The Fanconi Anemia Pathway Promotes Replication-Dependent DNA Interstrand Cross-Link Repair

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The Fanconi Anemia Pathway Promotes Replication-Dependent DNA Interstrand Cross-Link Repair

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Fanconi anemia is a human cancer predisposition syndrome caused by mutations in 13 *Fanc* genes. The disorder is characterized by genomic instability and cellular hypersensitivity to chemicals that generate DNA interstrand cross-links (ICLs). A central event in the activation of the Fanconi anemia pathway is the mono-ubiquitylation of the FANCI-FANCD2 complex, but how this complex confers ICL resistance remains enigmatic. Using a cell-free system, we showed that FANCI-FANCD2 is required for replication-coupled ICL repair in S phase. Removal of FANCD2 from extracts inhibits both nucleolytic incisions near the ICL and translesion DNA synthesis past the lesion. Reversal of these defects requires ubiquitylated FANCI-FANCD2. Our results show that multiple steps of the essential S-phase ICL repair mechanism fail when the Fanconi anemia pathway is compromised.

Cells derived from Fanconi anemia (FA) patients are hypersensitive to agents that induce DNA interstrand cross-links (ICLs) and exhibit ICL-induced chromosomal instability (*1*, *2*). Eight FANC proteins form a nuclear "core complex," which mono-ubiquitylates the FANCI-FANCD2 complex after DNA damage (*3*–*5*). Ubiquitylated FANCI-FANCD2 is recruited to the chromatin where it colocalizes with DNA repair factors (*6*, *7*). Mutation of the ubiquitin acceptor site in FANCD2 prevents FANCI-FANCD2 chromatin binding and sensitizes cells to ICL-inducing agents (*4*). Although the FA pathway might play a minor role in ICL repair during G_1 phase (*8*, *9*), its primary function is exerted in S phase (*6*, *10*–*13*). These results suggest that ubiquitylated FANCI-FANCD2 controls ICL repair in S phase, but the underlying mechanism is unknown.

Xenopus egg extracts support replication-dependent repair of a plasmid containing a cisplatin ICL (pICL) (*12*) (fig. S1A). Initially, two replication forks converge on the ICL, with their leading strands stalling 20 to 40 nucleotides (nt) from the lesion (Fig. 1A, i). One of the two leading strands then approaches the ICL, stalling again 1 nt from the cross-linked base [the "–1" position (Fig. 1A, ii)]. Subsequently, incisions on the other parental strand uncouple the cross-link (Fig. 1A, ii, green scissors), and lesion bypass occurs in two steps. First, a nucleotide is inserted across from the damaged template base ["insertion" (Fig. 1A, iii)], after which the strand is extended beyond the ICL in a DNA polymerase ζ–dependent manner ["extension" (Fig. 1A, iv)]. The final steps in repair are thought to involve excision repair and/or homologous recombination (Fig. 1A, v).

To examine the function of the *Xenopus laevis* FANCI-FANCD2 complex in ICL repair, we coexpressed xlFANCI (fig. S2) and xlFANCD2 (*10*, *14*) in insect cells and purified a stable 1:1 FANCI-FANCD2 complex (Fig. 1B and fig. S3A). Using antibodies to FANCI (fig. S3B) and

