

Figure 1. Biochemical characterization of XPFR153P-ERCC1 and XPFR799W-ERCC1 mutants. (A) Gel filtration profiles from the purification of recombinant XPF-ERCC1, XPF R153P-ERCC1 and XPF R799W-ERCC1 from baculovirus-infected Sf9 insect cells using a His₆ tag on ERCC1. Aggregated proteins elute at ~45 ml in the void volume of the column; heterodimeric XPF-ERCC1 elutes at ~65 ml corresponding to ~200 kD, and monomeric ERCC1 elutes at ~78 ml (~50kD). (B) SDS-PAGE analysis of purified protein complexes. Lane 1, 3 and 5 (D): XPF-ERCC1, XPFR153P-ERCC1 and XPFR799W-ERCC1, respectively, after purification over NTA-agarose, gel filtration and heparin columns. Lanes 2 and 4 (A) show the proteins present in the fractions eluting at 45 ml in the gel filtration column step of XPFR153P-ERCC1 and XPFR799W-ERCC1, respectively. (C) Immunodetection of XPF in normal (CSRO) and XPFR153P mutant cells. The star indicates the migration of a cross-reactive band demonstrating equal loading [32]. (D) Incision activities of XPFR153P-ERCC1, XPFR153P-ERCC1 and XPFR799W-ERCC1 (200 fmol) on a 5'-32P-labeled stem-loop DNA substrate (100 fmol) in the presence of either 0.4 mM MnCl₂ (lanes 2, 4 and 6) or 2 mM MgCl₂ (lanes 3, 5 and 7). Reactions were analyzed on a 15% denaturing polyacrylamide gel. The 46-mer substrate and 9-10-mer products are indicated. doi:10.1371/journal.pgen.1000871.g001
Figure 2. Differential immunofluorescence of cells from patients with XPF mutations. Fibroblasts from patients with mutations in XPF and a normal control were grown in the presence of different size beads. After 24 hr the cultures were washed to remove extracellular beads, mixed and co-plated on glass coverslips. The next day, the cells were fixed and immunostained as indicated. Cells were stained with Dapi to identify nuclei and examined by phase contrast microscopy to identify the cell type by their bead content and by fluorescence microscopy for immunodetection of XPF or ERCC1. (A) Analysis of XPF protein sub-cellular localization. Cells from an unaffected individual were labeled with 2 μM beads; XPF mutant cells were labeled with 0.8 μM beads. (B) Analysis of ERCC1 sub-cellular localization in patients with mutations in XPF. (C) Immunodetection of XPF and ERCC1 in nuclear and cytoplasmic fractions of normal fibroblasts (CSRO) and XPF mutant cells (XP51RO). Tubulin is used as a loading control of the cytoplasmic fraction. Nucleophosmin is used as a loading control for the nuclear fraction. (D) Quantitation of the fraction of cells containing exclusively nuclear XPF-ERCC1, XPF-ERCC1 in the nucleus and cytoplasm (pancellular) or exclusively cytoplasmic complex, as determined from immunofluorescence images (n \( \geq \) 100 cells per cell line).

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Remarkably, cytoplasmic XPF-ERCC1 is not detected by immunofluorescence in all XPF mutant cells within a population. To quantify the phenomena, the fractions of cells with exclusively nuclear, exclusively cytoplasmic, or pancellular XPF-ERCC1 were determined from immunofluorescence images (Figure 2D). In wt fibroblasts, 93% of cells have XPF-ERCC1 only in the nucleus. Seven percent of cells show pancellular XPF-ERCC1. But never is the complex seen exclusively in the cytoplasm. In all of the XPF mutant cell lines, XPF-ERCC1 was detected exclusively in the cytoplasm of a fraction of cells ranging from 3–46% of the total population. Thus all known XPF mutations lead to a reduction in nuclear XPF-ERCC1 and an increase in the amount of the complex detected in the cytoplasm.

