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Human XPG nuclease structure, assembly, and activities with insights for neurodegeneration and cancer from pathogenic mutations

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Xeroderma pigmentosum group G (XPG) nuclease structure, assembly, and activities with insights for neurodegeneration and cancer from pathogenic mutations

Significance

DNA repair is essential to life and to avoidance of genome instability and cancer. Xeroderma pigmentosum group G (XPG) protein acts in multiple DNA repair pathways, both as an active enzyme and as a scaffold for coordinating with other repair proteins. We present here the structure of the catalytic domain responsible for its DNA binding and nucleolytic activity. Our analysis provides structure-based hypotheses for how XPG recognizes its bubble DNA substrate and predictions of the structural impacts of XPG disease mutations associated with two phenotypically distinct diseases: xeroderma pigmentosum (XP), skin cancer prone (or Cockayne syndrome (XP/CS), severe progressive developmental defects).

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, http://www.pdb.org (PDB ID code 6V6H).

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The hallmark of CS is loss of the preferential repair of DNA damage in transcribed strands by TCR. While the mechanistic connection of XPG loss to CS is not entirely clear, it is evident that it extends beyond the enzymatic function of XPG in NER. The requirement for XPG in TCR likely involves its demonstrated interactions with RNA polymerase II (RNAPII), TFIIH, and CS group B (CSB) proteins, and biochemical evidence suggests that XPG may participate in allowing lesion removal in the presence of stalled RNAPII (3, 4, 9).

XPG also plays important roles in other DDR pathways through multiple protein interactions distinct from its NER functions. XPG depletion causes DNA double-strand breaks, chromosomal abnormalities, cell-cycle delays, defective HR repair (HRR), sensitivity to poly(ADP-ribose) polymerase inhibition, inability to overcome replication fork stalling, and replication stress (10). These phenotypes reflect its function in HRR mediated by its direct interactions with BRCA1, BRCA2, PALB2, and the RAD51 recombinaise, which likely promote loading of RAD51 onto RPA-coated ssDNA. In BER, XPG stimulates glycosylase activity of NTH1 in vitro (11–13). XPG interacts with and stimulates Werner syndrome helicase (WRN) in vitro, and the two proteins colocalize during replication (14).

XPG’s incision activity is implicated in cleavage of R-loops, including those generated by loss of WRN in the premature aging disorder Werner syndrome (15–17). While roles in the multiple roles of XPG in DDR depend on its many protein–protein interactions, some require its enzymatic activity but others apparently do not. It is less clear how its specific binding to ss/dsDNA junctions might be involved.

The type of mutations that lead to cancer-prone XP (XP-G) and the fatal neurodevelopmental disorder CS (XP-G/CS) are distinct, although both are rare autosomal recessive (18, 19). All of the ERCC5 changes causing XP-G are missense point mutations, whereas the originally described XP-G/CS mutations were predicted to be severely truncating, leading to the model that loss of XPG protein causes XP-G/CS, whereas XP-G results from an incision-defective mutant protein causing loss of global NER (20). This idea received strong support from mouse models, since mice with knockin catalytically inactivating point mutations in ERCC5 are UV-sensitive but otherwise normal (21, 22), whereas knockout mice display CS-like progressive progeroid features, extensive neurodegeneration, and early death (8, 23). More recently, however, several new XP-G/CS patients have been described with missense mutations in ERCC5 (24, 25), suggesting that the situation is complicated with respect to causal changes in XPG protein for disease outcome (i.e., XP vs. XP/CS). It is also presently unclear to what extent the requirement for participation of XPG in DDR processes other than NER or TCR, in particular BER and HRR, is relevant to development of CS when XPG function is lost.

XPG binding and incision of Y and bubble DNA structures is well studied (1, 4, 26–28), yet the structural mechanisms underlying XPG activities including DNA binding in DDR are not known. XPG is a founding member of the primordial FEN superfamily, also known as the 5′ nuclease or Rad2 superfamily, that includes human FEN1, EXO1, and GEN1 (29, 30), and yeast ortholog Rad2 (31–34). FEN1, involved in resolution of 5′ flaps in lagging-strand replication, is one of the most well-studied in the family and can serve as an example to broadly understand 5′ nuclease mechanisms. In FEN1, incision requires at least a four-step process of: 1) Initial binding to dsDNA regions of the 5′ flap; 2) specific substrate validation of target features (dsDNA, single-strand 5′ flap, and a 1-nt 3′ flap) with concomitant conformation shifts in both the protein and DNA conformations; 3) shifting the scissile phosphate into the catalytic site; and finally 4) two-metal–based phosphodiester bond cleavage (29, 35–39). Thus, only after substrate testing by coordinated DNA and protein conformational changes (35) is FEN1 licensed to cut DNA, making it distinct from other nucleases where substrate validation is at the binding step. Furthermore, for XPG some substrates are bound but not incised (4, 28). As incision appears not to be required for all XPG functions, could some DDR pathways involve its structured DNA binding capability?