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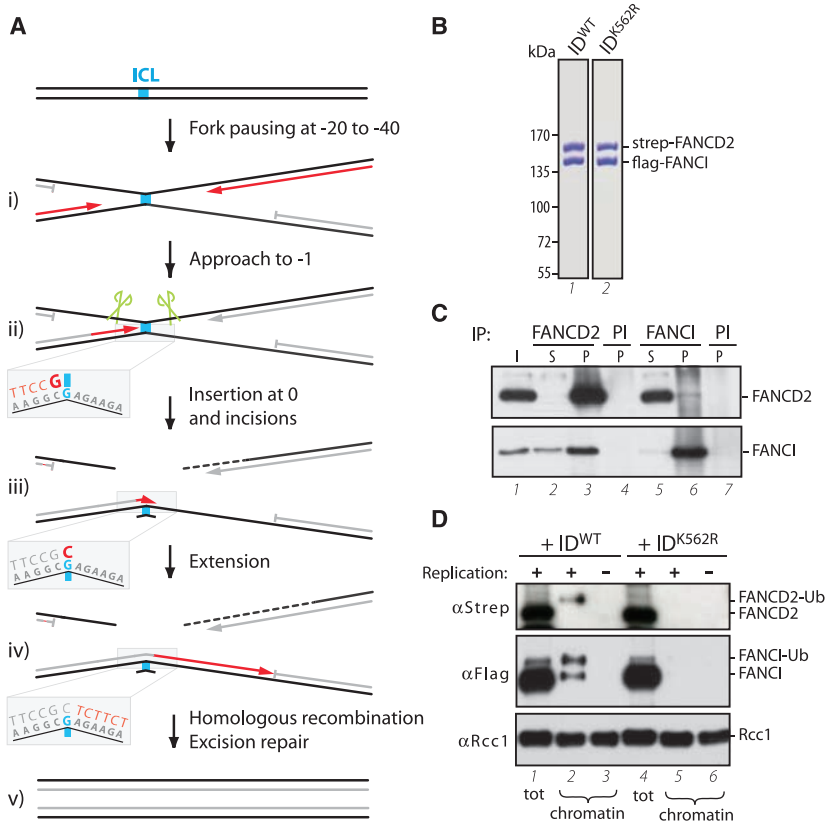

Fig. 1. (**A**) Schematic representation of lesion bypass in ICL repair (*12*). (**B**) Purified FANCI-FANCD2^{WT} and FANCI-FANCD2^{K562R} stained with Coomassie blue. (**C**) Reciprocal co-immunoprecipitation of xlFANCI and xlFANCD2 from *Xenopus* egg extract. Input (I) and supernatant (S) (0.2 μl extract), or precipitated proteins (P, from 1 μl extract) were blotted for FANCI and FANCD2. PI, preimmune serum. (**D**) Replication-dependent binding of FANCI-FANCD2 to damaged chromatin. Cross-linked sperm chromatin was replicated in extracts supplemented with FANCI-FANCD2^{WT} or FANCI-FANCD2^{K562R} (310 nM). Chromatin-bound fractions (from 2 μl extract) or total extract (0.2 μl), were analyzed by Western blotting with antibodies against a strep tag (recombinant FANCD2), a FLAG tag (recombinant FANCI), and RCC1 (loading control). Where indicated, replication was inhibited with Geminin. Note that only ubiquitylated FANCD2 binds chromatin, whereas both ubiquitylated and unubiquitylated FANCI bind [see also (*16*)].

FANCD2 (*12*), we showed that, in *Xenopus* egg extracts, FANCI and FANCD2 interact (Fig. 1C) and that the proteins undergo replication-dependent mono-ubiquitylation and chromatin binding, both of which are greatly enhanced by the presence of an ICL (fig. S3, C to E) (*10*). We also purified FANCI-FANCD2^{K562R}, in which the ubiquitin acceptor lysine at position 562 in FANCD2 is replaced by an arginine (Fig. 1B). Unlike FANCI-FANCD2^{WT}, FANCI-FANCD2^{K562R} did not bind to chromatin (Fig. 1D, compare lanes 2 and 5). In summary, *Xenopus* FANCI-FANCD2 binds chromatin dependent on DNA damage, FANCD2 ubiquitylation, and DNA replication.

