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C. elegans FANCD2 responds to replication stress and functions in interstrand cross-link repair

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ABSTRACT

One of the least well understood DNA repair processes in cells is the repair of DNA interstrand cross-links (ICLs) which present a major obstacle to DNA replication and must be repaired or bypassed to allow fork progression. Fanconi anemia (FA) is an inherited genome instability syndrome characterized by hypersensitivity to ICL damage. Central to the FA repair pathway is FANCD2 that is mono-ubiquitylated in response to replication stress and ICL damage through the action of the FA core complex and its E3-ubiquitin ligase subunit, FANCL. In its mono-ubiquitylated form FANCD2 is recruited to repair foci where it is believed to somehow coordinate ICL repair and restart of impeded replication forks. However, the precise mechanism through which the FA pathway and mono-ubiquitylation of FANCD2 promotes ICL repair remains unclear. Here we report on a functional homologue of FANCD2 in *C. elegans* (FCD-2). Although *fcd-2* mutants are homozygous viable, they are exquisitely sensitive to ICL-inducing agents, but insensitive to ionizing radiation (IR). *fcd-2* is dispensable for meiotic recombination and activation of the S-phase checkpoint, indicating that ICL sensitivity is likely due to a repair rather than a signalling defect. Indeed, we show that FCD-2 is mono-ubiquitylated in response to ICL damage and is recruited to nuclear repair foci. Consistent with the sensitivity of *fcd-2* mutants, FCD-2 focus formation is induced in response to ICL damage and replication stress, but not following IR, suggesting that FCD-2 responds to lesions that block DNA replication and not DNA double strand breaks *per se*. The realization that the FA pathway is conserved in a genetically tractable model system will permit the comprehensive analysis of the interplay between the FA, homologous recombination (HR), translesion synthesis (TLS) and nucleotide excision repair (NER) pathways, critical to the understanding of ICL repair.

1. Introduction

Fanconi anemia (FA) is a rare human autosomal recessive disorder associated with congenital abnormalities, bone marrow failure and an increased early incidence of acute myeloid leukaemia and solid tumours. Somatic cell fusion studies using cells derived from FA patients have thus far identified 12 genetic complementation groups: FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, and FANCM, and the corresponding genes have been cloned for all except FANCI [1,2]. Sequence analysis provides little insight to the function of these genes,

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and informative protein motifs are only observed for the most recently identified factors FANCJ (DNA helicase), FANCM (DNA helicase/translocase), and FANCL (PHD/RING ubiquitin ligase) [3–8].

All FA deficient cells are characteristically sensitive to chemotherapeutic agents that form DNA interstrand cross-links (ICLs), such as diepoxybutane, mitomycin C (MMC) and cisplatin (CDDP). ICLs are complex lesions that tether the strands of DNA presenting an absolute block to replication, yet ICL repair is currently one of the least well understood repair processes in cells [1,2]. Treatment of FA cells with ICLs results in increased chromosome breakage and late S/G2 cell cycle arrest, consistent with an inability to repair the ICL lesion and restart replication [9].

A key factor in the FA pathway is FANCD2, which is mono-ubiquitylated after ICL damage at lysine 561 [10]. A multi-subunit nuclear core complex comprising eight of the known FA proteins (A, B, C, E, F, G, L and M) is absolutely required for the ubiquitination of FANCD2 [1,2] and absence of any one of these core components appears to destabilise the complex and prevent FANCD2 activation. However, recent studies have eluded to additional DNA repair functions for certain FA core subunits independent of their role in FANCD2 mono-ubiquitylation [11]. The ubiquitylation activity of the FA core complex is provided by the FANCL subunit that encodes a PHD/RING containing Ub-ligase [7], whereas de-ubiquitylation of FANCD2 following ICL repair is mediated by USP-1 [12].

At the cellular level, FANCD2 mono-ubiquitylation is important for chromatin association and recruitment to DNA damage sites where it forms nuclear foci that co-localize with a number of DNA repair proteins, including BRCA1 and BRCA2/FANCD1 [10]. Current evidence supports a role for FANCD2 and the FA pathway in orchestrating lesion repair via homologous recombination (HR) and/or translesion bypass pathways. This view is supported by observations that cell lines derived from FA-A, -G and -D2 patients, and *fancg* and *fancc* mutant chicken DT40 cell lines are severely compromised for homology-directed repair via the HR pathway [13–15]. Furthermore, FANCD2 directly interacts with BRCA2/FANCD1 which initiates HR processes by targeting the Rad51 recombinase to DNA lesions and promoting the subsequent nucleation of the Rad51 filament on processed DNA ends [16,17]. However, the precise mechanism through which the FA pathway and mono-ubiquitylation of FANCD2 promotes DNA repair remains unclear.

Further elucidation of the role of the FA pathway would be greatly enhanced by the identification of a simple conserved model system. The nematode *C. elegans* is highly amenable to both genetic and biochemical analysis, and cytological studies of the germline exploit the temporal and spatial separation of mitotic and meiotic nuclei. Most DNA repair pathways appear to be conserved in *C. elegans*, including key human disease genes like BRCA1 and BRCA2, which are absent from lower eukaryotes such as yeast [18–21]. A potential sequence homologue of human FANCD2 has been identified in *C. elegans* (*fcd-2*), however, obvious orthologues of the FA core complex components are currently lacking. Here we show that *C. elegans fcd-2* deletion mutants are specifically sensitive to ICLs, analogous to human FA-derived cells. Furthermore, FCD-2 is ubiquitylated in response to ICL damage, and is recruited to repair foci. This implies that the essential elements of the FA pathway are conserved in the nematode worm, which will enable use of *C. elegans* as a model system to facilitate dissemination of FA repair activity.

