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Science 333, 84 (2011);
DOI: 10.1126/science.1204258

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Mechanism of RAD51-Dependent DNA Interstrand Cross-Link Repair

David T. Long, Markus Räschle, Vladimir Joukov, Johannes C. Walter

DNA interstrand cross-links (ICLs) are toxic DNA lesions whose repair in S phase of eukaryotic cells is incompletely understood. In *Xenopus* egg extracts, ICL repair is initiated when two replication forks converge on the lesion. Dual incisions then create a DNA double-strand break (DSB) in one sister chromatid, whereas lesion bypass restores the other sister. We report that the broken sister chromatid is repaired via RAD51-dependent strand invasion into the regenerated sister. Recombination activates RAD51-dependent strand invasion into the ICL (Fig. 1B, i). The leading strand of one fork is then extended to within one nucleotide of the ICL (Fig. 1B, ii). Next, dual incisions surrounding the ICL create a DNA double-strand break (DSB) in one sister chromatid, and translesion DNA synthesis restores the other sister by first inserting a nucleotide across from the adjoined base (Fig. 1B, iii), followed by strand extension beyond the ICL (Fig. 1B, iv). Ultimately, 5 to 25% of replicated pICL is fully repaired, as measured by regeneration of a SapI site that was originally interrupted by the cross-link (2, 10). In the absence of ubiquitylated FANCD2, DNA incisions, lesion bypass, and SapI site regeneration are greatly diminished (2). Given the established links between HR and ICL repair and the inefficient removal of the unhooked ICL in egg extracts (10), we postulated that SapI site regeneration is coupled to DNA replication via homologous recombination in vitro.

In vertebrate cells, DNA interstrand cross-link (ICL) repair is coupled to DNA replication and involves structure-specific endonucleases, translesion DNA polymerases, recombinases, and numerous proteins mutated in the human disease Fanconi anemia (FA) (1). FA is characterized by genomic instability and cellular sensitivity to DNA interstrand cross-linking agents. A central event in the FA pathway is the ubiquitylation of the FANCI-FANCD2 heterodimer, which activates it for ICL repair (2, 3). Extensive evidence indicates that homologous recombination (HR) is essential for ICL repair (4–9). However, the precise role of HR in ICL repair remains conjectural, and the functional connection between the FA and HR pathways is unclear.

Using *Xenopus* egg extracts, we established a cell-free system for replication-dependent repair of a plasmid containing a single, site-specific cis-platin ICL (pICL) (Fig. 1A) (2, 10). Upon addition of pICL to egg extracts, replication initiates at a random site, and two replication forks converge on the ICL (Fig. 1B, i). Fibrous leading strands form in a few minutes (Fig. 1B, ii), and dual incisions surrounding the ICL create a DNA double-strand break (Fig. 1B, iii). Upon addition of ubiquitylated FANCD2, DNA incisions, lesion bypass, and SapI site regeneration are greatly diminished (2). Given the established links between HR and ICL repair and the inefficient removal of the unhooked ICL in egg extracts (10), we postulated that SapI site regeneration is coupled to DNA replication via homologous recombination in vitro.

**Fig. 1.** The X-arc contains intermediates of ICL repair. (A) pICL schematic. (B) Model of ICL repair in *Xenopus* egg extracts (2, 10). (C) pControl or pICL was replicated in egg extract, digested with HincII, and analyzed by 2DGE. Arrowheads, see main text. (D) Cartoon of 2DGE patterns and relevant DNA intermediates. (E) ICL repair of samples from (C) was analyzed under normal and branch migration (+BM) conditions. Background, SapI fragments from contaminating un–cross-linked plasmid. For primary data, see fig. S1, E and F. All graphed experiments were performed at least three times, and a representative example is shown.
eration involves repair of the broken sister chromatin by recombination with the intact sister (Fig. 1B, v).