To address questions regarding XPG structural mechanisms in the DDR, we solved the crystal structure of the human XPG catalytic domain (XPGcat). We furthermore combined biochemical, small angle X-ray scattering (SAXS), and electron microscopy (EM) analyses of full-length protein with experimental data on disease-causing mutations both in XPG in patient cells and in bacterially expressed XP-Gcat. We show that XPG structure shares key metal-binding and basic residues with FEN1 and is likely to work through an analogous disorder-to-order transition requiring the properly folded XPGcat structure for an analogous multistep process to that associated with specificity in FEN1 and EXO1. We furthermore found that human XPG unexpectedly formed a stable dimer in solution and on DNA that appears to be required for catalytic activity and for protein stability. Rotary shadowing EM data showed that XPG bound specifically to bubble DNA and replication forks and strongly bent bubble DNA. We examine XP-G and XP-G/CS missense mutations and characterize the impact of these mutations on expression and endonuclease activity, prompting a reexamination of how these mutations result in the different disease phenotypes. The collective findings provide fundamental mechanistic implications for the molecular roles of XPG in multiple DDR pathways.

**Results**

XPGcat Crystal Structure Reveals 5′ Nuclease Fold with XPG-Specific Features. To reveal the structural basis for XPG’s DNA incision and binding activities, we created a construct “XPGcat” with the XPG catalytic domain for crystallographic analysis. Between the superfamily-conserved catalytic domain composed of two regions, N and I, XPG has an additional ~680-amino acid insert (called the “R-domain” or spacer region) as well as an extended C terminus following the I region (Fig. 1A) (4, 40). The R-domain and the C terminus are predicted to contain coiled coils and large regions of disorder that would likely interfere with crystallization (41). For XP-Gcat, we replaced the XPG R-domain with the corresponding 40-amino acid helical gateway/cap or arch region from an archaeal FEN (4, 42) and truncated the C terminus (Fig. 1A). To show that XPGcat is active for incision, we compared incision activity of full-length XPG or XPGcat on Y DNA (DNA forming a Y-like structure with one dsDNA and two ssDNA regions) or 15-nt bubble DNA, where 15 nt refers to a central, unpaired region between two dsDNA arms (SI Appendix, Fig. S1 A–C). As previously observed, full-length XPG could incise both, with greater activity for bubble DNA (4). XPGcat had robust incision activity for Y DNA but no detectable activity on 15-nt bubble DNA, consistent with the inserted FEN cap limiting active site access to only those substrates having a free 5′ terminus (43).

We crystallized XPGcat, solved the structure, and refined the model to a resolution of 2.1 Å with R and Rfree of 0.22 and 0.25, respectively (Fig. 1B and SI Appendix, Table S1). The overall structure is a mixed α/β single domain containing a central seven-stranded twisted β-sheet core with α-helices on either side. This fold resembles members of the FEN superfamily and the catalytic domain of its yeast ortholog Rad2 (RMSD 1.0 Å) (33), with which it shares 30% identity (29). A highly conserved α15/random coil (resides 937 to 955) on the back side (not fully visible in Fig. 1B) connects one side of the protein to the other. The XPGcat structure contains the two C-terminal helices (α16 and α17) found in Rad2 and FEN1, but not in EXO1 nor GEN1 (31, 33, 34, 36). These helices form the interface for DNA recognition on the nonincised strand near the junction. For FEN1, this is a dsDNA arm with a 1-nt 3′ flap. For XPG, this would be
the ssDNA region of its bubble DNA substrate. In EXO1, this region is not a required feature of its substrate.

Structurally conserved in all FEN superfamily members, seven carboxylates in the β-sheet core and basic residues in the gateway helices (α2 and α4) in XPG are similarly positioned in the active site (29). As observed for FEN1 and EXO1, mutation of one of the conserved basic residues (K84A) in XPGcat drastically reduced incision activity on Y DNA compared to WT XPGcat, with only a relatively minor effect on DNA binding (SI Appendix, Fig. S1D and E). Although we cannot assess part of α4 and α5 derived from the FEN protein, residue conservation and the similarity of the XPG α2 support a gateway-based mechanism for selection of the ssDNA on the strand that will be incised. Suggesting a similar disorder-to-order regulation of the active site, α2 is partially disordered as found for DNA-free FEN1 (44). XPGcat also includes the helix-2-turn-helix (H2TH) that helps form the K+ binding site and the β-pin implicated in DNA binding in FEN (36). No K+ was bound, not unexpectedly given the absence of DNA substrate.

All FEN superfamily members interact with ss/dsDNA junction substrates, primarily bind the dsDNA portions, and position the 5' ssDNA region near to the active site (29). As FEN1 is similar to XPG in overall structure and a FEN1 crystal structure exists with DNA on either side of the scissile phosphate, we modeled our XPGcat domain with FEN1 DNA. The alternative of modeling with DNA in the ortholog Rad2 structure would not be ideal, as Rad2 crystal structures with DNA contained only one dsDNA arm (33). Like other superfamily members, our structure is similar to XPG in overall structure and a FEN1 crystal structure serves as part of a FEN1-like substrate validation (SI Appendix, Fig. S1H). Elimination of these aromatic side chains reduces distortion and enhances DNA binding. However, substrate validation can no longer occur properly, so the gateway helices remain disordered and incision activity in full-length XPG is reduced. In the XPGcat construct, however, substrate validation has been bypassed by replacing the disordered gateway helices in XPG with the ordered thermophilic FEN helices.