2. Results

2.1. *C. elegans fcd-2 mutants are sensitive to cross-linking agents but insensitive to IR*

The demonstration that the FA complementation group D1 results from biallelic inactivation of BRCA2 [22] coupled with the recent identification of a functional homologue of BRCA2 in *C. elegans* [20] led to us to search for other putative FA gene homologues in the nematode. A putative FANCD2 homologue is encoded by Y41E3.9 on chromosome IV that shares homology to the chicken (GgFancD2) and human (HsFANCD2) proteins (Fig. 1A and B [44]). Y41E3.9 possesses a conserved C-terminal region (CCR) of approximately 300 amino acids that shares 24%/23% identity and 45%/44% similarity to the chicken (GgFancD2) and human (HsFANCD2) proteins, respectively. Y41E3.9 also shares homology over an additional 448 amino acid span (279–727aa) to the corresponding region in chicken FancD2 (435–920) (Fig. 1B).

To determine if Y41E3.9 is a functional FANCD2 homologue in *C. elegans*, we obtained two independently derived deletion mutants in Y41E3.9 (*tm1298* and *ok1145*). *tm1298* carries a 238 bp deletion in exon 5 and *ok1145* carries a 1221 bp deletion spanning exons 3 and 4 (Fig. 2A and B). Although both alleles are predicted to produce truncated proteins, Western blotting and immunofluorescence with antibodies raised against the N-terminus of FCD-2 failed to detect FCD-2 protein in either mutant strain suggesting that both deletions are null alleles (Fig. 5 and data not shown). Both *fcd-2* mutants are homozygous viable and fertile, with a normal average brood size of 315 ± 36 and 302 ± 42 at 20 °C, respectively. One of the hallmarks of FA deficient cells is their exquisite sensitivity to DNA interstrand cross-linking (ICL) agents, but only modest sensitivity to ionizing radiation (IR) [1,2]. To determine if Y41E3.9 is a functional *C. elegans* FANCD2 homologue we compared the sensitivity of N2(Wt), *tm1298* and *ok1145* mutants worms to a range of DNA damaging agents, including ICL-inducing agents and IR. In contrast to N2(Wt), progeny survival of *tm1298* and *ok1145* mutants is significantly compromised following treatment with cisplatin (CDDP), a bifunctional chemotherapeutic drug that produces cytotoxic intrastrand and interstrand DNA cross-links (ICLs; Fig. 2C). The sensitivity of *tm1298* and *ok1145* mutants to CDDP is likely due to an inability to repair ICL lesions, as both *fcd-2* mutants also exhibit sensitivity to trimethylpsoralen (TMP)/UVA, which can only produce ICLs due to the planar nature of TMP (Fig. 2D). Moreover, *fcd-2* mutants also exhibit sensitivity to the ICL-inducing agents nitrogen mustard and mitomycin C (data not shown). In contrast, *tm1298* and *ok1145* mutants are largely insensitive to treatment with ionizing radiation (IR) compared with N2(Wt) (Fig. 2E). This data demonstrates that deletion mutations in Y41E3.9 confer sensitivity to DNA cross-linking agents, but not to IR, analogous to human FANCD2 deficient cells. Based on sequence similarity and deficiency in ICL repair we propose