To investigate whether FANCD2 is required for ICL repair, >95% of FANCD2 was immunodepleted from *Xenopus* egg extracts, resulting in ~75% codepletion of FANCI (fig. S4A), but no significant defect in total DNA synthesis on pICL (fig. S4B). Repair efficiency was determined by measuring the regeneration of a Sap I restriction

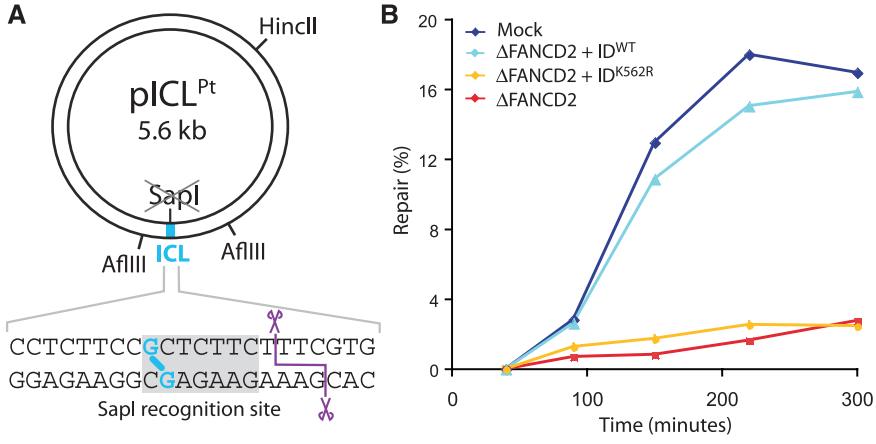

Fig. 2. FANCD2 and its ubiquitylation are required for ICL repair. (**A**) Sequence surrounding the ICL and relevant restriction sites of pICL. (**B**) pICL (2.3 ng/μl) was replicated in the presence of [α-^{32}P]deoxyadenosine triphosphate in mock-depleted extract, FANCD2-depleted extract (ΔFANCD2), or ΔFANCD2 extract supplemented with 375 nM FANCI-FANCD2^{WT} or FANCI-FANCD2^{K562R}, and repair efficiency was plotted. For primary data and calculation of repair efficiency, see fig. S5.