**Human XPG Forms a Dimer.** Earlier immunoprecipitation experiments suggested that XPG may be a multimer, although that conclusion was contradicted by biophysical measurements (45). We revisited this issue by examining full-length XPG recombinantly expressed in insect cells. We designed XPG with two different C-terminal tags, FLAG and GFP, so that the differentially tagged proteins could be distinguished by their mobility in SDS/PAGE. When the two tagged XPG proteins were coexpressed, anti-FLAG resin pulled down both XPG-FLAG and XPG-GFP, suggesting a complex had formed (Fig. 3A). The greater quantity of XPG-FLAG would be consistent with the stochastic presence of both homomeric XPG-FLAG multimers and heteromeric XPG multimers with both tags. Consistent with this interpretation, protein eluted from the FLAG beads and reisolated on GFP binder resin contained equivalent amounts of XPG-FLAG and XPG-GFP proteins (Fig. 3A and B). These results establish XPG as a multimer. Significantly, when the differentially tagged XPGs were expressed separately in two different insect cell cultures and then mixed before lysis, we did not observe preferential association, suggesting that XPG exists as detergent-resistant multimers in cells.
not observe XPG-GFP bound with anti-FLAG resin nor XPG-FLAG bound to GFP binder beads (Fig. 3B), indicating that XPG multimers are relatively stable and do not readily exchange under these conditions.

We measured the stoichiometry of purified XPG in solution by SAXS, from which the mass of a protein can be calculated within ∼10% error (46). Prior to SAXS data collection, we purified XPG through size-exclusion chromatography (SEC). We observed two peaks of XPG (SI Appendix, Fig. S2A and B), which had different mobility in native PAGE (SI Appendix, Fig. S2C). We collected SAXS data on the second, major peak, the only one for which we had enough protein concentration to get a reliable SAXS signal (SI Appendix, Figs. S2 and S3 and Table S3). The molecular mass calculated from the SAXS data was 265 kDa, consistent with a dimer (theoretical mass 266 kDa). The Porod exponent, a measure of the protein volume and thereby protein flexibility, was 2.9 (47). Globular, well-folded proteins have a Porod exponent of 4, flexibly linked globular regions 3, and unstructured proteins 2. Thus, a Porod exponent of 2.9 signifies a partially structured protein, in agreement with disorder prediction analyses. Indicating that XPG alone is always in a dimer or higher-order multimer in solution, we found no peak in SEC consistent with a monomer. To map the dimerization interface, we tested XPG truncation constructs (Fig. 1A and SI Appendix, Figs. S2 and S3 and Table S3). Elimination of the C terminus (XPGΔC) did not alter the dimerization state. Elimination of the R-domain (XPGΔR) gave mixed populations of monomer and dimer. Addition of 10-nt bubble DNA to XPGΔR increased the ratio of the dimer (SI Appendix, Fig. S3B). The DNA-induced dimerization for this construct was corroborated by multiangle light scattering (SI Appendix, Fig. S3C). The Porod exponents for XPGΔC and XPGΔR increased to 3.2, indicating a reduction in flexibility. The XPGcat construct without the R- and C-terminal domains had greatly reduced flexibility with a Porod exponent of 3.9. The experimental SAXS curve generally matched that predicted from the crystal structure (SI Appendix, Fig. S2E). Based on the residual differences between experimental and model SAXS curves, the best match came with an ensemble of monomer and dimer models, indicating that XPGcat is mostly monomeric with a small population of dimer. Together, these mass measurement results suggest that multiple regions in the R-domain, XPGcat, and the C terminus participate together in dimer stabilization.

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**Fig. 2.** Sequence alignment of XPGcat, Rad2, FEN1, EXO1, and GEN1.
As the crystal lattice can reveal protein–protein interfaces, we examined the XPGcat crystal lattice. A symmetric dimer interface (Fig. 3C) is formed by hydrophobic residues Leu955, Phe978, and Leu981 in α14 and α15 in one molecule with the same region from a crystallographically related molecule, and between the acidic side chain of Asp956 N-capping the positively charged N-terminal end of α14 (Figs. 2 and 3C). Automated assembly analysis independently identified the same dimer (48). These four residues are conserved in XPG but not in related superfamly member FEN1, although the helical structures themselves are conserved. FEN1 does not form a crystallographic contact at this position (36) and is a monomer in solution. In the yeast Rad2 ortholog of XPG, crystal contacts did not occur with this exact interface, but did form with nearby residues (33) (SI Appendix, Fig. S4A). The superfamly member GEN1 is a homodimer, but the C terminus to C terminus dimer interface in XPGcat is on a different side from the GEN1 dimer, where the gateway helices protecting the active site stack end-to-end and where helices α14 and α15 are packing with GEN1’s C-terminal chromodomain (32).

Comparison of XPGcat structure to that of FEN1 bound to its DNA substrate indicated that XPGcat as a monomer could bind to only one dsDNA arm (i.e., a Y-structure) and not to both dsDNA arms of a bubble DNA. As a dimer (albeit at a low ratio relative to monomer), XPGcat might interact with both dsDNA arms of a full bubble DNA, even if it cannot incise (SI Appendix, Fig. S1 A–C). To test this possibility, we compared binding of XPGcat to Y DNA and to 15-nt bubble DNA. Although we showed earlier that XPGcat could not incise the bubble DNA (SI Appendix, Fig. S1 A–C), surprisingly it bound to bubble DNA up to fourfold better than to Y DNA (Fig. 3D and SI Appendix, Fig. S4B), albeit still more weakly than does full-length XPG (4). This seemingly antithetical DNA binding with no incision is consistent with FEN superfamly members physically separating their initial DNA binding from positioning for incision (35). An XPGcat dimer, which our SAXS mass measurements suggest exists at low frequency, would create two dsDNA-interacting surfaces to preferentially bind bubble DNA over Y DNA.