To look for evidence of HR in ICL repair, pICL or an undamaged control plasmid (pControl) was replicated in egg extract, digested with HincII (Fig. 1A), and analyzed by two-dimensional gel electrophoresis (2DGE) (Fig. 1, C and D) (11). After 5 min, both plasmids generated the expected double-Y and bubble replication intermediates (Fig. 1C and Fig. 1D, i). As replication neared completion, pControl produced unit-sized linear DNA molecules (Fig. 1C, red arrowheads, and Fig. 1D, i), whereas pICL yielded a discrete X-shaped intermediate that results from the convergence of two forks on the ICL (Fig. 1C, blue arrowheads, and Fig. 1D, i). Two simple-Y intermediates also accumulated (3 to 7% of total signal), suggesting that a small number of converging forks undergo aberrant breakage or cleavage (Fig. 1C, green arrowheads, and Fig. 1D, ii). By 60 min, the X-shaped intermediates showed signs of processing, likely due to resection of the nascent lagging strands (10). Even later, the X- and Y-shaped intermediates were replaced by an “X-arc” (Fig. 1C, purple arrowheads, and Fig. 1D, iii). Formation of the X-arc coincided with SspI site regeneration (Fig. 1E, red trace), suggesting that the X-arc contains intermediates of ICL repair.

X-arcs are generally formed when two unit-sized linear molecules are joined by a mobile branch point, such as a Holliday junction or hemicatenane (Fig. 1D, iii) (12). Under conditions that promote resolution of mobile junctions through branch migration (13), the X-arc intermediates of pICL were resolved primarily into the predicted unit-sized linear molecules, whether magnesium was present or not (fig. S1, A to C). Magnesium inhibits migration of Holliday junctions, which implies that the X-arc intermediates contain hemicatenanes. Consistent with this, X-arc intermediates were resistant to cleavage by the Holliday junction resolvase, RuvC (fig. S1D). Exposing replicated pICL to branch migration conditions increased the yield of SspI repair fragments (Fig. 1E, purple trace), further indicating that X-arcs contained intermediates of ICL repair.

If repair of pICL involves HR, inhibiting the RAD51 recombinase should block repair. To test this prediction, we used a ~35–amino acid “BRC” peptide derived from BRCA2 that binds RAD51 and, when supplied at high concentrations, inhibits RAD51 nucleoprotein filament formation (14). As recently reported (15), addition of a BRCWT (where WT is wild type) peptide (fig. S2A) to Xenopus egg extracts abolished RAD51 loading onto damaged chromatin (Fig. 2A). Two variants of the peptide that were mutated in one (BRC*) or three (BRC***) amino acids (fig. S2A) were increasingly compromised for RAD51 binding (Fig. 2B) and inhibition of RAD51 loading (Fig. 2A), demonstrating that BRCWT specifically targets RAD51. Without affecting DNA replication (fig. S2B), the BRC peptides inhibited X-arc formation proportional to their effect on RAD51 binding and filament formation (compare Fig. 2, A and B, with Fig. 2C). Thus, X-arc intermediates are RAD51-dependent, indicating...
they represent products of HR. Although BRC\textsuperscript{WT} almost completely abolished Sapl site regeneration, BRC\textsuperscript{*} caused intermediate inhibition and BRC\textsuperscript{***} had no effect (Fig. 2D). These results demonstrate that replication-coupled ICL repair requires RAD51-dependent HR. Because dual incisions result in a two-ended DSB (Fig. 1B, iii), recombination likely first yields a double Holliday junction, which is then converted to a mobile hemicatenane.