Direct detection of XPF\textsuperscript{R153P}

To further rule out the possibility that the cytoplasmic XPF-ERCC1 detected was an artifact generated by non-specific antibodies, human XPF\textsuperscript{R153P} and XPF\textsuperscript{WT} were tagged with YFP and expressed in Xpf mutant hamster cells (UV41) for direct detection of XPF protein. The expression of fusion proteins was confirmed by immunoblot using antibodies against human XPF and GFP (Figure 3A). Immunodetection of XPF revealed overexpression of both fusion proteins relative to endogenous XPF protein levels in normal fibroblasts (C5RO). Numerous breakdown products of XPF were also observed, likely due to its overexpression. But only a single fusion protein migrating at the expected molecular mass of full length XPF-YFP was detected using an antibody that detects GFP. To determine if the fusion proteins were functional, transiently transfected cells were tested for their sensitivity to UV to measure NER and mitomycin C (MMC) to measure interstrand crosslink repair (Figure 3B). Wild-type XPF-YFP yielded near complete correction of the hypersensitivity of UV41 mutant cells to UV and MMC. By contrast, despite the fact that XPF\textsuperscript{R153P}-YFP was overexpressed to the same extent as XPF\textsuperscript{WT}-YFP, this protein was unable to correct either DNA repair defect (Figure 3B), as expected based on the hypersensitivity of the XPS1RO patient cell lines [32]. To determine the sub-cellular localization of XPF\textsuperscript{R153P}, cells expressing the YFP-tagged protein were plated on glass coverslips and the protein detected by fluorescence microscopy (Figure 3C). XPF\textsuperscript{WT}-YFP was exclusively in the nucleus. However, XPF\textsuperscript{R153P}-YFP was detected in the cytoplasm of 95% of the transfected cells. This confirms the immunodetection data indicating that mutant XPF is cytoplasmic.

Characterization of XPF\textsuperscript{R153P}-ERCC1 activity in living cells

Unscheduled DNA synthesis (UDS) measures the incorporation of radiolabeled nucleotides into the genome of non-S phase cells after exposure to UV radiation and is a direct measure of NER [35]. Previously, UDS in cells from patient XP42RO (XPF\textsuperscript{R709W}) and XP51RO (XPF\textsuperscript{R153P}) was reported to be 20% and <5% of that in normal fibroblasts, respectively (Table 1). UV-induced UDS was measurable in all of the mutant XPF cell lines except XP51RO. This demonstrates that all of the mutant XPF proteins, with the exception of XPF\textsuperscript{R153P}, retain catalytic activity in vivo.

To ask if XPF\textsuperscript{R153P} is also catalytically active in vivo, recombinant purified XPF\textsuperscript{R153P}-ERCC1 was microinjected into the nuclei of NER-deficient XP51RO primary fibroblasts to determine if UV-induced UDS could be restored. XP51RO cells were first fused on slides by treatment with inactivated Sendai virus to produce homopolykaryons (multinucleate cells). Only homokaryons were injected with protein, to permit identification of those cells that were injected with protein. The slides were irradiated with 10 J/m\textsuperscript{2} UV-C, cultured in the presence of...
Table 1. Characteristics of XPF mutant cell lines.