To test the functional relevance of this dimer interface, we made quadruple mutations (dimer mutant 4A: L955A/D956A/ F978A/L981A) in full-length XPG. We observed significantly lower expression levels of the mutant protein when expressed in insect cells, suggesting protein destabilization. Although we were unable to purify enough mutant protein for SAXS or other biophysical analyses for protein stability, we were able to test for incision activity. Consistent with the dimerization interface being functionally relevant, the 4A dimer interface mutant showed an approximately twofold decrease in 15-nt bubble DNA incision activity (Fig. 3E and SI Appendix, Fig. S4C). We reasoned that incision of bubble DNA would be more affected than of Y DNA. Indeed, we observed a 40% smaller loss in incision activity with the Y DNA (Fig. 3F and SI Appendix, Fig. S4D). Nonetheless, the dimer interface was important for protein activity even with Y DNA. We observed a similar protein destabilization of the dimer interface mutant in the XP-G/CS patient cell line XPCS1RO that lacks detectable XPG protein. We transfected these cells with WT XPG expression construct, the 4A mutant, or a quadruple dimer interface mutant “A3E” with three glutamate substitutions, instead of alanine, at the hydrophobic residues. Western analysis done at 48 h after transfection revealed greatly diminished XPG protein amounts expressed in the human cells from the two dimer interface mutants compared to WT (SI Appendix, Fig. S4E). This destabilization is specific to the full-length protein, as we saw no apparent destabilization of the XPGcat dimer interface mutant, and the Porod exponent was 3.9 for both XPGcat and the XPGcat 4A mutant (SI Appendix, Figs. S2 and S3).

**EM Analysis of DNA Binding.** Previous EMSA revealed stable binding of XPG to bubble DNA but did not show where XPG was bound along the DNA nor whether binding might bend the DNA (4). To address these questions, we used direct EM with tungsten metal shadow casting to visualize XPG/DNA complexes (Fig. 4). XPG from the SEC dimer peak C3 (SI Appendix, Fig. S2 A and B) was incubated with a 300-bp dsDNA with a central 10-nt bubble (Fig. 4A). We purified XPG/DNA complexes by a second SEC. We adjusted the concentration of XPG such that only ~50% of the DNA was bound by XPG as seen by EM, as higher ratios generated confounding multimolecule aggregates. We used mass ratios of 4 to 6 μg of XPG per microgram of the DNA substrate indicated that XPGcat as a monomer could bind to only one dsDNA arm (i.e., a Y-structure) and not to both dsDNA arms of a bubble DNA. As a dimer (albeit at a low ratio relative to monomer), XPGcat might interact with both dsDNA arms of a full bubble DNA, even if it cannot incise (SI Appendix, Fig. S1 A–C). To test this possibility, we compared binding of XPGcat to Y DNA and to 15-nt bubble DNA. Although we showed earlier that XPGcat could not incise the bubble DNA (SI Appendix, Fig. S1 A–C), surprisingly it bound to bubble DNA up to fourfold better than to Y DNA (Fig. 3D and SI Appendix, Fig. S4B), albeit still more weakly than does full-length XPG (4). This seemingly antithetical DNA binding with no incision is consistent with FEN superfamly members physically separating their initial DNA binding from positioning for incision (35). An XPGcat dimer, which our SAXS mass measurements suggest exists at low frequency, would create two dsDNA-interacting surfaces to preferentially bind bubble DNA over Y DNA.

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DNA substrate, equivalent to three to four XPG dimers per 300-bp DNA.

Examination of fields of molecules revealed three species. The predominant species 1 was 300-bp DNA bound by protein particles ~10 to 12 nm in diameter, including the metal coating (n = 100 measurements) (Fig. 4C). This size, roughly 8 to 10 nm when corrected for metal coating, is most consistent with the particles being XPG dimers. Species 2 was DNA bound by discrete protein particles that appeared larger, and species 3 was linear protein-free 300-bp bubble DNA molecules (Fig. 4B). We
interpret the smaller protein particles as representing dimers and the larger particles as higher-order XPG multimers. Of the several thousand XPG/DNA complexes visually examined, no XPG bound the DNA ends; rather, all XPG bound the DNA center, where the bubble was located.

XPG/DNA images were collected sequentially at the EM, and every DNA or XPG/DNA complex not in an aggregate was photographed. Images were then analyzed using Gatan digital Image software, and the length of DNA was determined for DNA alone or with XPG (n = 100 each). For measuring DNA within XPG/DNA complexes, the DNA path through the complex was taken to be straight from entry to exit. The lengths of DNA alone centered ~80 to 85 nm, whereas that of the DNA in complexes centered at ~75 to 80 nm, with skewing toward lower values (Fig. 4D). If the DNA had wrapped around the protein complex in a manner similar to that in nucleosomes, we would have expected a greater foreshortening of 10 to 20 nm. Therefore, we conclude that DNA does not wrap around the protein when bound by XPG.

