ARTICLES

Identification of Holliday junction resolvases from humans and yeast

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Four-way DNA intermediates, also known as Holliday junctions, are formed during homologous recombination and DNA repair, and their resolution is necessary for proper chromosome segregation. Here we identify nucleases from *Saccharomyces cerevisiae* and human cells that promote Holliday junction resolution, in a manner analogous to that shown by the *Escherichia coli* Holliday junction resolvase RuvC. The human Holliday junction resolvase, GEN1, and its yeast orthologue, Yen1, were independently identified using two distinct experimental approaches: GEN1 was identified by mass spectrometry following extensive fractionation of HeLa cell-free extracts, whereas Yen1 was detected by screening a yeast gene fusion library for nucleases capable of Holliday junction resolution. The eukaryotic Holliday junction resolvases represent a new subclass of the Rad2/XPG family of nucleases. Recombinant GEN1 and Yen1 resolve Holliday junctions by the introduction of symmetrically related cuts across the junction point, to produce nicked duplex products in which the nicks can be readily ligated.

In 1964, Robin Holliday proposed the formation of four-way DNA junctions to account for the types of products that are formed during meiotic recombination in fungi[1]. Since that time, the Holliday junction has been invoked as a central intermediate in many models for recombination and the recombinational repair of DNA double-stranded breaks[2]. Because they provide a covalent linkage between chromosomes, their resolution is necessary for chromosome segregation. Enzymes that resolve Holliday junctions by endonucleolytic cleavage have been isolated from bacteriophages T4 and T7, bacteria and archaea[3]. These proteins are generally small homodimeric nucleases that show both structural and sequence specificities. Resolution occurs by the introduction of symmetrically related nicks in two strands of like polarity, such that the nicked duplex DNA products can be ligated without need for further processing.

The *E. coli* Holliday junction resolvase, RuvC protein, represents the model for the involvement of this class of enzyme in recombination and repair[4]. So far, little is known about eukaryotic Holliday junction resolvases. Holliday junction resolvases have been identified from *S. cerevisiae* (Cce1) and *Schizosaccharomyces pombe* (Ydc2), but these are active only in mitochondria[5,6]. Mammalian cell-free extracts contain a nuclear Holliday junction resolvase activity (designated ResA)[7–11], but its identity remains unknown despite almost two decades of study. This is due to its low abundance, and the fact that mammalian cells (and all eukaryotes) possess alternative means to process Holliday junctions. For example, Holliday junctions can be 'dissolved' by the Bloom's syndrome complex (BLM helicase–topoisomerase IIIα–RMI1/2)[12], or be cleaved asymmetrically by MUS81–EME1 (asymmetric cleavage produces flapped and gapped duplex products that cannot be ligated without further processing)[10,13–15].

Here, we used a two-pronged approach to identify eukaryotic resolvases that fit the classical Holliday junction resolvase model. First, large quantities of cell-free extracts were prepared from human cells and extensively fractionated to enable the identification of ResA by mass spectrometry. Second, a tandem affinity purification (TAP) fusion library of *S. cerevisiae* genes was screened for proteins that promote Holliday junction resolution. Notably, these parallel approaches led us to identify the same protein: *Homo sapiens* GEN1 and its *S. cerevisiae* orthologue Yen1. We show that GEN1 and Yen1 are highly specific Holliday junction resolvases as defined by the RuvC model.

Identification of the human Holliday junction resolvase

Nuclear extracts prepared from 200 litres of HeLa cells (at 1×10^6 cells ml^{-1}) were fractionated and assayed for Holliday junction resolution activity using the small 5′-^{32}P-end-labelled synthetic Holliday junction X26, which has a 26-base pair (bp) homologous core through which the junction can migrate. The products of resolution, ^{32}P-labelled nicked duplex DNA, were visualized by neutral polyacrylamide gel electrophoresis (PAGE) and autoradiography. The purification scheme used a combination of six column matrices that separate proteins according to their size, charge and specific chemical affinities, and was designed to separate ResA away from the MUS81–EME1 complex (Supplementary Fig. 1). Robust Holliday junction resolution activity was present in the peak fractions that eluted from the final monoS column (Fig. 1a, fractions 28–33), but SDS–PAGE analysis showed that these fractions remained quite impure, despite extensive fractionation (Fig. 1b). Indeed, mass spectrometry (MS) revealed that peak fraction 29 contained 75 distinct proteins.