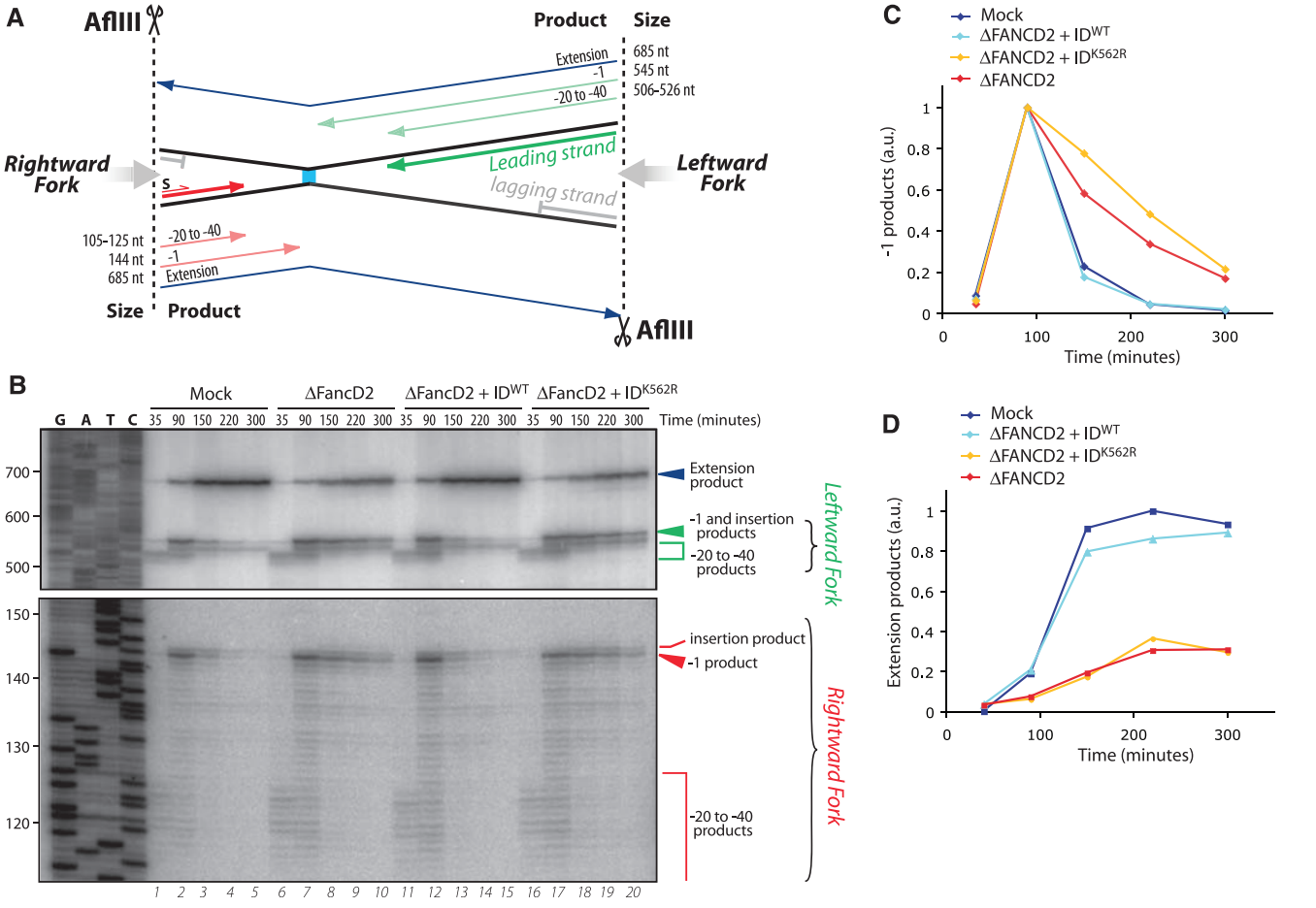

Fig. 3. Insertion of a nucleotide across from the damaged base is compromised in FANCI-FANCD2–deficient extracts. (**A**) Schematic representation of leading strand intermediates generated after pICL digestion with Afl III. (**B**) Samples from the reactions described in Fig. 2B were digested with Afl III, separated on a sequencing gel alongside a ladder derived from extension of primer S [S in (A)] on pControl, and visualized via autoradiography. Nascent strands generated by the rightward (red) and leftward (green) replication forks are indicated to the right and illustrated in (A). The –1 and extension products observed in (B) were quantified and graphed in (**C**) and (**D**), respectively.

site that coincides with the cross-link (Fig. 2A and fig. S5). In mock-depleted extracts, 15 to 24% of the replicated DNA became cleavable by Sap I (Fig. 2B and fig. S6). Sap I site regeneration is not 100% efficient because of significant destruction of the incised sister chromatid (*12*), incomplete removal of the unhooked ICL (*12*), and possibly some mutagenic lesion bypass events. In FANCD2-depleted extracts, regeneration of the Sap I site was reduced by a factor of 14, on average (Fig. 2B and fig. S6). The residual Sap I cleavable products might arise from incomplete FANCD2 depletion or FANCD2-independent ICL repair. Addition of recombinant FANCI-FANCD2 (Fig. 1B) rescued the repair defect (Fig. 2B and fig. S6), which ruled out the possibility that FANCD2 depletion non-specifically inactivated repair. In contrast, FANCI-FANCD2^{K562R}, which is not ubiquitylated (fig. S4C), did not rescue repair (Fig. 2B and fig. S6), which demonstrated a role for ubiquitylated FANCI-FANCD2 in replication-coupled ICL repair. A recent study concluded that FANCL depletion reduces origin-dependent ICL repair in *Xenopus* egg extracts, but the defect was minor (30%) and was not rescued with recombinant proteins (*8*). Our experiments demonstrate that the Fanconi anemia pathway is essential for replication-dependent ICL repair and, together with previous observations (*13*, *15*), provide powerful evidence that Fanconi anemia is a bona fide DNA repair disorder.

The ATR (ataxia telangiectasia mutated and Rad3-related) signaling pathway confers ICL resistance in vertebrate cells (*2*, *16*), and replication of pICL in *Xenopus* egg extracts activates ATR as measured by Chk1 and Rad1 phosphorylation (*12*) (figs. S1B and S7). However, the repair defects we observed in FANCD2-depleted extracts were not due to defective checkpoint activation (fig. S7).