**XPG Bends Bubble DNA Substrate.** We collected images of ~200 examples each of DNA alone or XPG-bound DNA sequentially as they were encountered at the EM. In these incubations, most protein particles were species 1 as contrasted to the larger species 2. Most of the protein-bound DNAs were observed to have a significant bend (Fig. 4C). We determined the DNA bend angle at the center of the XPG/DNA complexes using ImageJ software (Fig. 4E). A 180° angle corresponds to a straight molecule with no bend or kink at the center, while a 0° angle would correspond to a molecule in which the two arms are folded fully back on themselves. Unexpectedly for a bubble DNA, the bending angle to a molecule in which the two arms are folded fully back on themselves. Unexpectedly for a bubble DNA, the bending angle for protein-free DNA fell predominantly within values of 160° to 180° (DNA being bent by 100° from the straight). The bending angle distribution for complexes of XPG with full-length XPG at equimolar concentration, we observed the ss/dsDNA junction in bubble DNA, as observed in the EM analysis.

**XPG Binding to Three-Way Junctions.** XPG plays a key role in maintenance of genome stability by HRR at stalled replication forks (10), raising the possibility that XPG may bind to forks as implied by EMSA on three-way DNA junctions (28). To test this possibility, we used EM (Fig. 4F). The substrate was an ~3-kb dsDNA circle with a displaced 400-bp arm that created a single replication fork (49). The 3° leading-strand terminus was near the fork, and the displaced (lagging) strand had an ssDNA gap of 15 nt. We found that XPG bound with high specificity to the fork, never along the duplex circle or at the end of the displaced arm (Fig. 4G). The ability to bind to substrates with discontinuous dsDNA may underlie XPG-specific functions in multiple DDR pathways in addition to NER.

**Structural Biochemistry of Pathogenic Mutation Sites.** To examine XPG pathogenic mutations in their molecular context, we mapped all known missense mutations onto our structure (nine XP-G, five XP-G/CS, and one mixed/uncertain) (24, 25, 50–59). All were deletions and truncations that cause XP-G/CS map throughout the ERCC5 gene, 14 of 15 missense mutations in both XP-G and XP-G/CS map to the N and I catalytic domains (Fig. 5A). Within the catalytic domain, mutations do not cluster by disease in sequence or location (Fig. 5A and B). Interestingly, none are of a known, conserved catalytic residue. For both XP-G and XP-G/CS mutations, the side chains are generally in well-packed areas with side chains pointing into the protein. All but two (residues 818 and 968) are located within or at the ends of helices (nine residues) or β-strands (three residues) (SI Appendix, Table S4). Similar to many XP and XP/CS mutations in XPD (60, 61), five mutations (of residues 2, 778, 792, 798, and 805) are at N- or C-terminal ends of helices or β-strands that control tertiary organization.

Analysis of our structure reveals that both XP-G and XP-G/CS mutations are positioned to perturb protein stability, but with distinct differences (SI Appendix, Table S4). First, XP-G mutations change residue interactions for sites adjacent in sequence that would cause local disruption. For example, an XP-G mutation might disrupt packing with another in the next helix. In contrast, XP-G/CS mutation sites lie at interfaces that connect sequence-distant regions likely to cause domain impact. Second, XP-G disease mutations substitute larger side chains that would be expected to disrupt structural elements in neighboring regions acting in DNA binding or incision. In contrast, XP-G/CS mutations are within secondary structures key for domain stability. Four of the five either mutate glycine or proline to another residue or vice versa, thereby causing dramatic changes in flexibility or rigidity. The fifth XP-G/CS mutation, W814S, would create a core destabilizing cavity. Two sets of XP-G and XP-G/CS mutations exemplify these features: 1) XP-G mutation A874T and XP-G/CS mutation L858P (Fig. 5C) and 2) XP-G mutation W968C and XP-G/CS mutation W814S (Fig. 5 D and E). Due to the β-branched Thr, XP-G mutation A874T is likely to break local packing in the H2TH motif acting in K° and dsDNA binding. In contrast, the proline in the XP-G/CS mutation L858P is likely to redirect the key turns and globally disrupt the N- and C-terminal interface between residues 9 and 858. Analogously in the second example (Fig. 5 D and E), the XP-G mutation W968C is likely to disrupt a surface-exposed hydrophobic channel and local binding of the 3° ssDNA region. In contrast, the XP-G/CS mutation W814S removes a buried Thr to form a hydrophilic cavity and is likely to disrupt the junction of N- and C-terminal regions (residues 7, 10, and 814). Thus, structure analysis predicts that XP-G mutations are locally disrupting and impact functional regions, whereas XP-G/CS mutations globally disrupt the XPG core domain, thereby impacting...
the full-length protein and consequently likely all of its interactions. To test these observations, we made five XP-G, four XP-G/CS, and one “mixed” missense mutations individually in XPGcat to test catalytic activity and protein stability. The XP-G mutations were A28D, L65P, A792V, A874T, and W968C. The XP-G/CS mutations were P72H, G805R, W814S, and L858P. The mutation L778P was defined as mixed, since it exhibits as XP in homozygous patients, but cells from a phenotypically uncharacterized patient in which L778P is heterozygous with a frameshift mutation in ERCC5 have biochemical properties that are CS-like (SI Appendix, Table S4) (24, 50). All 10 pathogenic mutations reduced recombinant expression of XPGcat to different extents compared to the WT (Fig. 5F, SI Appendix, Fig. S6), consistent with all 10 destabilizing the structure (Fig. 5F and SI Appendix, Fig. S6). We were able to purify a small amount of protein for two XP-G mutants for biochemical analysis. The generally accepted model is that XP-G mutations will directly disrupt the incision activity and that it is this reduction in catalytic activity that leads to defects in NER (45). XP-G mutations in XPGcat did cause decreased incision activity on Y DNA compared to an equal protein amount as WT, but not as dramatically as expected (Fig. 5G) (45). It is quite possible that pathogenic mutations may have a bigger effect in full-length XPG than in XPGcat, due to the folded archaeal FEN arch in place of the unstructured R-domain (SI Appendix, Fig. S1H). However, if both XP-G and XP-G/CS mutations destabilize XPG protein in vivo, we should observe lowered expression of full-length XPG in cells from both patient categories. Reduced expression in XP-G/CS cells has been observed (20), but it has not previously been documented for XP-G.