To identify the nuclease responsible for resolution, the proteins in fraction 29 were separated by SDS–PAGE, and the gel was cut into small slices (Fig. 1c, slices a–l). We found that Holliday junction resolution activity could be renatured from a single gel slice (slice e), in which proteins had an expected mass of approximately 60 kDa. MS analyses revealed the identity of 20 proteins in gel slice e, but this was reduced to 12 by subtraction of those also present in other gel slices that lacked resolution activity. One of the remaining candidate proteins, identified by the presence of five peptides by MS analyses, caught our interest as it corresponded to an amino-terminal fragment of an uncharacterized 103 kDa protein (FLJ40869) that

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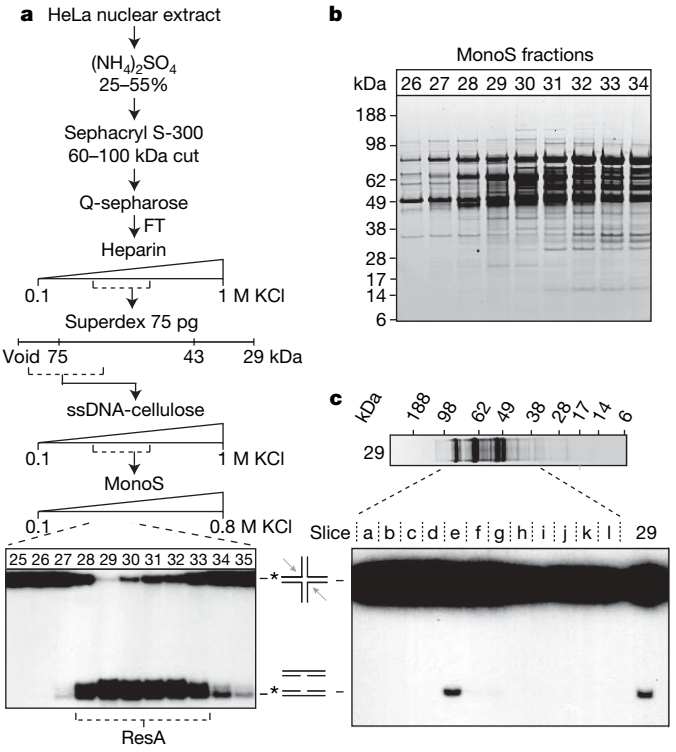

Figure 1 | Identification of human Holliday junction resolvase activity. **a**, Purification scheme for the Holliday junction resolvase ResA. Symmetrical cleavage of ^{32}P-labelled Holliday junction X26 (arrows) gives rise to nicked duplex products. Peak fractions 25–35 of the final monoS column are shown. Asterisks indicate ^{32}P-labels. FT, flow through. **b**, SDS–PAGE of fractions 26–34 from monoS, stained with SYPRO Ruby. **c**, Fraction 29 from **b** was cut into slices (a–l), and proteins were renatured and analysed for Holliday junction resolution using ^{32}P-labelled Holliday junction X0. Control lane (right): fraction 29 diluted 50-fold.

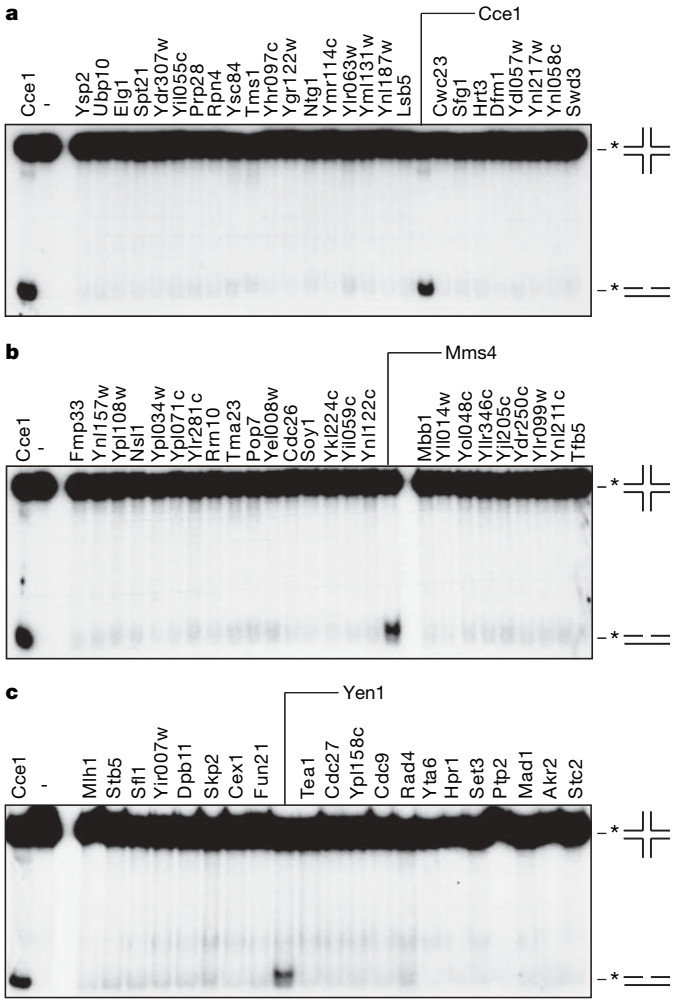

Figure 2 | Yeast screen for Holliday junction resolution activities. **a**–**c**, Lysates were prepared from 1,100 yeast cultures containing *S. cerevisiae* TAP fusion proteins. The TAP fusion proteins were immunoprecipitated using IgG sepharose and analysed for their ability to promote the resolution of ^{32}P-labelled Holliday junction X26. Three representative panels of the high-throughput screen are shown. Each lane represents one candidate protein, indicated by protein name or open reading frame code.

contains a Rad2/XPG nuclease domain (Supplementary Fig. 2). This human protein was identified as GEN1, which is encoded by a gene at chromosome location 2p24.2.