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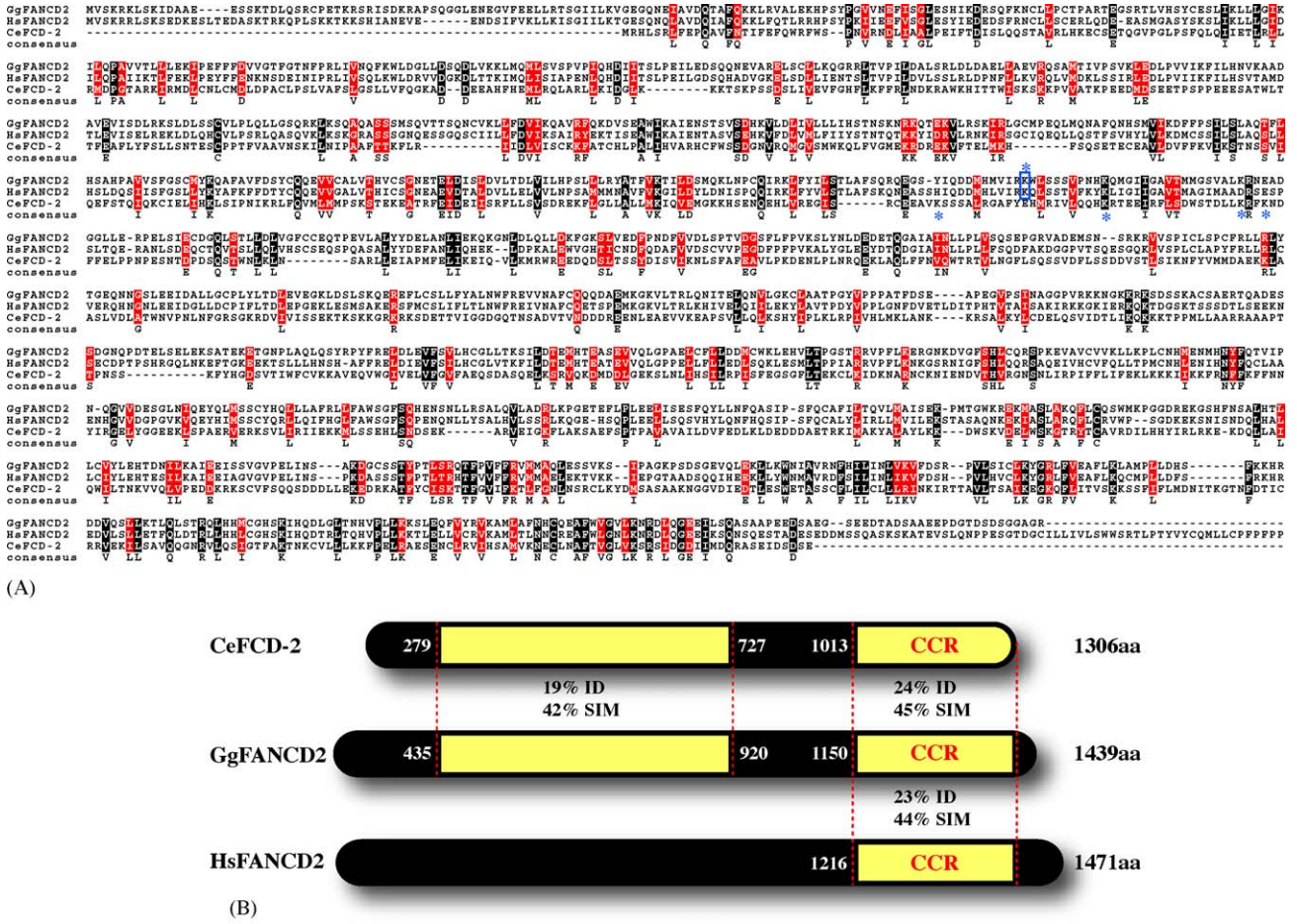

Fig. 1 – Protein sequence alignment and comparison of *C. elegans* FCD-2 to its chicken and human counterparts. (A) Protein sequence alignment of *C. elegans* FCD-2 (CeFCD-2), chicken FANCD2 (GgFANCD2) and human FANCD2 (HsFANCD2). Conserved amino acid residues are shown in white with a black background. Semi-conserved amino acid residues are shown in white with a red background. The consensus amino acid sequence is shown underneath. The position of lysine 561 (site of mono-ubiquitylation) in HsFANCD2 is boxed. Lysine residues in CeFCD-2 with the corresponding region are shown (*). (B) A scaled representation of *C. elegans* FCD-2 compared with GgFANCD2 and HsFANCD2. The various conserved domains, including the C-terminal conserved domain (CCD), are indicated with their percentage identity and similarity between species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

that Y41E3.9 (referred to from now on as FCD-2) is a functional homologue of FANCD2 in *C. elegans*.

2.2. *fcd-2 is dispensable for SC assembly and crossover recombination during meiotic prophase*

Consistent with the fact that *fcd-2* mutants are homozygous viable, analysis of the *C. elegans* germline reveals that meiotic prophase nuclei are cytologically normal (data not shown). Furthermore, RAD-51 foci form at sites of meiotic DSBs and the synaptonemal complex (SC) correctly assembles at the interface between homologue pairs as normal, as shown by immunostaining with antibodies against the strand-exchange protein RAD-51 and the core SC component, SYP-1 [20,23] (Fig. 3B). Indicative of successful crossover recombination, *tm1298* and *ok1145* mutant strains also exhibit six DAPI-stained bivalent chromosomes in oocyte nuclei arrested at diakinesis (data not shown). These data indicate that *fcd-2* is dispensable for recruitment of RAD-51 to meiotic DSBs, SC assembly, and successful crossover recombination during meiosis I in *C. elegans*.

2.3. *fcd-2 is dispensable for the S-phase checkpoint*

It is feasible that the sensitivity of *fcd-2* mutants (*tm1298* and *ok1145*) to ICL-inducing agents may be caused by a defect in the S-phase checkpoint that is required for sensing the presence of DNA damage during replication and signalling to illicit cell cycle arrest [24,25]. We therefore assessed the integrity of the S-phase checkpoint response in *fcd-2* mutants by subjecting L4 staged animals to treatment with hydroxyurea (HU), an inhibitor of ribonucleotide reductase that leads to replication fork stalling and activation of cell cycle arrest [26,27]. HU treatment of wild type (N2) animals leads to S-phase check-