Indeed, as we saw for XPGcat mutant proteins, we observed greatly reduced XPG expression in two XP-G patient cell lines (XP125LO, XP65BE) compared to a WT cell line VA13 (Fig. 5H and I). This is especially surprising given that both patients were only mildly affected. Three XP-G/CS cell lines (XP124LO, XP72MA, and XP165MA) had more dramatic changes: All lacked detectable XPG protein, with the exception of a potential trace amount in XP165MA. XP40GO, a cell line with no associated patient information but biochemical analysis of which was consistent with XP-G/CS (24), also lacked detectable XPG. We did not have cells for analysis from sibling patients XP174-1 and -2, which carry the same L788P mutation as XP40GO but are homozygous for it and are XP in phenotype (SI Appendix, Table S4) (50). Nonetheless, our results suggest that both XP-G and XP-G/CS mutations disrupt XPG protein stability.

**Discussion**

Multiple specialized structure-specific nucleases act in DDRs and maintenance of genome integrity, and the key to their different activities and functions is in their DNA and protein binding, catalytic specificities, and regulation. Besides its important roles in NER, XPG has key functions in BER (11–13) and HRR (10), and its loss is implicated in accumulation of R-loops (15–17, 62). A molecular understanding of the diverse XPG functions will be greatly facilitated by the combined XPGcat crystal structure and solution measurements (SAXS, EM, and biochemical, mutational, and cell biology analyses) presented here that reveal DNA-specificity elements, dimer assembly, bubble DNA bending, and the location of pathogenic mutations.

**DNA Sculpting and a Unified Basis for XPG DDR Activities.** The reported findings and implications from XPG nuclease...
superfamily DNA complexes support sculpting of dsDNA having unpaired regions (discontinuous dsDNA) as a likely foundational basis for diverse XPG activities (Fig. 6). In NER (Fig. 6, panels 1 and 2), the DNA bubble is the discontinuous dsDNA that XPG binds, bends, validates as a substrate, and then incises, analogous to nucleases such as FEN1 and MRE11 that sculpt DNA for specific incision (36, 63). Notably, XPG’s substrate interaction is quite distinct from the XPC substrate recognition that initiates NER. In NER, XPD holds the lesion-containing ssDNA, while RPA binds the undamaged ssDNA. In BER (Fig. 6, panel 3), where XPG directly stimulates hNTH1 glycosylase activity, disrupted stacking by thymine glycol in the substrate (SI Appendix, Fig. S7) would be transiently recognized and sculpted by XPG to promote glycosylase loading. In this connection it is noteworthy that DNA bending by FEN superfamily members does not require stable binding (35). In HRR (Fig. 6, panel 4), discontinuous DNA could be three- or four-way junctions representing stalled replication forks, chicken foot structures from fork reversal, or Holliday junctions. In these situations, XPG binding may position dsDNA arms for downstream processing, as observed in the promotion of specific repair pathways by the alkylated DNA-binding protein Atl1 in fission yeast (64). Analogous to other FEN superfamily members, XPG activity can be disconnected from DNA binding, thus allowing DNA binding-only functions (4, 28).

**XPG Dimer and DNA Recognition.** For the MRE11 nuclease dimer, a helix-bundle interface similar in size to that in the XPGcat crystal structure was assumed to be only a crystal contact, but it was later found functionally key for DNA binding and for distinguishing two-ended breaks versus one-ended stalled replication forks (65). Our combined results (from coimmunoprecipitation analysis, mutational analysis, EM, and SAXS) establish that XPG without TFIH exists as a stable dimer, which appears suitable for its NER functions. Notably, it is not excluded that during NER, the second XPG dimer subunit could be exchanged with a TFIH subunit in a heteromeric interaction, as seen for TFIH p52 and p8 subunits (66). Distinguishing between these possibilities will require further structural information on the NER preincision complex. Nonetheless, possible participation of the previously unrecognized XPG dimer in NER has implications for bubble recognition that merit consideration.

First, rather than an XPG monomer binding only to the bubble junction 3′ to the lesion where it incises, an XPG dimer could bind both bubble junctions, explaining XPG preference for bubble DNA over γY DNA (4).