To address which step in ICL repair is dependent on FANCI-FANCD2, we replicated pICL in mock-depleted or FANCD2-depleted extract and examined lesion bypass. To this end, DNA samples were withdrawn at various times; digested with Afl III, which cuts on either side of the ICL (Fig. 3A); and nascent products were analyzed on a sequencing gel. In mock-depleted extract, the leading strands of the leftward and the rightward forks initially stalled 20 to 40 nt from the ICL (Fig. 3B, lane 1, green and red brackets), as previously described (*12*) (Fig. 1A). Then, one of the two forks resumed synthesis and stalled again 1 nt before the cross-linked base, causing a peak of "–1" products at ~90 min (Fig. 3B, lane 2, green and red arrowheads). Finally, insertion of a nucleotide across from the adducted base followed by extension resulted in formation of a 685-nt extension product (Fig. 3B, lanes 3 to 5, blue arrowhead). In FANCD2-depleted extracts, there was a marked persistence of the –1 product and a reduction in extension products, indicating an insertion defect (Fig. 3B, lanes 6 to 10; for quantification, see Fig. 3, C and D, and fig. S8). Extension products continued to accumulate slowly in the absence of FANCI-FANCD2 (Fig. 3D and fig. S8), but this was not accompanied by a proportional increase in repair products (Fig. 2B and fig. S6), suggestive of a FANCD2-independent, error-prone lesion-bypass process. The defects observed in FANCD2-depleted extracts were fully rescued by recombinant FANCI-FANCD2^{WT} but not by FANCI-FANCD2^{K562R} (Fig. 3, B to D, and fig. S8). In the samples lack-