| Patient | Mutation Allele 1 | Mutation Allele 2 | Age (yr) | Skin Cancer | Clinical features | UDS | UV sensitivity | % of cells with non-nuclear XPF-ERCC1 | Ref |
|---------|------------------|------------------|---------|-------------|------------------|-----|---------------|---------------------------------------|-----|
| CSRO    | none             | none             |         | -           | normal           | 100%| 1X            | 7%                                    | [33]|
| Father of XP42RO | R799W       | none             |         | -           | photosensitivity without skin lesions | 100%| 1X            | rare                                  | [33]|
| XP23OS  | 45Sfs           | ?               | 45      | -           | mild XP          | 45% | 4X            | rare                                  | [60]|
| XP7NE   | P379S           | silent           | 28      | -           | mild XP          | 30% | 2X            | 27%                                   | [61]|
| XP62RO* | R799W           | R799W           | 62      | +           | mild XP with late onset neurodegeneration | 20% | not reported | 65%                                   | [33]|
| XP42RO* | R799W           | R799W           | 62      | +           | mild XP with late onset neurodegeneration | 20% | 2X            | 33%                                   | [33]|
| XP2YO   | T567A           | 7*              | 65      | +           | mild XP          | 17% | 3X            | n.d                                   | [46]|
| AS871   | R589W           | del exon3       |         | -           | severe XP with neurodegeneration | 15% | 2X            | 59%                                   |      |
| XP26BR  | R799W           | R799W           |         | -           | mild XP          | 15% | not reported  | 33%                                   |      |
| XP32BR  | R589W           | P379S           | 12      | -           | mild XP          | 10% | 2X            | 39%                                   |      |
| XP24BR  | R799W           | R589W           | 29      | -           | severe XP with neurodegeneration | 5%  | 3X            | 74%                                   | [61]|
| XP24KY  | R799W           | 537fs + 7bp     | 50      | -           | XP with late onset neurodegeneration | 7%  | 3X            | 45%                                   | [46]|
| XP51RO  | R153P           | R153P           | 16      | -           | neurodegeneration severe progeria | <5% | 10X           | >33%                                  | [30]|

UDS unscheduled DNA synthesis

*The patient had normal levels of XPF transcript, suggesting one allele encodes a full-length mRNA.

*Mutation could not be confirmed on genomic DNA.

Siblings.

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3H-thymidine and nuclear grains indicating sites of thymidine incorporation in non-S phase cells measured (Figure 4). As expected, delivery of XPF<sup>WT</sup>-ERCC1 to the nuclei of cells led to a significant increase in the number of grains detected in homopolykaryons relative to individual cells in the same culture (Figure 4A). A significant increase in UV-induced UDS was also detected in homopolykaryons injected with XPF<sup>R153P</sup>-ERCC1 (Figure 4C). This confirms the in vivo activity data and establishes that XPF<sup>R153P</sup> is catalytically active in vivo if it is delivered to the nucleus. UDS levels were not recovered to the same extent as when WT protein was injected (Figure 4D). But injection of XPF<sup>R153P</sup>-ERCC1 or XPF<sup>R799W</sup>-ERCC1 led to a similar increase when WT protein was injected (Figure 4D). But injection of XPFWT-ERCC1 to the nuclei of cells led to a significant increase in the number of grains detected in homo- polykaryons relative to individual cells in the same culture (Figure 4C). This confirms the detected in homopolykaryons injected with XPF R153P-ERCC1 polykaryons relative to individual cells in the same culture significant increase in the number of grains detected in homo-

Clinical correlation

To determine if the severity of disease associated with a particular mutation in XPF could be predicted by the amount of XPF-ERCC1 detected in cell nuclei, the results in Figure 2D were compared to the clinical information available about the patients from which the cells were derived. In Table 1, the cell lines are listed in order of decreasing UDS. Patients with mild disease tend to have greater UDS or DNA repair. Patients XP32BR and XP26BR could be exceptions, but they are too young to know the full extent of their disease. In Figure 2D, the cell lines are clustered into those from patients in which severe disease/neurodegeneration was documented (right) or not yet observed (left). There is a trend towards those with severe disease to have more cells with non-nuclear XPF-ERCC1, but this trend did not reach significance (p = 0.06, unpaired Student’s t-test), likely due to the small sample size. Therefore, the detection of cells with cytoplasmic XPF-ERCC1, while maybe useful to screen for patients with XPF mutations is not sufficient to predict patient prognosis.

Discussion

Classically, inherited mutations in a gene are associated with a single disease. However, mutations in several genes involved in the NER pathway can result in more than one disease. The most prominent example is XPD, which if mutated can cause the cancer-prone disease XP but also Cockayne Syndrome (CS) characterized by photosensitivity, growth retardation, developmental abnormalities and profound neurodegeneration, as well as trichohydrotrophy (TTD), which is similar to CS, but also involves the skin and nails [36]. Similarly, mutations in XPB can also cause XP, TTD and a combined XP-CS [37] and mutations in XPG can lead to XP or XP-CS [38]. Of all the genes whose products are required for NER, only XPB, XPD and XPG are required for the proper function and stability of the basal transcription factor TFIIH [39]. Thus the more severe symptoms of CS and TTD are attributed to a combined defect in NER as well as transcription [36,40–42]. Mutations in XPF were recently linked to a second disease in addition to XP, a disease of systemic accelerated aging termed XFE progeroid syndrome [32]. In this study, we sought to determine how mutations in XPF can lead to such a wide variety of symptoms.
Mutations in XPF do not ablate catalytic activity