We next used BRC peptides to investigate which step in ICL repair involves RAD51. Replicating pICL was digested with AflIII (Fig. 1A) and subjected to denaturing gel electrophoresis to examine leading strand products of the rightward and leftward replication forks as they converge on the ICL (Fig. 3A). In the presence of BRC\textsuperscript{WT}, the arrival of leading strands 20 to 40 nucleotides from the ICL (Fig. 3, B and C), and nucleotide insertion across from the ad-ducted base (Fig. 3, B and E) were all unaffected. When BRC\textsuperscript{*} samples were analyzed by native 1D- and 2DGE, there was no change in the rate at which dual-stalled fork structures disappeared (Fig. 3, A to D), indicating that DNA incisions were unaffected. In contrast, BRC\textsuperscript{***} specifically reduced full-length extension products twofold (Fig. 3, B and F), consistent with an absence of RAD51-mediated restoration of the broken sister chromatid (Fig. 1B, v). Time addition of BRC peptides to ICL repair reactions indicated that RAD51 completes its repair function late, just before Sapl site regeneration (Fig. S4, A and B). Additionally, plasmid competition experiments showed that recombination occurred primarily between sister chromatids (Fig. S5). Together, the data indicate that RAD51-dependent HR between the broken and intact sister chromatids is a crucial, late step in ICL repair.

We used chromatin immunoprecipitation (ChIP) to examine the binding of RAD51 and replication protein A (RPA) to three locations on pICL during repair (Fig. 4A). Initially, RPA and RAD51 accumulated near the ICL (Fig. 4B, site I). After a delay, they also bound ~700 base pairs from the ICL (Fig. 4B, site II), likely due to a decrease in nucleosome concentration at the ICL. ATP-dependent RAD51 remained bound near the ICL (Fig. 4B, site III), because dual-stalled fork structures persisted in the absence of FANCD2 (Fig. 4D, red arrow). Thus, in the presence of ICL repair, RAD51 is loaded onto stalled replication forks before DSB formation.

We next examined the connection between the FA and HR pathways. In FANCD2-depleted extracts, the X-arc disappeared, and it was rescued by the readdition of recombinant FANCI-FANCD2 (Fig. 4E, purple arrowheads). As expected, the dual-stalled fork structure persisted in the absence of FANCD2 (Fig. 4E, blue arrowhead), consistent with a defect in incisions (2). The data indicate that HR acts downstream of FANCI-FANCD2 during ICL repair, explaining previous epistasis experiments (7). Consistent with some analyses of RAD51 foci [reviewed in (8)], RAD51 binding to ICLs was not reduced in the absence of FANCD2 (Fig. 4F and fig. S7, A to C). Conversely, inhibition of RAD51 function using BRC\textsuperscript{WT} peptide had no effect on FANCD2 recruitment or
ubiquitylation (Fig. 2A and fig. S7D). Together, our data show that FANCI-FANCD2 acts upstream of HR in the context of replication-coupled ICL repair, but that it is not required for RAD51 recruitment to chromatin. Instead, the requirement for FANCI-FANCD2 in promoting HR can be explained by its role in promoting the incisions that underlie DSB formation (2). The FA pathway may also enhance HR via more direct mechanisms, because FA proteins also stimulate HR in the context of preformed DSBS (6, 7, 9, 16, 17).

Here, we report that in the context of replication-coupled ICL repair, the DSB generated in one sister chromatid through the action of FANCI-FANCD2 is fixed via strand invasion into the intact sister (fig. S8). We find that RAD51 binds efficiently to ICLs before a DSB has been generated (fig. S8, ii). Although lesion bypass likely displaces RAD51 from one sister chromatid, incisions and resection of the other sister creates a new docking site for RAD51, such that both ends of the DSB are coated with the recombinase (fig. S8, iv). The interaction of RAD51 with ICL-stalled forks before DSB formation may function to prevent fork breakage/degradation (5, 15, 18) in favor of regulated incisions and/or to initiate strand invasion as soon as possible once the DSB has been formed.

A major obstacle impeding our understanding of DSB repair has been the absence of cell-free systems. Combined with ChIP and the ability to inactivate or remove essential proteins, the system described here represents a powerful tool to elucidate the complex mechanism underlying DSB repair.