Second, XPG could bind the same ss/dsDNA junction as XPF, on opposite sides. FEN superfamily members bind the dsDNA region of their substrate at two distinct parts of the dsDNA helix, leaving the intervening DNA region arching out from the protein surface (29). As our structure implies that XPG binds DNA similarly, XPF-ERCC1 could bind this intervening region, which would contain the proper site for its incision (67) (Fig. 6). Thus, both XPG and XPF-ERCC1 could bind the same dsDNA arm, but at different places. Precedence within the superfamily for interacting on opposites sides of the same DNA comes from FEN1 and DNA ligase (68). Colocalization to the same junction might be a part of XPG licensing of XPF incision.

Third, only one ss/dsDNA junction of the bubble DNA could be incised by an XPG dimer. In our dimer model, one subunit positions the 5′ ssDNA near the active site, while for the other it is the 3′ ssDNA (Fig. 6). In the FEN1 superfamily, the spacing between the DNA binding sites (K′/H2TH and the active site) positions the substrate 5′ ssDNA properly for incision, but not 3′ ssDNA (36). Thus, only one junction of the bubble would be positioned for XPG catalysis.

Fourth, the XPG dimer could help define the distance and ssDNA path between the two bubble junctions, independent of bubble size from 10 to 30 nt. The distance between XPG dimer dsDNA regions is fixed and forces the ssDNA to expand outward in a U-shape during NER bubble expansion. This U-shape DNA conformation was observed in an RPA crystal (69) (Fig. 6).

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**Fig. 6.** Unifying models for XPG function, pathways, and cancer. Hypothesized unifying structure-specific mechanism based on XPG binding to discontinuous dsDNA and breaking base stacking.
Although human RPA/ssDNA did not form this shape on its own in solution (70), an XPG dimer would be suitable to promote this conformation for RPA/DNA.

Imlications from XP-G and XP-G/CS Mutations. Based on observed ERCC5 frameshift mutations (20) and mouse models (8, 21, 22), XP-G/CS mutations are postulated to result in loss of XPG protein, which we have now confirmed. In contrast, XP-G mutations are all missense mutations and have been assumed primarily to cause defective nucleic acid activity with consequent NER loss, UV sensitivity, and cancer predisposition, but no presumed major destabilization of the protein (20). Indeed, a mouse model with incision-defective mutant protein exhibits XP characteristics (22). Surprisingly, our XPGcat biochemistry shows that many XP-G mutations also destabilize the catalytic domain, and a significant reduction in XPG protein was observed in two XP-G patient cell lines. Low XPG protein availability could itself contribute to reduced NER activity in XP-G patient cells in addition to the effect of the mutation on catalysis per se. A related implication is that the residual levels of mutant protein in XP-G patients must still protect against CS. A possible partial explanation may relate to the known up-regulation of XPG in response to replication stress (10), particularly if it is also regulated by transcription stress. However, to what extent the effect of disease mutations on protein stability in XPGcat mirrors the effect of the same mutations on the endogenous human protein remains to be fully determined. In our construct, XP-G mutations generally appear to allow more stable XPGcat protein than do XP-G/CS mutations, but there is overlap between the range of expression levels. If this situation reflects the mutation effects on XPG in humans, it would not be possible to differentiate XP-G and XP-G/CS mutants simply by how much protein exists. Furthermore, given the genetic and environmental diversity in the human population, many other factors are likely involved in determining the disease outcome of particular mutations. It is also possible that heteroallelic effects may contribute to disease outcome. Indeed, the mutation L778P exhibits as XP in homozygous patients, but cells from a patient in which that missense mutation is heterozygous with a truncating frameshift mutation in XPG have biochemical properties that are CS-like (SI Appendix, Table S4) (24, 50).

The structure of a protein is intimately linked to its stability, function, and interactions, and the effect of many human missense variants can be accurately interpreted from an analysis of experimental structures (71). Based on our XPGcat structure, we hypothesize that the residual proteins in missense mutants have different degrees of “intactness.” We predict that XP-G mutants will have limited local disruption of the catalytic domain, while XP-G/CS mutations, located at interfaces between distant sequence regions of the protein or in the β-sheet core, will disrupt much larger portions of the catalytic domain. These disease-related predictions are generally analogous to XP and XP/CS mutations in XPD, in that XP mutations appeared to locally related predictions are generally analogous to XP and XP/CS mutations. From these collective findings, we propose that XPGcat-enforced discontinuous dsDNA binding, unstacking, and bending provide a testable unified mechanism (Fig. 6) whereby XPG, in combination with different protein partnerships, may determine repair function and cell fate in multiple key DDR transactions.

Materials and Methods

See SI Appendix for further materials and methods.

Antibodies. Antibodies used in this study include α-GAPDH (Millipore), α-tubulin (Calbiochem), α-Flag (Sigma), α-XPG R2 #97727 (4) and α-XPGcat.

Protein Constructs and Purification. XPGcat was cloned with residues 79 to 785 replaced with residues 89 to 128 from Pyrococcus furiosus FEN-1 (42) and with a C-terminal truncation after residue 987 and recombiantly expressed in bacteria and purified by Q, SP, Heparin, and SEC. XPGcat and untagged full-length XPG were purified as previously described (4). Full-length XPG with a C-terminal FLAG tag was expressed in High Five insect cells and purified by anti-FLAG M2 resin (Sigma), SP Sepharose, and Superose 6.