Since CS and TTD are attributed to defects in transcription [34,43], the prediction is that mutations in XPB, XPD or XPG that cause CS or TTD should affect basal transcription in addition to NER, whereas mutations that affect only NER cause XP. Indeed, mutations in the catalytic domain of XPG, for example A792V, disrupt the endonuclease activity of XPG, but not its interaction with TFIIH and therefore causes classical XP [44]. Similarly, a mutation in the helicase domain of XPD, D234N, affects NER, but not basal transcription and therefore leads to XP [45]. By analogy, we examined the enzymatic activity of XPF<sup>R799W</sup> and XPF<sup>R153P</sup>, which cause XFE progeroid syndrome and XP respectively, and discovered that neither mutation ablates the catalytic activity of the protein. Recombinant protein complexes harboring either mutation are able to incise a stem-loop substrate in vitro (Figure 1) and to restore NER in vivo (Figure 4). This is in keeping with the fact that patients with XP-F have residual UDS or NER (Table 1).

Intriguingly, XP-F patients tend to have much milder photosensitivity and later onset skin cancer than XP patients from other complementation groups with the same level of UDS [46]. One explanation for this is that NER occurs in XP-F cells but at a much slower rate [47], making UDS a relatively poor reflection of the true DNA repair capacity of a cell. In total, these data provide clear evidence that viable mutations in XPF do not ablate catalytic activity of the XPF-ERCC1 nuclease. Of note, all XP patients for which the mutation in XPF was confirmed by sequencing genomic DNA harbor one of three recurrent point mutations (R799W, R589W or P379S). The rarity and limited repertoire of only hypomorphic point mutations in patients strongly suggests that XPF-ERCC1 nuclease activity is essential for normal embryonic development.

Stability of XPF<sup>R799W</sup>-ERCC1 and XPF<sup>R153P</sup>-ERCC1 is reduced

Mutations in a single gene could lead to diverse clinical outcomes if mutations differentially affect the stability of the gene product. For example, mutations affecting the stability of the TFIIH complex are linked with TTD but not XP [34]. Total cellular XPF and ERCC1 are dramatically reduced in cells from a patient with XFE progeroid syndrome (Figure 2C). However, XPF levels are reduced to the same extent in whole cell extracts from a patient with mild disease and 20% of the normal level of NER (Figure 1C). Therefore, mutations in XPF clearly affect protein level, which undoubtedly contributes to reduced DNA repair and disease. However, the level of XPF-ERCC1 in patient cells, as detected by immunoblotting, is inadequate to explain the differences in the severity in the DNA repair defect and disease between patients with different mutations in XPF. Interestingly, in at least a subset of XP-F patients, XPF mRNA levels are normal, but XPF protein level is low [46], indicating that mutant XPF is unstable.

Mutations in XPF affect protein subcellular localization

The novel and unexpected finding is that mutation in XPF leads to increased cytoplasmic localization of the XPF-ERCC1 nuclease complex (Figure 2 and Figure 3) and that this aberrant subcellular localization is what prevents XPF-ERCC1 from participating in DNA repair (Figure 4). This was demonstrated by immunofluorescence detection of the complex using multiple antibodies. The results were confirmed by examining the subcellular localization of fluorescently tagged recombinant XPF (Figure 2). In further support of this, ERCC1 is also mislocalized to the cytoplasm of cells from the one patient reported with a mutation in ERCC1 [48].
Cytoplasmic localization of XPF-ERCC1 is not observed in all cells harboring XPF or ERCC1 mutations, suggesting the possibility that mutations affect proper folding of XPF-ERCC1 and that misfolded proteins are preferentially sequestered in the cytoplasm through interactions with other proteins or preferentially degraded. Alternatively, there may be tremendous selection for cells with nuclear XPF-ERCC1. This is consistent with the notion that the repair complex is essential for viability. Indeed, continuous passaging of XP51RO cells over years leads to a striking increase in the fraction of cells with nuclear XPF-ERCC1 and reduced sensitivity to the crosslinking agent mitomycin C (Ahmad, Bhagwat and Niedernhofer, unpublished data). Thus the fraction of cells with cytoplasmic XPF-ERCC1 may be underrepresented in Figure 2D, although only early passage cells were used in this study.