Identification of the yeast Holliday junction resolvase

In parallel with the above approach, a *S. cerevisiae* TAP fusion library[16] was screened for Holliday junction resolution activities. The library contains 4,247 open reading frames tagged and expressed from their genomic locus. Strains harbouring TAP fusion proteins were individually grown in 100 ml cultures and TAP fusion proteins were immunoprecipitated from their lysates and tested for resolution using ^{32}P-labelled Holliday junction X26. The screen was biased towards the low expression categories (50–1,500 molecules per cell), based on our prediction that a Holliday junction resolvase would not be an abundant protein. We also excluded proteins that have well established and unrelated functions (for example, membrane proteins). In total we screened 1,100 strains.

Representative results are shown in Fig. 2. The screening procedure was optimized with a yeast strain expressing TAP-tagged Cce1 (present at approximately 50 molecules per cell), which was used as an internal control in all analyses. Cce1 was the first TAP fusion strain in the library (strain 76) to give a positive result in the Holliday junction resolution assay (Fig. 2a), which generally gave clear negatives for almost all other TAP fusions. We next identified a Holliday junction processing activity associated with Mms4 (strain 152; Mms4 is known as Eme1 in *S. pombe* and EME1 in mammalian cells and is a component of the Mus81–Mms4 heterodimeric nuclease) (Fig. 2b), Mus81 itself (strain 520) and Slx4 (strain 582), which is known to form a complex with Slx1 (data not shown). Although Slx1–Slx4, a flap nuclease, has detectable activity with Holliday junctions (like Mus81 the cuts are asymmetric) they are not thought to represent its physiological substrate[17].

We also isolated novel candidate Holliday junction resolvases, of which a protein known as Yen1 (strain 570) looked the most promising (Fig. 2c). Yen1 is encoded by open reading frame YER041W, and its function is unknown (*yen1* mutants are viable). During further study, Yen1–TAP displayed consistent Holliday junction cleavage activity at levels comparable to Cce1 and Mus81–Mms4 (Supplementary Fig. 3). Further isolates included Cef1, Mgs1, Apn2, Rad16 and Slx8, but subsequent tests failed to confirm these proteins as capable of Holliday junction resolution (Supplementary Fig. 3). This left us with Yen1 as the only viable candidate for a novel Holliday junction resolution activity. Notably, Yen1 is the *S. cerevisiae* orthologue of human GEN1 (Supplementary Fig. 4), the Holliday junction resolution activity identified from HeLa cells. Thus, two completely different approaches to identify Holliday junction resolvases led us to the same protein. We therefore focused our efforts on Yen1 and GEN1.

Holliday junction cleavage by Yen1 and GEN1

Yen1 and GEN1 are members of the Rad2/XPG family of structure-specific nucleases that are characterized by the presence of three distinct sequence motifs: the N-terminal (N) and internal (I) XPG

nuclease domains, together with a helix–hairpin–helix domain (Supplementary Fig. 5)[18]. The Rad2/XPG family contains several well-characterized nucleases, but in contrast to Yen1–TAP, we did not observe specific Holliday junction resolution by other family members such as Rad2–TAP, Rad27–TAP or Exo1–TAP (Supplementary Fig. 6). These results suggest that Yen1 and GEN1 may represent a subclass of the Rad2/XPG family that has evolved to resolve Holliday junctions.

To determine whether Holliday junction cleavage occurred by symmetric (RuvC-like resolution) or asymmetric (like Mus81–Eme1) cutting, we prepared vectors that expressed recombinant forms of full-length Yen1 and GEN1. First, *YEN1* was placed under the control of the *GAL1* promoter and Yen1–Flag was overexpressed in yeast strain W303. When cell-free extracts were assayed for Holliday junction resolution activity using a Holliday junction with a fixed branch point (Holliday junction X0), we found that Yen1 expression resulted in efficient Holliday junction resolution (Fig. 3a, lane 3). In contrast, a mutant form of Yen1, created by mutating two conserved glutamic acid residues (residues 193 and 195) to alanine (Supplementary Fig. 4), or extracts prepared from cells carrying the empty vector, failed to resolve X0 (Fig. 3a, lanes 2 and 4). Analogous mutations abolish[19] or severely reduce[20] the nuclease activities of other Rad2/XPG family proteins. Second, human *GEN1* was transiently transfected into human 293T cells, and GEN1–Flag protein was partially purified by Flag affinity chromatography. We found that GEN1–Flag also resolved Holliday junction X0 efficiently (Fig. 3b, lane 3).