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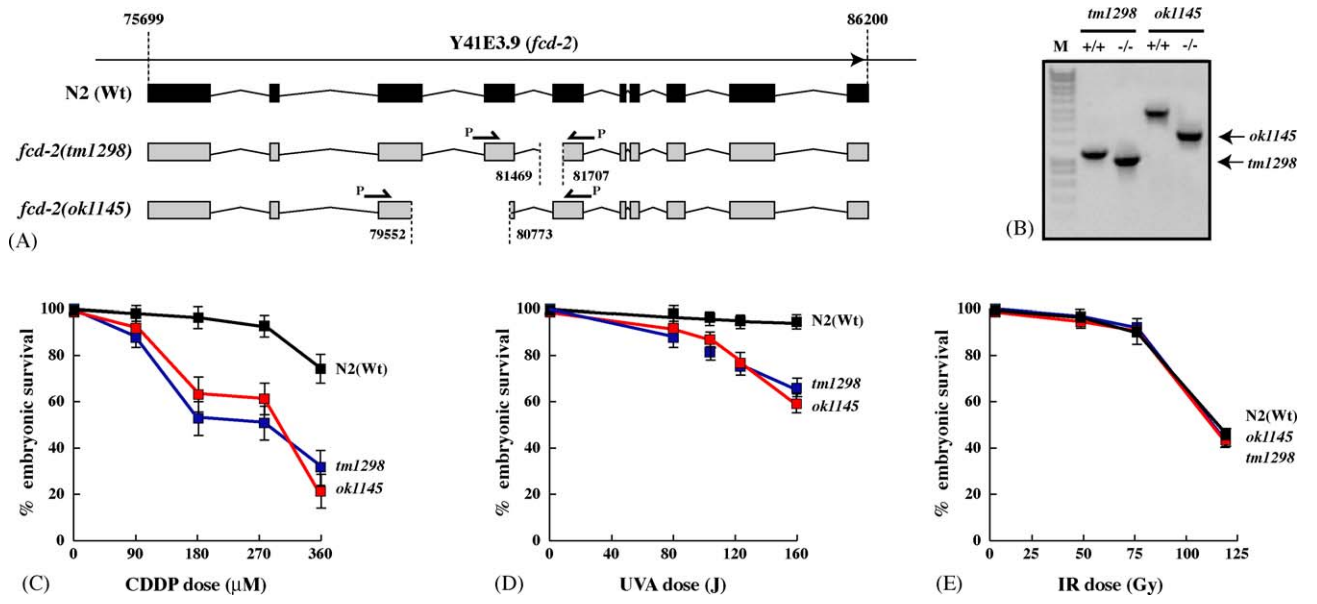

Fig. 2 – *C. elegans* FANCD2 mutants (*fcd-2*) are exquisitely sensitive to ICL lesions. (A) Schematic of the gene structure of *fcd-2* (Y41E3.9) comparing the wild type (black) with two *fcd-2* deletion mutants, *tm1298* and *ok1145* (grey). *tm1298* carries a 238 bp deletion in exon 5. *ok1145* carries a 1221 bp deletion spanning exons 3 and 4. The breakpoints and the position of the internal primers (p) used for nested PCR to detect the *tm1298* and *ok1145* deletions are shown. (B) Nested PCR using *fcd-2* allele-specific primers (marked by "p" in A) on a single N2(Wt), *tm1298* and *ok1145* animal. Arrows indicate the PCR product obtained in *tm1298* (−/−) and *ok1145*(−/−) deletion strains. (C–E) Percentage progeny survival of N2(Wt), *fcd-2*(*tm1298*) and *fcd-2*(*ok1145*) animals treated with the indicated dose of cisplatin (CDDP; C), 10 μg/ml trimethylpsoralen/UVA (TMP/UVA; D) and ionizing irradiation (IR; E). Error bars indicate standard error of the mean (S.E.M.) from at least 24 adult worms over two independent experiments.

point arrest that manifests as both enlarged nuclei and an overall reduction in the number of nuclei in the mitotic compartment of the germline, as previously described (Fig. 3C [26,27]). Enlarged mitotic nuclei and a reduced total number of nuclei indicative of cell cycle arrest is also detected in *tm1298* and *ok1145* (*fcd-2*) mutant strains (Fig. 3C). Quantification of cell cycle arrest supports our cytological observation that *fcd-2* is dispensable for the S-phase checkpoint: N2(Wt) exhibit 68.4.3 ± 3.1 mitotic nuclei in a given volume versus 15.4 ± 1.6 after treatment ($P < 0.01$). *tm1298* and *ok1145* mutants exhibit 72.3 ± 2.1, 66.3 ± 4.2 versus 19.3 ± 1.6, 17.3 ± 3.4 after treatment, $P < 0.05$, respectively (Fig. 3D). In contrast, *atl-1*(*tm853*) mutants fail to arrest the cell cycle following HU treatment and show no measurable decrease in the number of nuclei in the mitotic compartment when compared with untreated animals (Fig. 3D) (53.7 ± 2.9 versus 51.4 ± 3.8 after treatment, $P < 0.05$), as previously shown [27]. These results indicate that *fcd-2* is dispensable for the S-phase checkpoint response. It is therefore likely that the sensitivity of *fcd-2* mutant strains to ICL-inducing agents is due to a defect in DNA repair, rather than a defect in sensing or signalling the presence of replication stalling lesions.