Disease caused by protein mislocalization

Many human diseases are caused by misrouting or mislocalization of proteins, ranging from metabolic disorders to cancer. Mislocalization of the tumor suppressors p53 [49], FOXO [50], p73 [51] and β-catenin [32] into the cytoplasm rather than the nucleus, leads to a loss of protein function and is associated with cancer. In contrast, mislocalization of NF-kB [53], BRCA1 and BARD1 [53,54] from the cytoplasm into the nucleus is also associated with a variety of tumors. A classic example of a disease caused by protein mislocalization is cystic fibrosis which is caused by retention of the cystic fibrosis transmembrane conductance regulator (CFTR) protein in the endoplasmic reticulum, instead of its localizing to the cell surface [55,56]. In addition, nephrogenic diabetes insipidus, retinitis pigmentosa, emphysema and α1-antitrypsin deficiency liver disease are also caused by mislocalized proteins [55].

Mislocalization of proteins may result from a mutated nuclear localization sequence (NLS) or nuclear export sequence (NES). Remarkably, the majority of the missense mutations in XPF is at arginine residues and leads to conversion of the arginine to a noncharged residue. So these mutations could affect a complex NLS. All of the point mutations (R→P, R→W and P→S) are also predicted to alter protein structure, supporting the notion that XPF mutations affect protein folding and/or protein:protein interactions that are critical for nuclear localization.

Our data add XPF to the list of proteins that if mislocalized contribute to disease. While this leads to novel insight into the regulation of XPF-ERCC1 and DNA repair in cells, the extent of XPF-ERCC1 mislocalization, as measured by immunodetection, does not predict the level of NER (UDS) or disease severity (Table 1). This could be because each mutation differentially affects folding of the protein and thereby differentially affects protein expression, protein degradation and/or cellular localization. Another possibility is that there are modifier proteins that influence disease severity, in particular in patients with homozygous mutations. However, we believe the former is of primary importance based on the observation that titration the level of expression of ERCC1-XPF in mice directly impacts lifespan and the severity of symptoms [57,58].

In the case of XPF, it is the absence of XPF and its binding partner ERCC1 in the nucleus leading to reduced repair of genomic DNA that is disease-causing, rather than toxicity of mislocalized protein. Our data illustrate a novel mechanism by which the DNA repair capacity of a cell is determined: by nuclear localization of XPF-ERCC1. The identification of proteins that regulate this could lead to novel targets for improving DNA repair to treat patients with mutations in XPF or reduce cancer risk after exposure to genotoxic agents. Alternatively, these proteins would be excellent targets for small molecule inhibitors that would reduce repair and thereby prevent tumor resistance to genotoxic cancer therapies.

Materials and Methods

Biochemical characterization of XPR153P-ERCC1 and XPR799W-ERCC1

Purification of recombinant XPF-ERCC1 was performed essentially as previously reported [27] from baculovirus-infected SF9 insect cells using a His6 tag on ERCC1. In brief, plasmids pFastBac1-XPF and pFastBac1-ERCC1-His were used to transfect SF9 insect cells, and to amplify the virus according to the manufacturer’s instructions (BAC TO BAC system; Life Technologies). Cell extracts were prepared 65 hr after infection with an MOI of 5 and highly purified protein was obtained using chromatography on Ni–agarose, gel-filtration and heparin columns. Only XPF-ERCC1 eluting as proper heterodimer on the gel filtration column at ~65 ml of eluant was collected. The aggregated protein, eluting in the void volume (~40–50 ml), was not used in experiments.