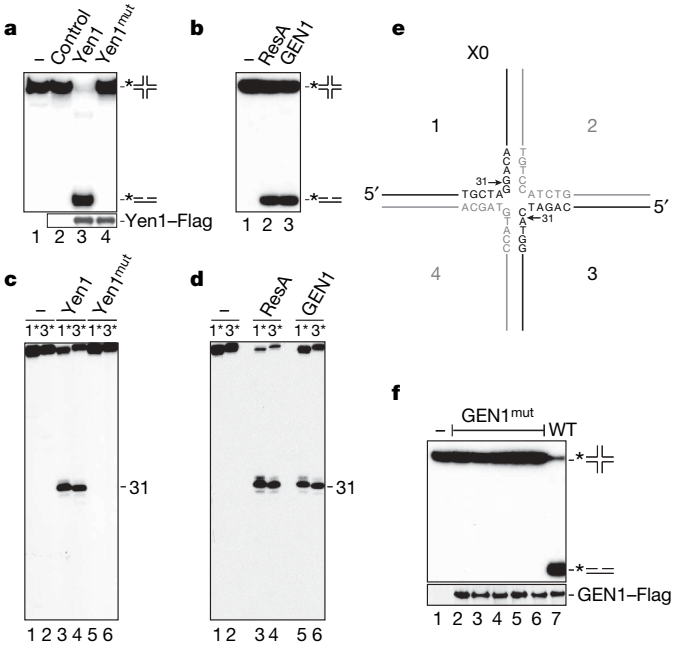

Figure 3 | Resolution of Holliday junctions by recombinant Yen1 and GEN1. **a**, ^{32}P-labelled Holliday junction X0 was incubated with cell-free extracts from yeast overexpressing Flag-tagged Yen1, or a catalytically inactive Yen1(E193A/E195A) mutant (Yen1^{mut}), and the products were analysed by neutral PAGE. Control: extracts from cells transformed with empty expression vector. Yen1–Flag and Yen1^{mut}–Flag were detected by western blotting using anti-Flag antibody. **b**, As **a**, but affinity purified GEN1–Flag, or ResA, was used. **c**, **d**, Holliday junction X0, 5′-^{32}P-end-labelled in strand 1 or 3, was treated as in **a** and **b**, and products were analysed by denaturing PAGE. Asterisks indicate the strand with the radioactive label. **e**, The cleavage sites. **f**, Wild-type and mutant derivatives of GEN1–Flag were assayed using Holliday junction X0. Lane 1, control lane; lane 2, GEN1(D30A); lane 3, GEN1(E134A); lane 4, GEN1(E136A); lane 5, GEN1(D157A); lane 6, GEN1(E134A/E136A); lane 7, wild-type GEN1. In the bottom panel, GEN1–Flag proteins were detected by western blotting.

Using Holliday junction X0, 5′-^{32}P-end-labelled in either strand 1 or 3, we observed that both Yen1 and GEN1 cut the immobile junction X0 at a unique site located one nucleotide to the 3′ side of the junction point. The nicks were introduced with perfect symmetry to produce fragments that were 31 nucleotides in length as determined by denaturing PAGE (Fig. 3c, lanes 3 and 4, and Fig. 3d, lanes 5 and 6). The cleavage pattern is summarized in Fig. 3e. Recombinant GEN1–Flag produced identical cuts to those produced by ResA purified from HeLa cells (Fig. 3d, compare lanes 3 and 4 with 5 and 6).

Yen1 and GEN1 also produced perfectly symmetric cleavage patterns within the region of homology in Holliday junction X26, with cleavage occurring at two preferred sites within the 26-bp homologous core (Supplementary Figs 7 and 8). Thus, like RuvC, the eukaryotic resolvases show some sequence preferences. The cleavage pattern produced by GEN1 was again identical to that produced by ResA (Supplementary Fig. 8), confirming that GEN1 is equivalent to ResA from HeLa cells. Importantly, the introduction of site-directed mutations in the nuclease domains of GEN1–Flag resulted in a complete loss of Holliday junction resolution activity (Fig. 3f).

Symmetrical cleavage of Holliday junctions by RuvC results in the formation of nicked duplex products in which the nicks can be ligated without need for further processing. To determine whether the eukaryotic Holliday junction resolvase gives rise to ligatable products, we constructed a derivative of the migratable Holliday junction X26 in which the arms were of different length (the junction was 5′-^{32}P-end-labelled in the 53-nucleotide-long strand 2S) such that nick ligation could be assayed by denaturing PAGE as the formation of a 60-nucleotide-long ^{32}P-labelled strand (shown schematically in Fig. 4a). When resolved by either RuvC or GEN1–Flag (Fig. 4b, lanes 3 and 5), and treated with T4 DNA ligase, equivalent levels of nick religation were observed (lanes 4 and 6). Thus, both RuvC and GEN1 leave ligatable nicks after symmetrical cleavage.