2.4. *FCD-2 is ubiquitylated in response to cross-linking agents*

To further explore the role of FCD-2 in ICL repair we generated antibodies against FCD-2 that recognise full-length FCD-2 recombinant protein expressed in *E. coli* (Fig. 4A). Given that ICL damage stimulates FANCD2 mono-ubiquitylation in human cells [10] we first assessed whether FCD-2 is also ubiquitylated following ICL damage. The mono-ubiquitylated form of HsFANCD2 is readily detectable by Western blotting in whole cell extracts [10]. However, we are unable to detect FCD-2 in whole *C. elegans* extracts which we attribute to the fact that FCD-2 focus formation (see below), and therefore its expression, is restricted to a small number of proliferating cells within the germline, that represent less than 2–5% of all cells in the adult animal (data not shown; Fig. 3A). We therefore opted to enrich for FCD-2 by immunoaffinity purification from 100 ml cultures of N2(Wt), *tm1298*, or *ok1145* strains, before and 18 h post-treatment with 180 μM CDDP. Western blotting with an ubiquitin antibody reveals that endogenous FCD-2 is subject to ubiquitylation in N2(Wt) animals in response to CDDP treatment (Fig. 4B, lane 2) but not in untreated N2(Wt) animals (Fig. 4B, lane 1) nor in *tm1298* and *ok1145* strains either before or after CDDP treatment (Fig. 4B, lanes 3–6). Moreover, a single ubiquitylated band is observed after ICL damage indicating that this modification is mono-ubiquitylation rather than poly-ubiquitylation (Fig. 4B). The detection of this modification is intriguing in light of the fact that the FA core complex, which controls FANCD2 mono-ubiquitylation in higher eukaryotes [1,10], is not obviously conserved in *C. elegans*. Potential acceptor lysine residues for ubiquitylation are located in the region of FCD-2 that aligns with the mono-ubiquitylation site (K561) in human FANCD2 ([10] Fig. 1A), but at present we do not know

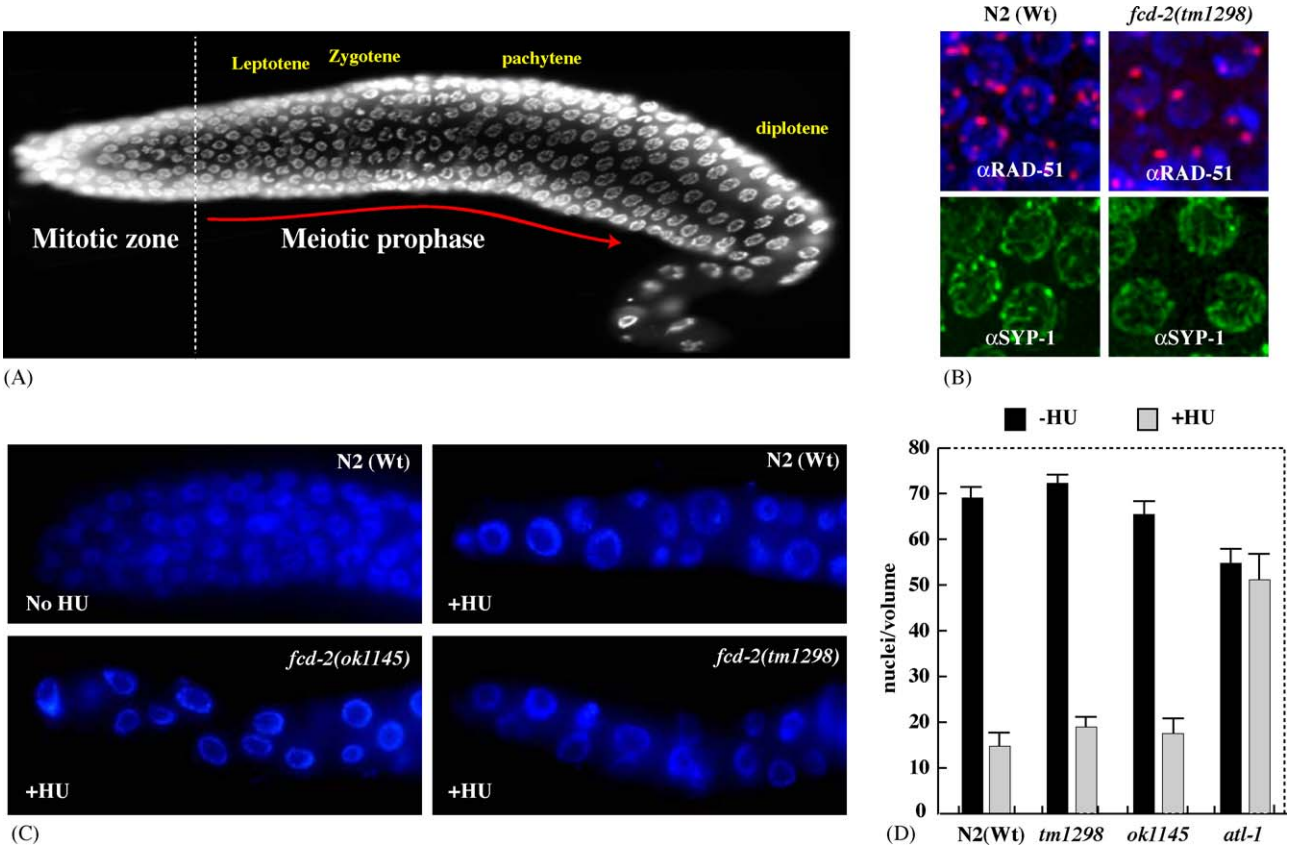

Fig. 3 – FCD-2 is dispensable for meiotic recombination and the S-phase checkpoint. (A) An annotated DAPI-stained image of the *C. elegans* germline indicating the position of the mitotic zone and progressive stages of meiotic prophase. (B) Representative images of fixed meiotic nuclei from strains of the indicated genotype immunostained with RAD-51 and SYP-1 antibodies [23]. The *ok1145* mutant is also normal for RAD-51 focus formation and SC assembly (data not shown). (C) Representative images of a single focal plane through the mitotic region of the germline before and 16 h after 40 mM HU treatment of L4 larval stage animals of the indicated genotype counterstained with DAPI. (D) Quantification of cell cycle arrest of mitotic germline nuclei was determined before and after treatment of L4 larval stage animals of the indicated genotype. The number of nuclei in a volume of 54,000 μm^3 16 h after exposure of to 40 mM hydroxyurea was scored in at least 10 germlines for each experiment, as previously described [26]. Error bars indicate standard error of the means (S.E.M.) from three independent experiments.