The endonuclease activity of wild-type and mutant XPF-ERCC1 was performed using a stem–loop substrate also as previously described [27]. A stem12–loop22 oligonucleotide (GCCAGGCGCTCGGTTGCGAGGCCTGGG) was 5'-32P endlabeled. Nuclease reactions were performed on 100 fmol of DNA substrate and 20–200 fmol of XPF-ERCC1 protein in a total volume of 15 μl in optimized nuclease buffer (25 mM HEPES pH 8.0, 40 mM NaCl, 10% glycerol, 0.5 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin and 0.4 mM MgCl2 or 2 mM MgCl2). The reactions were incubated at 30°C for 2 h and stopped by adding 15 μl of 90% formamide/10 mM EDTA and heating at 95°C for 5 min. Samples were loaded onto 15% denaturing polyacrylamide gels and reaction products were visualized by autoradiography and quantified on a PhosphorImager (STORM860; Molecular Dynamics).

Cell lines and culturing

Human fibroblasts immortalized with hTert were cultured in Ham’s F10 with 10% fetal calf serum and antibiotics and incubated at 3% oxygen as described previously [32]. Cell lines included those derived from a normal individual (CSRO) [59], the parent of a patient, heterozygous for a mutation in XPF [33], XP-F patients (XP42RO) [33], XP23OS [60], XP24KY [46], XP7NE [61], XP32BR, XP26BR, XP24BR [61], and XP62RO, and a patient with XFE progeroid syndrome caused by a mutation in XP (XP51RO) [32]. Unscheduled DNA synthesis (UDS) in these cells lines was previously reported as referenced above and confirmed in mixed cultures (XP-F cells co-cultured with normal cells using a more accurate click-staining method, as recently described [62]).

Immunodetection of XPF in patient cells

Cells were trypsinized, washed twice with PBS and lysed with 1 ml NETT buffer (100 mM NaCl, 50 mM Tris base pH 7.5, 5 mM EDTA pH 8.0, 0.5% Triton X-100) containing Complete™ mini protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Molecular Biochemicals). Then the lysates were freeze-thawed twice in liquid nitrogen to disrupt nuclear membranes. From each sample, 50 μg of protein was resolved on 10% SDS-PAGE gels after boiling for 10 min in the presence of loading buffer. XPF was detected using a human XPF monoclonal antibody (clone 219; Neomarkers, Fremont, CA) at a dilution of 1:10000.
Differential immunofluorescence of fibroblasts isolated from patients with mutations in XPF

 Cultures of primary human fibroblasts from patients with mutations in XPF or a normal individual were grown in the presence of different size beads (2 μm or 0.8 μm; Sigma). After 24 hr the cultures were trypsinized and washed extensively with phosphate-buffered saline to remove any extracellular beads. The cells were then mixed in various combinations and co-plated on glass coverslips to provide internal controls of normal XPF-ERCC1 protein levels [34]. After 16 hr, the cells were fixed with 2% paraformaldehyde in sodium phosphate buffer, pH 7.4, for 15 min then permeabilized with 0.1% Triton X-100 in PBS. The samples were immunostained with polyclonal anti-ERCC1 (1:2000; [63]) or polyclonal anti-XPF (1:1000; [16]) followed by goat anti-rabbit ALEXA 488 (1:500; Molecular Probes). Samples were stained with Dapi to identify nuclei and examined by phase contrast microscopy to identify the genotype of the cells according to their bead content and by fluorescence microscopy for immunodetection of repair proteins.