Substrate specificity

Given that Rad2/XPG family members are structure-specific nucleases, we next determined the specificity of GEN1–Flag. For this, the protein was incubated with equimolar amounts of a variety of substrates including splayed-arm DNA, 3′- and 5′-flap DNAs, and model replication fork DNA. GEN1–Flag showed a strong preference for the Holliday junction (Fig. 4c). Time course experiments indicated that the initial rate of Holliday junction cleavage was 7- and 20-fold greater than that observed with 5′-flap or replication fork substrates, respectively (Fig. 4d). GEN1 failed to cut splayed-arm or 3′-flap substrates.

To determine whether GEN1 alone was capable of Holliday junction resolution, or whether cleavage required the presence of other co-purifying factors, we took advantage of the fact that the N-terminal region of GEN1 contains the Rad2/XPG nuclease domains and purified GEN1(1–527) to near homogeneity from *E. coli* (Fig. 4e). Recombinant GEN1(1–527) was an active Holliday junction resolvase (Fig. 4f), and showed the same structure specificity as affinity-purified human GEN1–Flag protein. This specificity for Holliday junctions, coupled with the observed symmetrical cleavage mechanism, shows that Yen1 and GEN1 are bona fide Holliday junction resolvases similar to RuvC.

Discussion

When the *E. coli* Holliday junction resolvase, RuvC protein, was discovered in 1991 (refs 21, 22), and analogous activities were detected in mammalian cell-free extracts[7], it seemed that the mechanisms of Holliday junction resolution were conserved throughout all kingdoms of life. However, this concept was thrown into question by the description of alternative ways to process Holliday junctions involving either topoisomerase-mediated junction dissolution[12], or asymmetric cleavage by MUS81–EME1 flap endonucleases[13,23]. The identification of the novel eukaryotic GEN1/Yen1 Holliday junction resolvases described here, and analysis of their mechanism of resolution, suggests that the RuvC model is indeed universal, while also

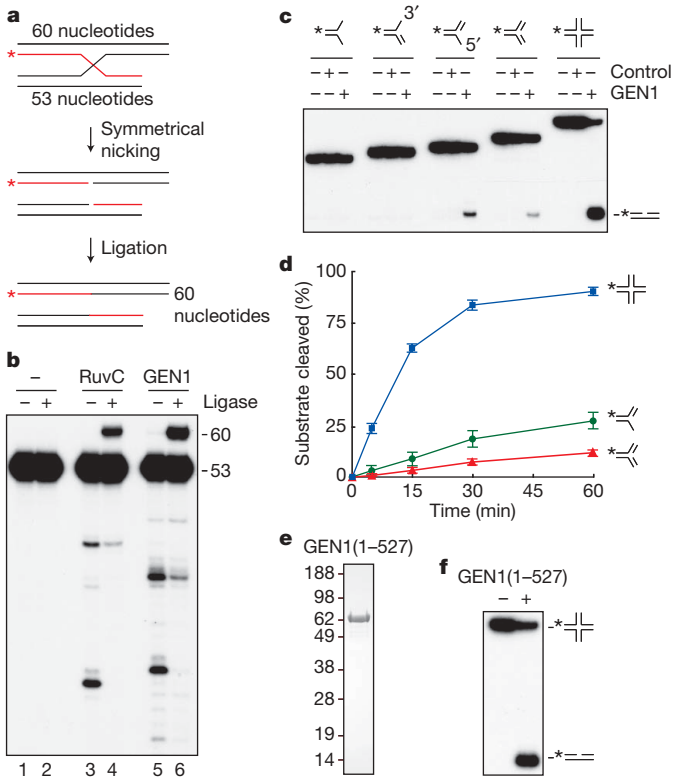

Figure 4 | Specificity of GEN1 Holliday junction resolvase. a, Assay for nick ligation. Holliday junction X26-S, ^{32}P-end-labelled in strand 2S (red: 53-nucleotides long), was treated with GEN1–Flag (or RuvC) and the resolution products were incubated with T4 DNA ligase. Symmetric cleavage followed by ligation produces a 60-nucleotide-long ^{32}P-labelled strand. **b**, Ligation of resolution products generated by either RuvC or GEN1–Flag, determined by denaturing PAGE. **c**, Substrate specificity of GEN1–Flag. The indicated substrates (from left to right: splayed arm, 3′-flap, 5′-flap, replication fork, and Holliday junction), ^{32}P-end-labelled on a common strand, were incubated with GEN1–Flag or a mock protein control. Products were analysed by neutral PAGE. **d**, Time course of reactions similar to those of **c**. Product formation was quantified by phosphorimaging and expressed as a percentage of total DNA (±s.d., $n = 3$). **e**, SDS–PAGE gel showing Coomassie-blue-stained His-tagged GEN1(1–527) (11 μg) purified from *E. coli*. **f**, Holliday junction resolution by His-tagged GEN1(1–527) (60 nM).

highlighting the diversification of Holliday junction processing mechanisms in eukaryotes.