Incision Assays. XPG is product-inhibited and protein was assayed using single turnover questions with enzyme excess. The DNA substrates were formed as outlined in SI Appendix, Table S2. For untagged XPG, WT XPG-FLAG, or a mutant version of XPG-FLAG, labeled 15-nt (100 fmol: 3.3 nM) bubble substrates were incubated at 37 °C with XPG protein (33.2 nM) in a buffer (25 mM Tris, pH 6.8, 10% glycerol, 2.5 mM BME, 0.5 mg/mL BSA, 30 mM KCl, and 4 mM MgCl₂) for 15, 30, 60, or 90 min. For XPGcat assay, 10-nM substrates were incubated with 25 nM XPGcat at 16 °C for 15, 30, 45, and 60 s in a buffer containing 25 mM Tris, pH 6.8, 10% glycerol, 2.5 mM BME, 0.5 mg/mL BSA, 0.3 mM EDTA, 30 mM KCl, and 2 mM MgCl₂. Product DNA was separated from substrate DNA by denaturing PAGE. Assays were done in triplicate, on different days.

EMSA for DNA Binding of XPGcat or Mutant Constructs. For EMSA, 5 nM of the 5′-32P-end-labeled bubble or Y-substrates were incubated with protein in a binding buffer containing 10 mM Hepes pH 7.5, 110 mM KCl, 1 mM EDTA, 1 mM DTT, 10 mM CaCl₂, 100 ng of poly dI-dC, and 4% glycerol at room temperature for 15 min. For DNA binding of WT XPG or various mutants, all of the above binding conditions are the same, except 10 mM CaCl₂ was replaced with 0.1% Nonidet P-40. Assays were done in triplicate on different days.

Crysallization and Structure Determination. Data from three crystals of human XPGcat were merged for structure refinement. XPGcat (27 mg/mL) was incubated 1:1 in one of three mother liquors: 1) 40% AmSO₄, 200 mM Imidizole/ Malate Buffer pH 4.2, 100 mM MgCl₂; 2) 24% AmSO₄, 200 mM Imidizole/Malate Buffer pH 4.2, 250 mM MgCl₂ 0.5 mM SmSO₄, 10 mM DTT; 3) 32% AmSO₄, 10 mM DTT, 200 mM Imidizole/Malate Buffer pH 4.2, and 50 mM MgCl₂. The crystal diffraction was highly anisotropic, which led to unusually high rejection of reflections (72).

Biochemical Analysis of Multimerization. XPG-FLAG and XPG-GFP (full-length XPG with either a C-terminal FLAG or GFP tag) were expressed together or separately in High Five insect cells. When we expressed the differently tagged XPG separately, we mixed the cells postexpression. Cells were lysed by Dounce homogenizers. XPG-FLAG was purified from the lysate by anti-FLAG M2 resin (Sigma) and eluted by 3x-FLAG peptide. The eluant from the anti-FLAG resin was sequentially incubated with beads attached to GFP binder nanobodies (73). Beads and eluant were analyzed by PAGE and Western analysis.

SAXS Analysis of XPGcat, XPGΔR, and Full-Length XPG. SAXS data on freshly purified full-length XPG-FLAG, XPGΔC-FLAG, XPGΔR, and XPGcat were collected at beamline 12.3.1 at the Advanced Light Source synchrotron on a MarCCD 165 detector (75) and included Porod exponent analysis for objective quantization of flexibility (76). SAXS data collection and data analysis details are provided in SI Appendix, Table S3.

Preparation of DNA Substrates for EM. The substrate resembling a replication fork was created as previously described (49). The bubble DNA substrate consisted of a 300-bp DNA constructed to have a 10 base unpaired bubble at
Preparation of XPG/DNA Protein Complexes for EM Visualization. XPG purified through SEC was incubated with 50-ng DNA in a buffer consisting of 10 mM Hepes pH 7.5, 110 mM KCl, 1 mM EDTA, and 4% glycerol. For most experiments these reactions contained the DNA at 2.5 μg/mL and XPG at 10 to 15 μg/mL equivalent to a ratio of three to four XPG dimers per 300-bp DNA. An equal volume of 1.2% glutaraldehyde was added for 5 min at room temperature followed by chromatography over a 2-μL column of 5% agarose beads (Agarose Bead Technology Inc Burgos, Spain) equilibrated in 10 mM Tris pH 7.5, 0.1 mM EDTA.

Preparation of Samples for EM Visualization. Samples were prepared for EM analysis as previously described (74). Bending angles were measured on the micrographs using ImageJ software and lengths measured with Gatan Digital Image software.

2AP Experiments. For 2AP experiments, 6 μM 20-nt bubble DNA with 2AP at the 3′-junction was incubated alone, with 6-μM untagged XPG protein, or protein and 10 mM CaCl2. The oligonucleotide containing 2AP was used for the ssDNA control. The experiment was done in triplicate.

Cell Extract Preparation and Western Blotting Method. Supernatants from immortalized WT VA13 or various XP-G or XP-G/C lines were analyzed by Western blot with rabbit anti-XPG 97727 antibody (R2) (4) and anti-GAPDH antibody (Millipore, MAB374) or anti-Actin (Calbiochem, Ab-1) to obtain loading controls. Westerns were done twice.

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