Cell fractionation. Cells were fractionated into nuclear and cytosolic fractions as described [64], with minor modification. In brief, cells were trypsinized, pelleted and washed twice with PBS. The pellet was vortexed at maximum speed for 15 sec with 200 μl of CERI reagent from the Pierce NE-PER fractionation kit (Pierce paraformaldehyde in sodium phosphate buffer, pH 7.4, for 48 hrs after transfection. High-speed cell sorter (Dako North America, Carpinteria, CA) 24–48 hrs after transfection of recombinant XPF-ERCC1

Subcellular localization of XPF–YFP in Chinese hamster Ovary (CHO) cells

XPF cDNA was cloned into pYF-P1 (BD Biosciences Clontech, Palo Alto, CA) such that YFP was expressed as fusion protein at the C-terminus of XPF. This construct, pXPF-YFP-P1, was then used to create XPF-YFP by QuickChange® Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions. The wild type and mutant constructs were transfected in XPF-deficient CHO cell lines UV41 or UV47 using lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Cells expressing YFP were flow sorted using Dako Cytomation MoFlo high-speed cell sorter (Dako North America, Carpinteria, CA) 24–48 hrs after transfection.

To study the subcellular localization of XPF, YFP-positive CHO cells were plated on glass coverslips and grown to 95% confluency. The next day, the samples were fixed with 2% paraformaldehyde in sodium phosphate buffer, pH 7.4, for 15 min. The cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered and nuclei were stained with Dapi-vector shield (Vector Laboratories, Inc. Burlingame, CA). XPF-YFP was visualized using an Olympus BX51 fluorescent 4 microscope at 60–100X magnification.

Clonogenic survival assays of wild-type and mutant CHO cells

Wild type (AA8), XPF-deficient (UV41), XPF-YFP and XPFR153P-YFP cells were seeded in 6 cm dishes in triplicate at 10^3–10^6 cells per plate, depending on the dose of genotoxicant. After 16 hr, the cells were irradiated with UV-C or exposed to mitomycin C (MMC). After approximately 1 week, the cultures were fixed and stained with 50% methanol, 7% acetic acid and 0.1% Coomassie blue. Colonies, consisting of at least 10 cells, were counted using a Nikon SMZ 2B 15 stereomicroscope microscope with 10X eyepiece. The data were plotted as the number of colonies that grew on the treated plates relative to untreated plates ± the standard error of the mean for 2–3 independent experiments.

Immunoblotting of XPF in Wt and mutant CHO cells

Whole cell extracts were prepared from C5RO and UV41 cells transfected with vectors expressing YFP, XPF-YFP or XPFR153P-YFP. Proteins were separated by SDS PAGE using a 10% gel. XPF was detected using a human XPF monoclonal antibody (clone 219; Neomarkers, Fremont, CA) at a dilution of 1:1000. YFP was detected using a GFP monoclonal antibody (Clones 7.1 and 13.1; Roche, Indianapolis, IN) at a dilution of 1:1000.

Correction of XPF mutant cell UV sensitivity by micro-injection of recombinant XPF-ERCC1

Microinjection of purified proteins was performed as previously described [65, 66]. Briefly, primary human fibroblasts from XP51RO were fused by treating cultures with inactivated Sendai virus and then plated on glass coverslips. Subsequently, purified, recombinant XPF-ERCC1 protein complex (wild type or containing the R799W or R153P substitution in XPF) was injected into the nuclei of homopolykaryons. The cultures were irradiated with 10 J/m^2 UV-C and pulse labeled for 3 hrs with [3H]-thymidine. Unscheduled DNA synthesis (UDS) was detected by autoradiography.

One to ten femtoliters of a 10–100 nM solution was injected into the nuclei of 10–20 homopolykaryons for each of the three recombinant proteins and the number of radiographic grains counted in at least 20 nuclei of the homopolykaryons and a similar number of nuclei of single cells in the same sample. The mean and standard deviation of the number of grains was calculated for each of the three proteins. An unpaired, two-tailed Student’s t-test was used to determine if there was a significant difference in unscheduled DNA synthesis between cells that were injected with recombinant XPF-ERCC1 and cells that were not injected.

Author Contributions

Conceived and designed the experiments: AA JHE NRB JHJH WV NGJJ ODS LJN. Performed the experiments: AA JHE NRB NW AR EA AFT ODS LJN. Analyzed the data: AA JHE NRB ODS LJN. Contributed reagents/materials/analysis tools: JHJH WV ODS LJN. Wrote the paper: NGJJ LJN.

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