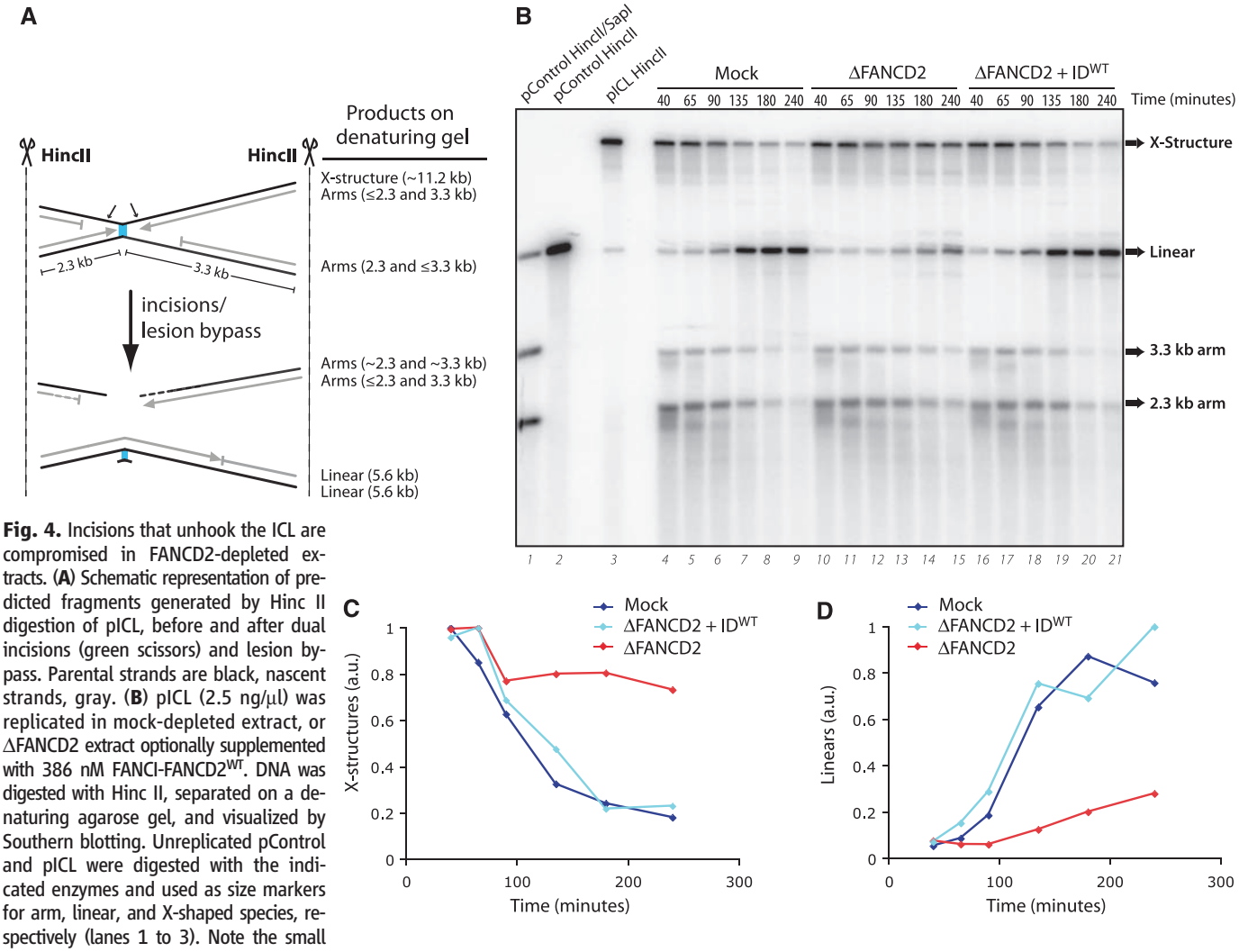

Fig. 4. Incisions that unhook the ICL are compromised in FANCD2-depleted extracts. (**A**) Schematic representation of predicted fragments generated by Hinc II digestion of pICL, before and after dual incisions (green scissors) and lesion bypass. Parental strands are black, nascent strands, gray. (**B**) pICL (2.5 ng/μl) was replicated in mock-depleted extract, or ΔFANCD2 extract optionally supplemented with 386 nM FANCI-FANCD2^{WT}. DNA was digested with Hinc II, separated on a denaturing agarose gel, and visualized by Southern blotting. Unreplicated pControl and pICL were digested with the indicated enzymes and used as size markers for arm, linear, and X-shaped species, respectively (lanes 1 to 3). Note the small amount of linear products in lane 3 (3% of the total), which represents contaminating non–cross-linked plasmids. X-shaped structures and linear species observed in (B) were quantified and graphed in (**C**) and (**D**), respectively.

ing functional FANCI-FANCD2, the level of –1 products did eventually decrease (Fig. 3, B and C, and fig. S8). However, since the extension products never accumulated to more than ~30% of the mock-depleted samples (Fig. 3D and fig. S8), we infer that this decline of –1 products is primarily due to degradation (*12*). We conclude that in the absence of FANCI-FANCD2, nucleotide insertion opposite the cross-linked base is inhibited. This differs from the effect of DNA polymerase ζ immunodepletion, which arrests lesion bypass immediately after the insertion step (fig. S9) (*12*).

We next investigated whether FANCI-FANCD2 is required for incisions, which are thought to occur on one of the parental strands on either side of the lesion (*2*) (Fig. 1A, ii). To visualize incisions, DNA repair intermediates were digested with Hinc II and analyzed by denaturing gel electrophoresis and Southern blotting (Fig. 4A). After 40 min, the most abundant species were the high-molecular-weight parental X-shaped molecules, as well as 2.3- and 3.3-kb species that represent stalled nascent strands (Fig. 4B, lane 4). Dual incisions surrounding the ICL are expected to convert the parental X-shaped molecule into a 5.6-kb linear product and 2.3- and 3.3-kb fragments (Fig. 4A). As expected, in mock-depleted extract, we observed a time-dependent decrease of X-shaped molecules and a concomitant increase in linear species (Fig. 4B, lanes 4 to 9; quantified in Fig. 4, C and D). The 2.3- and 3.3-kb species declined over time because of lesion bypass and/or resection (Fig. 4B). The kinetics of X-shaped molecule disappearance in this assay confirmed our previous conclusion (*12*) that the majority of incisions occurs after forks reach the –1 position (fig. S10C).