GEN1 and Yen1 promote Holliday junction resolution by a symmetrical cleavage mechanism analogous to that shown by RuvC, Cce1, Hjc and the bacteriophage resolvases. However, they do not show any sequence or structure conservation with these proteins, but instead belong to the Rad2/XPG family of nucleases[18]. The presence of the eukaryotic Holliday junction resolvases in the Rad2/XPG nuclease family emphasizes the wide-ranging functions of this group of proteins. The phylogenetic tree (Supplementary Fig. 9) shows the family divided into four subclasses, consistent with their cellular functions: class I, characterized by XPG (*H. sapiens*) and Rad2 (*S. cerevisiae*), promote endonucleolytic incision at the 3′ side of bubble structures formed during nucleotide excision repair[24]; class II, characterized by FEN1 (*H. sapiens*) and Rad27 (*S. cerevisiae*), use their 5′-flap endonuclease activities for Okazaki fragment processing during DNA replication and also act in a variety of specialized DNA repair reactions that require the trimming of 5′ flaps[25]; class III comprise the EXO1-related proteins, which exhibit 5′–3′ exonuclease and weak 5′-flap endonuclease activities, that are required for DNA replication, DNA repair and meiotic recombination[26]; and the results described here indicate that class IV comprise the eukaryotic Holliday junction resolvases.

The yeast *YEN1* and human *GEN1* genes encode proteins of 87 kDa and 103 kDa, respectively. However, our studies show that a 60 kDa N-terminal fragment of GEN1 (GEN1(1–527)) purified from *E. coli* promotes efficient Holliday junction resolution, as does the truncated form of GEN1 purified from HeLa extracts. It is therefore unclear whether the protein is functionally active as a full-length protein. One possibility is that the resolvase is activated by proteolytic cleavage or by interaction with other factors. In this regard, it is interesting that extracts prepared from cell lines defective in the RAD51C or XRCC3 recombination/repair proteins show reduced levels of Holliday junction resolution activity[11]. However, although RAD51C and XRCC3 have been reported to associate with ResA, we did not detect the presence of these proteins in the most purified ResA/GEN1 fraction from HeLa cells, or in the affinity-purified GEN1–Flag fractions (Supplementary Fig. 10). These data indicate that no strong physical link exists between RAD51C–XRCC3 and GEN1, and that these proteins are not directly required for Holliday junction resolution by purified GEN1. Further work will be required to define the relationships between RAD51C–XRCC3 and GEN1.

Eukaryotic cells process recombination intermediates in several different ways, and the outcome is likely to be dependent on pathway choice (Supplementary Fig. 11). In mammalian cells Holliday junction intermediates may be dissolved by BLM–topoisomerase IIIα–RMI1/2 complex, to produce non-crossovers (in which there is no exchange of flanking markers), or be cleaved by MUS81–EME1 complex to produce crossovers with an exchange of flanking markers. The efficient Holliday junction resolution activities described here are indicative of a third pathway, Holliday junction resolution by Yen1/GEN1, which by analogy with other resolvases would be expected to give rise to crossovers and non-crossovers with equal efficiency, depending on the orientation of Holliday junction cleavage.

S. pombe mus81 and *eme1* mutants have pronounced meiotic recombination defects consistent with defects in the processing of recombination intermediates[23,27]. Indeed, Mus81–Eme1 seems to provide the primary means to generate crossovers in this organism, although its actions may not be restricted to Holliday junctions[28]. In contrast, *S. cerevisiae mus81* and *mms4* mutants have only mild defects in crossover formation[29], and *Mus81*$^{-/-}$ mice fail to show any obvious meiotic defects[30,31]. The heightened dependence that *S. pombe* shows for Mus81–Eme1 may be due to the fact that *YEN1* is absent from the *S. pombe* genome, whereas it is present in *S. cerevisiae* and other eukaryotes. Consistent with the concept of alternative mechanisms by which joint molecules can be resolved, it was recently shown that crossover and non-crossover products still form at high levels in *S. cerevisiae sgs1* (an orthologue of BLM) *mms4* double mutants[32,33]. Preliminary studies in *S. cerevisiae* indicate that *yen1 mms4* double mutants fail to complete meiosis and contain unresolved interhomologue joint molecules (L. Jessop, E. Kolar, J. Haber and M. Lichten, personal communication), suggestive of functional redundancy between Mus81–Mms4 and Yen1.