References and Notes

A Key Enzyme in the Biogenesis of Lysosomes Is a Protease That Regulates Cholesterol Metabolism
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Mucolipidosis II is a severe lysosomal storage disorder caused by defects in the α and β subunits of the hexameric N-acetylgalosamine-1-phosphotransferase complex essential for the formation of the mannose 6-phosphate targeting signal on lysosomal enzymes. Cleavage of the membrane-bound α/β-subunit precursor by an unknown protease is required for catalytic activity. Here we found that the α/β-subunit precursor is cleaved by the site-1 protease (S1P) that activates sterol regulatory element–binding proteins in response to cholesterol deprivation. S1P-deficient cells failed to activate the α/β-subunit precursor and exhibited a mucolipidosis II–like phenotype. Thus, S1P functions in the biogenesis of lysosomes, and lipid-independent phenotypes of S1P deficiency may be caused by lysosomal dysfunction.

More than 50 soluble enzymes are targeted to lysosomes in a mannose 6-phosphate (M6P)–dependent manner. The formation of M6P residues on newly synthesized lysosomal enzymes is catalyzed by two multimeric enzyme complexes, N-acetylgalosamin (GlcNAc)-1-phosphotransferase and GlcNAc-1-phosphodiester-α-N-acetylgalosaminidase, allowing binding of the enzymes to M6P-specific receptors (I). The receptor–enzyme complexes are then transported to the endosomal compartment, followed by low pH–induced dissociation and delivery of lysosomal proteins to lysosomes and recycling of receptors to the Golgi apparatus. The GlcNAc-1-phosphotransferase comprises a hexameric complex of three subunits (α2β2γ) with a molecular mass of 540 kD (2). The α and β subunits are encoded by a single gene, GNPTAB, and synthesized as a 190-kD precursor protein in a hairpin orientation that contains cytoplolic N and C termini and a complex luminal modular structure (3, 4). Mutations in GNPTAB cause a severe lysosomal storage. disorder, mucolipidosis II (MLII; I-cell disease) (5, 5). The loss of GlcNAc-1-phosphotransferase activity leads to the synthesis of lysosomal enzymes lacking M6P residues, resulting in missorting and intracellular deficiencies of multiple lysosomal hydrolases, and lysosomal storage of nondegraded material, which are used as diagnostic markers in MLII patients (6). Clinically, these patients are characterized by skeletal abnormalities, chondrodysplasia, cardiomegaly, and motor and mental retardation, leading to early death (6). Cleavage of the α/β-subunit precursor between Lys929 and Asp1056 by an unknown protease is a prerequisite for the enzymatic activity of the GlcNAc-1-phosphotransferase complex (7). Treatment with brefeldin A, a drug that disrupts Golgi trafficking, prevents the cleavage of the α/β-subunit precursor, suggesting that this reaction takes place in the Golgi apparatus (8).

To identify the protease responsible for the cleavage, we generated an α/β-subunit precursor miniconstruct that allows its efficient expression, spans the membrane twice, and lacks amino acids 431 to 819 (Fig. 1A). Antibodies to the human β subunit (9) allowed the detection of both the α/β-subunit precursor constructs and the cleaved 45-kD β subunit in baby hamster kidney (BHK) cells (Fig. 1B). Analysis of additional miniconstructs with stepwise deletions showed that 20 amino acids proximal to the cleavage site were required for proper proteolysis (Fig. 1B). Construct 3 was the best substrate and was used for all further experiments. To define structural requirements for efficient cleavage of the α/β-subunit precursor in more detail, we substituted residues Thr923 to Ser934 (according to the numbering of the full-length precursor) individually with alanines, Arg925, Leu927, and Lys928 were the most critical for cleavage of the phosphotransferase

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1Supporting Online Material
www.sciencemag.org/cgi/content/full/333/6038/84/DC1

Accessed 12 May 2011
10.1126/science.1202585