In the absence of FANCD2, incisions were severely inhibited, as seen from the persistence of X-shaped species and a severe delay in the accumulation of linear molecules (Fig. 4B, lanes 10 to 15; quantified in Fig. 4, C and D). In addition, the 2.3- and 3.3-kb fragments persisted longer, likely because of inhibition of lesion bypass. These effects were rescued by FANCI-FANCD2^{WT}. Tracking only the parental strands in this assay confirmed that incisions are inhibited in the absence of FANCD2 and showed that the defect is not rescued by FANCI-FANCD2^{K562R} (fig. S11). Together, these data show that ubiquitylated FANCI-FANCD2 is required for efficient incisions surrounding the cross-link.

Finally, we examined the precise timing of FANCI-FANCD2 ubiquitylation. As shown in fig. S12C, FANCI and FANCD2 ubiquitylation correlated with the arrival of leading strands at the –1 position, consistent with a role for the FANCI-FANCD2 complex in the insertion and incision steps, which occur after forks reach the –1 position (*12*) (figs. S10 and S12).

Using a chemically homogeneous cisplatin ICL and a bona fide repair assay, we show that the Fanconi anemia pathway is required for DNA replication–coupled ICL repair. These results explain why Fanconi anemia cells treated with ICL-inducing agents arrest late in S phase (*11*) and eventually die. We further demonstrate that FANCI-FANCD2 must be ubiquitylated to support repair, which suggests that its role in this process involves direct binding to the lesion. In the absence of FANCI-FANCD2, incisions near the ICL and translesion synthesis (TLS) past the lesion are severely inhibited, defining two critical steps in ICL repair that fail when the Fanconi anemia pathway is compromised. Although at present we cannot determine whether the insertion or incision steps occur first, it is widely envisioned that incisions must precede insertion (*2*). In this view, FANCI-FANCD2 might directly promote incisions and thereby affect TLS indirectly (fig. S13A). For example, FANCI-FANCD2, which contains no apparent nuclease domains, could promote dual incisions by recruiting the Slx4 nuclease complex to the lesion (*17*). However, we cannot rule out the converse scenario, in which TLS precedes incisions (fig. S13B), which would involve translesion DNA synthesis past an ICL (*18*). In this case, the primary function of FANCI-FANCD2 might be to promote TLS, perhaps via interaction with the ubiquitin-binding domains of Rev1. This model is consistent with genetic epistasis between TLS polymerases and the FA pathway (*19*), as well as reduced damage-dependent mutagenesis in FA cells (*20*). Finally, FANCI-FANCD2 might directly control both the incision and insertion steps (fig. S13C). Future experiments will be required to establish a molecular model of how FANCI-FANCD2 regulates the incision and/or TLS machineries during replication-coupled ICL repair.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1182372/DC1
Materials and Methods
Figs. S1 to S13
References

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Indirect Punishment and Generosity Toward Strangers

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Many people incur costs to reward strangers who have been kind to others. Theoretical and experimental evidence suggests that such "indirect rewarding" sustains cooperation between unrelated humans. Its emergence is surprising, because rewarders incur costs but receive no immediate benefits. It can prevail in the long run only if rewarders earn higher payoffs than "defectors" who ignore strangers' kindness. We provide experimental evidence regarding the payoffs received by individuals who employ these and other strategies, such as "indirect punishment" by imposing costs on unkind strangers. We find that if unkind strangers cannot be punished, defection earns most. If they can be punished, however, then indirect rewarding earns most. Indirect punishment plays this important role, even if it gives a low payoff and is rarely implemented.

Indirect reciprocity is widespread in human societies. It occurs when we incur costs to reward those we know have been kind to others or punish those we know have been unkind to others. Indirect reciprocity is based on reputation and helps to enforce trustworthy behavior between individuals who do not know each other and who may not meet again. Such encounters form a substantial part of our interactions and are especially frequent in online commerce.