Previously, it was shown that weak ssDNA and 5′-flap/fork endonuclease activities were associated with GEN1 orthologues from rice[34,35] and insects[36,37], and that RNA-interference-mediated gene silencing of rice *GEN1* resulted in male sterility[35]. Additionally, the messenger RNA levels of a rice GEN1 orthologue were found to be induced by DNA-damaging agents such as ultraviolet light, methyl methanesulphonate or H_2O_2, consistent with a role in DNA repair[34]. Genome-wide sequencing of all coding regions in tumours derived from a cohort of breast cancer patients revealed deletions/frameshift mutations in *GEN1* (FLJ40869) in two individuals[38]. As the mutations found within *GEN1* have a high probability of being pathogenic, rather than passenger mutations, *GEN1* was classified as a cancer gene. These mutations were 785delC and 824–827_delGTAA, which would give rise to GEN1 truncations without Holliday junction resolution activity. Thus, *GEN1* joins the growing list of genes involved in recombinational repair, such as *BRCA1*,

BRCA2, *FANCJ* (also called *BACH1*) and *FANCN* (*PALB2*), that are linked with tumorigenesis.

METHODS SUMMARY

DNA substrates, 5′-^{32}P-end-labelled on oligonucleotides X26-2 or X0-1, were made by annealing oligonucleotides (Supplementary Table 1) and purified by PAGE[39]. Yeast TAP fusion library[16] was purchased from Open Biosystems, and screened as described[39]. Yen1–Flag was expressed in yeast and soluble lysates were analysed for Holliday junction resolution activity. Human GEN1–Flag was purchased from Origene as a pCMV-Entry expression vector, transfected into 293T cells, and affinity purified using anti-Flag M2 affinity gel (Sigma). A mock protein control was made from untransfected 293T cells using the same procedure. Mutant derivatives of GEN1–Flag were made by site-directed mutagenesis. Carboxy-terminal His-tagged GEN1(1–527) was purified after overexpression in *E. coli*, using HisTrap, heparin, ssDNA and monoS columns. Holliday junction resolution and substrate specificity assays were performed under similar conditions. For the ligation experiment, cleavage reactions were supplemented with 400 U T4 DNA ligase (NEB) and incubation continued at room temperature for 1 h. ^{32}P-labelled products were analysed by 10% neutral PAGE, or by electrophoresis through a 12% denaturing gel containing 7 M urea.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Identification of human Holliday junction resolvase. HeLa S3 cells (200 l at 1×10^6 cells ml^{-1}) were grown[10], harvested and washed three times with iced PBS. The cell pellet was re-suspended in 2 vol hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT)), left on ice for 1 h and lysed using an 'A' pestle (25 strokes) in the presence of complete EDTA-free protease inhibitor cocktail (Roche). After centrifugation (1 h at 2,500*g*), the nuclear pellet was resuspended in 2 vol high-salt extraction buffer (10 mM Tris-HCl pH 8.0, 300 mM KCl, 1 mM EDTA, 2 mM DTT) and incubated on ice for 1 h before being centrifuged again (1 h at 35,000 r.p.m., Beckman 45Ti rotor). Solid $(NH_4)_2SO_4$ (25%; 134 g l^{-1}) was added to the supernatant and dissolved by stirring on ice. After 30 min insoluble material was removed by centrifugation (30 min at 12,000*g*). The $(NH_4)_2SO_4$ concentration was raised to 55% (an additional 179 g l^{-1}), and precipitated proteins were recovered by centrifugation and re-suspended in 32 ml buffer A (50 mM K_2HPO_4/KH_2PO_4 pH 6.8, 10% glycerol, 1 mM EDTA, 1 mM DTT and 0.01% NP40) containing 250 mM KCl. After dialysis against the same buffer, proteins were loaded onto a 320 ml sephacryl S-300 column in four separate runs. Fractions (112 ml) with resolution activity were pooled, dialysed against buffer B (50 mM K_2HPO_4/KH_2PO_4 pH 7.4, 10% glycerol, 1 mM EDTA, 1 mM DTT and 0.01% NP40) containing 100 mM KCl flowed through a 1 ml HiTrap Q sepharose (GE healthcare) and loaded onto a 5 ml HiTrap heparin column (GE healthcare). After washing, proteins were eluted with a 75 ml gradient to 1 M KCl. Resolvase fractions were loaded onto a 120 ml superdex 75 column, pre-equilibrated with buffer A plus 250 mM KCl, eluted and dialysed against buffer B plus 100 mM KCl before loading onto a 300 µl ssDNA cellulose column (Amersham). The column was eluted with a 4.5 ml gradient to 1 M KCl, and fractions were dialysed against buffer B plus 100 mM KCl before loading onto a monoS PC 1.6/5 column (GE healthcare). Resolution activity was eluted in a 1 ml gradient to 800 mM KCl. Assays were carried out using Holliday junction X26 in the presence of nonspecific competitor DNA (10 ng μl^{-1} poly(dI-dC)).