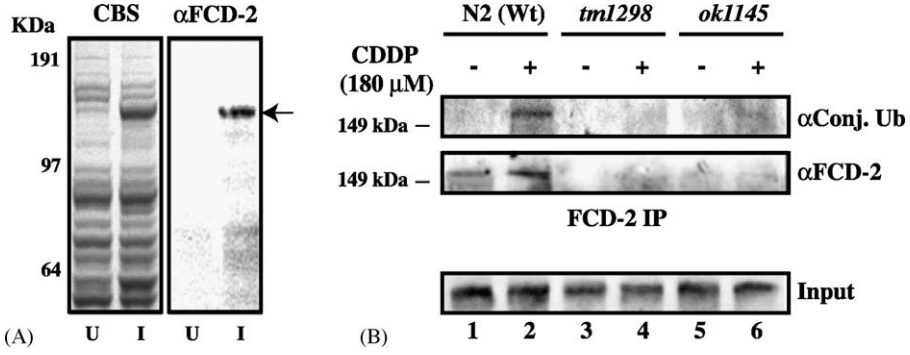

Fig. 4 – FCD-2 is mono-ubiquitylated in response to ICL damage. (A) SDS-PAGE of un-induced (U) and induced (I) expression of His-tagged FCD-2 in *E. coli* stained with coommasie blue stain (CBS; left panel) or Western blotted for FCD-2 (right panel). (B) Western blotting for conjugated ubiquitin (conj. Ub; FK2) and FCD-2 following immunoprecipitation for FCD-2 from N2(Wt), *fcd-2*(*tm1298*) and *fcd-2*(*ok1145*) mutants before (–) and 18 h after treatment with 180 μM CDDP (+). Extracts from the different genotypes were normalized for protein concentration prior to immunoprecipitation (IP). Western blotting for actin was used to show consistent protein level in the input prior to IP.

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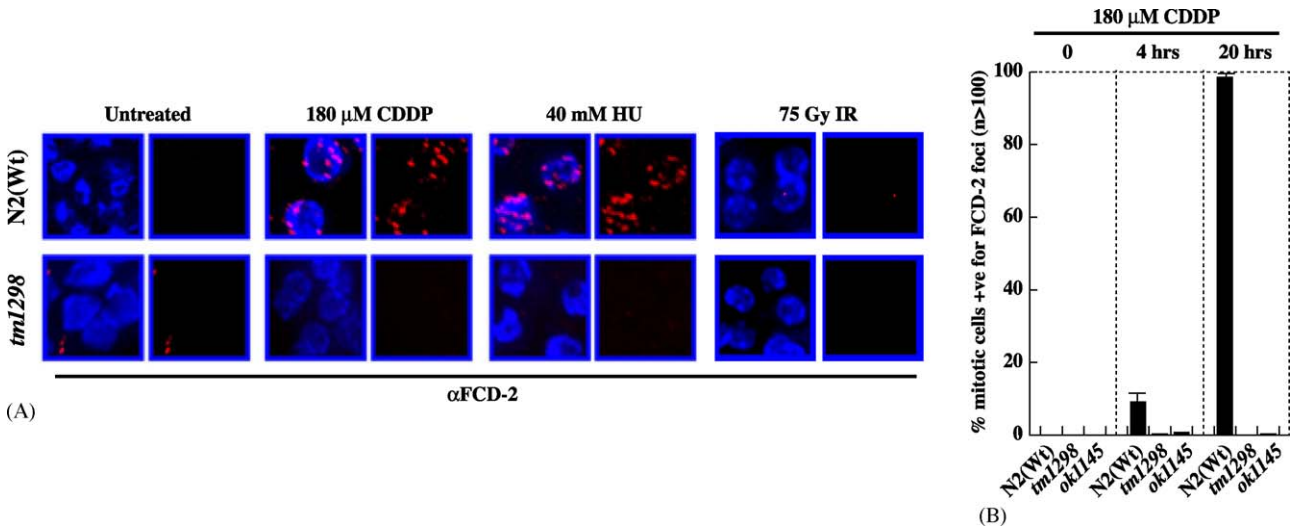

Fig. 5 – FCD-2 is recruited to ICL-lesions at sites of replication stress. (A) Representative images of FCD-2 immunostaining in the mitotic region of the germline in N2(Wt) and *fcd-2(tm1298)* mutants before (untreated) and 18 h post-treatment with 180 μM CDDP, 40 mM hydroxyurea (HU) and 2 h post-treatment with 75 Gy ionizing radiation (IR). (B) Quantification of FCD-2 focus formation in mitotic nuclei at 0, 4 and 20 h post-treatment with 180 μM CDDP in strains of the indicated genotype. Error bars indicate S.E.M. from at least 100 nuclei from each genotype.

which of these lysines is mono-ubiquitylated in response to ICL damage. However, the recent demonstration that a fusion of mono-ubiquitin to the C-terminus of FANCD2 is able to rescue the vast majority of the defects caused by mutation of K561 (the site of mono-ubiquitylation in the wild type FANCD2 protein) indicate that whilst mono-ubiquitylation is critically important for FANCD2 function the site of modification on the protein is largely irrelevant [11].