An aliquot of the peak ResA fraction was heated for 10 min at 72 °C in NuPAGE LDS sample buffer and run on a Novex 4–12% Bis-Tris PAGE gel in MES SDS running buffer (Invitrogen). The gel was washed twice in 20 ml elution-renaturation buffer (60 mM K_2HPO_4/KH_2PO_4 pH 7.4, 1 mM DTT, 100 µg ml^{-1} BSA, 50 mM KCl, 1% Triton X-100), and cut into small slices. Each slice was homogenized in 3 vol of elution-renaturation buffer and incubated at 4 °C for 5 h with gentle mixing. Supernatants containing eluted proteins were analysed for Holliday junction resolution activity by overnight incubation at 37 °C with ^{32}P-labelled Holliday junction X0 (~1 nM) and 5 mM $Mg(OAc)_2$.

Peptides for analysis were generated by *in situ* tryptic digestion of protein/gel bands. LC-ESI/MS/MS analysis of the peptides was carried out on a SYNAPT HDMS mass spectrometer (Waters) and the data searched against a concatenated, non-redundant protein database (UniProt 13.6), using the Mascot search engine (Matrix Science).

Resolution assays. Holliday junction resolution reactions (10 µl) contained ^{32}P-labelled DNA substrates (~1 nM) and 0.5 µl aliquots of the indicated mammalian fractions in phosphate buffer (60 mM Na_2HPO_4/NaH_2PO_4 pH 7.4, 1 mM DTT, 100 µg ml^{-1} BSA, 2 mM ATP) supplemented with 5 mM $Mg(OAc)_2$. For Yen1, reactions contained ^{32}P-labelled DNA (~1 nM) and protein extract (5 µg) in 40 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$ and 10 mM NaCl. Reactions with RuvC (100 nM), purified as described[40], were carried out in phosphate buffer supplemented with 15 mM $Mg(OAc)_2$. Incubation was for 30 min at 37 °C (ResA/GEN1) or 30 °C (Yen1), and the DNA products were deproteinized for 15 min at 37 °C using 2 mg ml^{-1} proteinase K and 0.4% SDS.

Preparation of Yen1−Flag. The *YEN1* gene was amplified from W303 yeast genomic DNA, a C-terminal Flag-tag was added, and cloned into pYES-DEST52 containing a *GAL1* promoter for overexpression in yeast. The coding sequence of *YEN1* differed from the *S. cerevisiae* database with a C175G change that resulted in alanine at amino acid 59. This sequence was found in two independent clones of W303 and also in strain BY4741, and is displayed in the NCBI website (Locus P40028). Yen1–Flag was overexpressed in pYEN1–Flag transformed W303 cells, and after 10 h soluble extracts were made by mechanical disruption followed by centrifugation.

Purification of GEN1(1–527). The GEN1(1–527) coding sequence was transferred to the Gateway destination vector pET-DEST42 for overexpression in *E. coli* BL21(DE3)-RIL (Stratagene). GEN1(1–527)-His expression was induced using 100 µM IPTG, and after 7 h cells were harvested and lysed. GEN1(1–527) was purified using a HisTrap FF Crude column, a HiTrap heparin column, a ssDNA cellulose column and finally a monoS PC 1.6/5 column (all GE healthcare).

Sequence analysis. Domains present in the Rad2/XPG family members were compared using the SMART database[41]. Alignments were carried out using JAligner (http://jaligner.sourceforge.net), using a BLOSUM90 matrix, a gap open value of 10 and a gap extension value of 0.5. The phylogenetic tree was constructed using one or two representatives from each of the classes of the Rad2/XPG family in *Arabidopsis thaliana*, *S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *H. sapiens* using sequences from the NCBI database (http://www.ncbi.nlm.nih.gov) or WormBase (http://www.wormbase.org). Accession numbers of the sequences are: *S. cerevisiae*: NP_011774 (Rad2), NP_012809 (Rad27), NP_014676 (Exo1), NP_010549 (Din7), NP_010959 (Yen1); *A. thaliana*: NP_566830 (XPG), NP_850877 (FEN1), Q8L6Z7 (EXO1), Q9LPD2 (GEN1); *C. elegans*: NP_491891 (XPG), WP:CE28239 (homologous to FEN1), WP:CE28728 (homologous to EXO1), WP:CE01401 (homologous to GEN1); *D. melanogaster*: AAD50779 (XPG), NP_523765 (FEN1), Q24558 (EXO1/tosca), NP_647943 (GEN1); *H. sapiens*: NP_000114 (XPG), NP_004102 (FEN1), NP_003677 (EXO1a), NP_872431 (GEN1). Sequences were aligned and analysed with ClustalX 2.0.9 (ref. 42) using default parameters. A neighbour-joining tree was generated and bootstrapped by 1,000 replications. The tree was visualized with Treeview[43].

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