2.5. *FCD-2 is recruited to sites of replication stress following exposure to ICL-inducing agents or hydroxyurea*

Mono-ubiquitylation of FANCD2 by the FA core in response to replication stress is important for its chromatin association and subsequent recruitment to DNA damage sites where it forms discrete nuclear foci [10]. Since FANCD2 focus formation is readily induced in complex eukaryotes following treatment with ICL-inducing agents or with hydrozyurea [10], we next assessed whether FCD-2 is recruited to sites of ICL repair or replication stress. FCD-2 immunostaining of the *C. elegans* germline from untreated N2(Wt) animals gave no detectable staining (Fig. 5A). In contrast, 18 h following treatment with 180 μM CDDP or 40 mM hydroxyurea robust FCD-2 nuclear foci are observed in N2(Wt) (Fig. 5A), exclusively in the mitotic zone of the germline, the sole region of the adult animal that is actively proliferating under normal growth conditions (Fig. 3A). Treatment of N2(Wt) animals with 75 Gy IR failed to induce significant numbers of FCD-2 focus formation (Fig. 5B), consistent with our previous result that *fcd-2* is dispensable for survival following IR (Fig. 2E). Importantly, focus formation following CDDP or HU treatments is abolished in *tm1298* and *ok1145* mutants (Fig. 5A and B) indicating that the nuclear foci detected by this antibody are specific for FCD-2. Consistent with the fact that formation of ICL lesions following CDDP treatment take several hours in mammalian cells [28], FCD-2 focus formation is marginally elevated in N2(Wt) animals 4 h after CDDP treatment, but is only readily detected in mitotic nuclei 20 h post-treatment (Fig. 5B). Collectively, these data suggest that FCD-2 responds to ICL-lesions that cause replication fork arrest but is not recruited to DSBs *per se*. Since FCD-2 focus formation is restricted to proliferating cells in the germline (mitotic zone; Fig. 3A), and foci are induced by replication fork stalling with HU or ICL-inducing agents, we conclude that FCD-2 foci correspond to sites of replication fork arrest, analogous to our previous findings with ATL-1, which is known to form foci at stalled replication forks [27]. Our observation that FCD-2 is subjected to mono-ubiquitylation and is recruited to sites of ICL repair strongly suggest that the FA pathway is functionally conserved in *C. elegans*.

3. Discussion

It is known that the FA pathway is required for ICL repair, yet how the FA pathway functions at the molecular level in resolving such lesions and promoting restart of stalled replication forks remains unresolved [1,2]. It is therefore important to establish model organisms that can aid in mechanistic studies as well as to elucidate the interplay between FA proteins and other pathways believed to be involved in ICL repair. To date, only higher eukaryotes such as mouse, chicken and more recently zebrafish have been shown to possess functional homologues of FANCD2 in addition to some other FA factors [11,29–32]. No FA homologues exist in yeast, hindering rapid genetic analyses that could be used to identify novel components and/or factors that regulate normal FA function. Here we demonstrate that a functionally conserved, albeit simplified, FA pathway exists in *C. elegans*, an organism in which powerful genetics and biochemistry are easily attainable.

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The hallmark of FA is an exquisite sensitivity to agents that generate DNA interstrand cross-link lesions [4,33]. We show that *C. elegans* mutants defective for FCD-2 are exquisitely sensitive to a range of different ICL-inducing agents, yet are relatively insensitive to IR, strongly suggesting that FCD-2 is a true functional homologue of human FANCD2 (Fig. 2 [34]). We also demonstrate that FCD-2 mutants are capable of eliciting checkpoint activation in mitotic cells in response to replication stress (Fig. 3), indicating that the increased sensitivity of these mutants to ICL-inducing agents is likely due to defective repair, rather than detection and signalling of the damage. The key event associated with activation of the FA pathway is the ubiquitylation of FANCD2 by the core complex and recruitment to sites of DNA damage and/or stalled replication [7,10]. To date, we have been unable to identify any potential *C. elegans* homologues of the FA core complex. However, we clearly detect mono-ubiquitylation of FCD-2 upon exposure to ICL-inducing agents (Fig. 4). These data suggest that this central feature of the FA pathway is conserved in the nematode and that a simplified pathway can carry out similar functions to its more complex human counterpart, as is the case for many lower eukaryotic systems. Such simplification will aid dissemination of the key functions of each individual protein involved in the *C. elegans* FA pathway, avoiding complications arising through redundancy. Given that *C. elegans* appears to lack obvious functional homologues of any of the proteins that comprise the FA core complex, it raises the question of what factors are involved the mono-ubiquitylation of FCD-2? At present, positive identification of a FANCL homologue has eluded us, although many ubiquitylation enzymes have been identified in the nematode [35]. Interestingly, a recent large-scale yeast two-hybrid study by Li et al. identified K01G5.1 as a potential interacting factor with FCD-2 [36]. Domain mapping based on sequence analysis shows that K01G5.1 possesses a PHD/RING finger domain, suggesting possible E3 ligase activity. Determining whether K01G5.1 is a true functional homologue of FANCL is the focus of an ongoing study within the lab. In addition to CeBRC-2(FANCD1), we have recently identified homologues of FANCJ and FANCM in *C. elegans* that will be described in detail elsewhere. Therefore, *C. elegans* possesses homologues of FA factors that function both upstream and downstream of FCD-2 mono-ubiquitylation and recruitment.

Consistent with a role for FCD-2 in the repair of ICL lesions, we observe FCD-2 recruitment to sites of ICL damage and stalled replication forks (Fig. 5). This response is specific to actively dividing cells of the adult worm, namely the mitotic region of the germline (Fig. 3A). This highlights a conserved role for FCD-2 in promoting the restart of replication forks that have stalled as a consequence of impeding ICL lesions. Also, discrete FCD-2 nuclear foci are only observed after several hours of cisplatin treatment (Fig. 5), which is consistent with the time taken for drug uptake and the formation of ICL lesions [28].

The importance of demonstrating a functional conserved FA pathway in *C. elegans* is highlighted by the fact that homozygous deletion mutants exist in many genes involved in the repair of ICL lesions that are inviable in other organisms, e.g. BRCA1/BARD1, BRCA2 and Rad51 [18–21,27,37]. Also, the rapid and powerful genetics available in *C. elegans* should augment future studies of the interplay between these factors and the FA pathway, which cannot easily be assessed in higher eukaryotes such as mice and mammalian cells. Indeed, work by others has now demonstrated that *C. elegans* possess functional homologues of the BLM1 helicase, the TLS polymerase polε, the exonuclease Exo3 and NER factor XPF [38–41]. Work in mammalian cells has shown that these factors play important roles in the repair of ICL lesions and likely co-operate with FANCD2 in resolving such lesions prior to restart of stalled replication forks. Furthermore, recent work in our laboratory has demonstrated that *C. elegans* proteins can be tagged to facilitate the biochemical purification of complexes associated with the tagged protein following treatment of animals with various DNA damaging agents [21,42]. This recent work highlights another advantage of the use of *C. elegans* as a model organism to study complex processes such as ICL repair in order to determine how multiple pathways interrelate with each other and how individual factors within these pathways interplay at the molecular level. We believe that use of *C. elegans* as a model organism in which to study both the FA pathway *per se* and the general process of ICL repair will aid in the rapid identification of novel components and further our understanding as to how mammalian cells deal with such complex lesions.

4. Materials and methods

4.1. Strains and culture conditions

C. elegans strains were cultured as described previously [43]. *fcd-2*(*tm1298*) and *fcd-2*(*ok1145*) strains were generated and kindly provided by Shoehi Mitani of the National Bioresource Project for the *nematode*, Japan, and the *C. elegans* knockout consortium, respectively. *atl-1*(*tm853*) was described previously [21,27]. Hydroxyurea (HU) treatment was performed by transferring L4 larva stage animals of the appropriate genotype to plates containing 40 mM HU for 16 h prior to analysis, as previously described [26]. To assess ICL sensitivity, young adult worms were exposed to 10 μg/ml trimethylpsoralen (TMP) in S basal for 1 h, before UVA irradiation (80–200 J) at a dose rate of 1 mW/cm^2. The progeny of individual worms were quantified for the period 22–26 h post-treatment, and unhatched eggs were identified after a further 48 h incubation. Sensitivity to cisplatin (CDDP), mitomycin (MMC), or nitrogen mustard (HN2), was performed in an analogous manner.

4.2. Sequence alignments

Protein sequences were aligned using pileup and refined using the lineup algorithm (Genetics Computer Group). Multiple sequence files were exported to ESPript 2.0 at http://www.prodes.toulouse.inra.fr/ESPript/cgi-bin/nph-ESPript_exe.cgi for box-shading analysis.

4.3. Antibodies, cytological preparation and immunostaining

Synthetic peptides were generated by the Peptide Synthesis Laboratory, Cancer Research UK, and used to generate affin-

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ity purified rabbit anti-FCD-2 polyclonal antibodies, as previously described [21]. FCD-2 N: (n-CTDISLQQSTAVRLHKEC-c) and FCD-2 C: (n-CSRCLKYDMSASAAKN-c). Immunoaffinity purification of FCD-2 prior to detection of mono-ubiquitylation (ubiquitin antibody; Affiniti, UK) was performed as previously described [21]. For immunostaining, gravid hermaphrodites were transferred to 30 μl PBS on a poly-L-lysine coated slides. The worms were washed in PBS before transferring to 50 μl 10 mM levamisole and germlines were extruded by removing the head and tail using a fine gauge needle (27 G). Levamisole was replaced with 1% para-formaldehyde (PFA) in PBS for 10 min and germlines were permeabilised for 5 min in TBSBT (TBS + 0.5% BSA + 0.1% Triton X-100), then washed in TBSB for at least 2× 5 min, followed by DAPI staining or blocking for 30 min. Primary antibodies were diluted in TBSB (1:500 for FCD-2, 1:200 for RAD-51, 1:200 SYP-1) and incubated overnight at 4 °C in a humid chamber. Germlines were subsequently washed at least 3× 5 min in TBSB before incubation with the secondary antibody for 1–2 h at room temperature (anti-rabbit Cy3 1:10,000; anti-guinea FITC 1:5000 (Sigma)). Finally germlines were washed at least 3× 5 min in TBSB before mounting with a cover slip on Vectashield containing DAPI (Vector Laboratories). Deltavision microscopy was used to examine germlines on an Olympus inverted microscope (IX71), and images captured using SoftWorx computer software (Applied Precision) as previously described.

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