A DOG’s View of Fanconi Anemia: Insights from C. elegans

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C. elegans provides an excellent model system for the study of the Fanconi Anemia (FA), one of the hallmarks of which is sensitivity to interstrand crosslinking agents. Central to our understanding of FA has been the investigation of DOG-1, the functional ortholog of the deadbox helicase FANCJ. Here we review the current understanding of the unique role of DOG-1 in maintaining stability of G-rich DNA in C. elegans and explore the question of why DOG-1 animals are crosslink sensitive.

We propose a dynamic model in which noncovalently linked G-rich structures form and un-form in the presence of DOG-1. When DOG-1 is absent but crosslinking agents are present the G-rich structures are readily covalently crosslinked, resulting in increased crosslinks formation and thus giving increased crosslink sensitivity. In this interpretation DOG-1 is neither upstream nor downstream in the FA pathway, but works alongside it to limit the availability of crosslink substrates. This model reconciles the crosslink sensitivity observed in the absence of DOG-1 function with its unique role in maintaining G-Rich DNA and will help to formulate experiments to test this hypothesis.

1. Introduction

The helicase, FANCJ, is required for the Fanconi Anemia (FA) pathway to function properly and thus maintain genome integrity. In humans, FANCJ mutations have been identified in early-onset breast cancer patients [1, 2] and FA complementation group J patients [3–5]. However, the role of FANCJ in the FA pathway of DNA repair is not fully understood. Some insights have been gained from research on DOG-1 (Deletions Of G-rich DNA), the Caenorhabditis elegans functional ortholog of FANCJ [6–9]. However, even in this relatively simple model system, important questions remain. An outstanding issue is the relationship between the relatively well-known function of DOG-1/FANCJ in preventing replication blocks at unresolved secondary structures and its function in resistance to interstrand crosslinks (ICLs). Previous work from our group has shown that DOG-1 acts upstream of, or parallel to, FCD-2 in the maintenance of G-tracts [7] but is dispensable for FCD-2 focus formation in response to ICL generating agents [8]. One possibility is that DOG-1 takes on two different functions, one in G4 DNA resolution and one in FA crosslink repair. On the other hand, it is possible that its ability to unwind G-rich secondary structure may be sufficient to explain its role in both situations.

Here we summarize the current understanding of DOG-1/FANCJ function and hypothesize how to reconcile the two known roles for this protein with its helicase function.

2. DOG-1 Is Required for Maintenance of G-Tracts

DOG-1 was discovered as being essential for the maintenance of G-rich DNA [6] and was subsequently shown to be the functional ortholog of FANCJ [8]. The value of C. elegans as a model for Fanconi Anemia and ICL repair has been thoroughly reviewed in Youds et al. [9]. An understanding of DOG-1’s role in replication and repair began with the observation that it is a mutator. This was immediately recognizable in C. elegans because of the appearance of spontaneous morphological mutants (described in Cheung et al. [6]) and further explored by the capture and characterization of mutational changes in genes essential for survival (lethal mutations) maintained using a genetic balancer [10]. In dog-1 mutants, the manifestation of the morphological Vab (Variable ABnormal) phenotype was linked to the gene vab-1. An examination of the molecular nature of the vab-1 mutations revealed small deletions that were detectable by PCR. These deletions initiated at the 5’ end of poly-C or
the 3' end of poly-G stretches of DNA and extended for variable distances. These observations led to the proposal that the deletions were occurring as a result of structural blocks to lagging-strand synthesis [6]. In this model, poly-G stretches present in the C. elegans genome form secondary structures. These secondary structures require the helicase function of DOG-1 to resolve them, allowing fork progression. In the absence of the helicase function, deletions are formed between the stalled fork and the upstream Okazaki fragment initiation. Another research group subsequently confirmed the prediction that Okazaki-sized deletions occurred on the lagging strand by using unbiased array comparative hybridization (aCGH) of DOG-1-minus genomes [11]. In this study, it was shown that deletions occurred exclusively at sequences that could form quadruplex structures (G4) at a frequency of 4% per site per animal generation. In the human genome, there are estimated to be >300,000 G4 forming sites [12], and these have potentially mutagenic properties implicated in development of cancer susceptibility in the absence of FANCJ function.

Further work from our laboratory revealed that in the absence of DOG-1 large chromosomal rearrangements occurred [10]. The rearrangements included larger deletions, duplications of chromosomal fragments, and translocations between chromosomes, in addition to the small deletions detectable by PCR. These large rearrangements were identified because they acquired lethal mutations, which could be isolated and characterized with the use of a balancer chromosome that provided a rescuing wild-type allele in a stable genetic construct (reviewed in [13]). The analysis showed that 1% of the chromosomes acquired lethal lesions [10], giving a forward mutation frequency greater than tenfold of the spontaneous frequency. The frequency is equivalent to that for 500 Rads of ionizing radiation [14]. Rearrangements derived from dog-1 mutant that were examined by aCGH revealed that in most (but not all) cases the breakpoints occurred in G-rich DNA. In one example, a translocation between chromosome V and the X-chromosome was formed. In this case, the right end of the X-chromosome was duplicated and attached to the left breakpoint of a deletion at left end of chromosome V (Figure 1). The breakpoint on chromosome V is in a 24 bp G/C tract, while the breakpoint on the X is in a “short” 13 bp G-rich sequence. In vertebrates, large rearrangements have also been observed in the absence of FANCJ function. In avian DT40 cell lines, large-scale genomic deletions occurred at the rearranged immunoglobulin heavy chain locus (IgH) in the absence of FANCJ, but not other FA genes [15]. These researchers found that in FANCJ mutant cells cultured for two months, G4 sequences detected by aCGH were found at the breakpoints of one deletion. However, not all breaks occurred in G-rich DNA, suggesting that other sequences are also susceptible to breakage in the absence of FANCJ.

3. Homologous Recombination and Translesion Synthesis Compensate for the Absence of DOG-1

Repair pathways that compensate for the absence of DOG-1 in C. elegans have been identified. These include homologous recombination (HR) repair and translesion synthesis (TLS), but not nonhomologous end joining (NHEJ) [7]. In human cell lines, monoubiquitylation of FANCD2 is followed by HR repair. Our genetic analysis has shown that DOG-1 mutants
that are also mutant for FCD-2 (FANCD2) exacerbate G-tract deletions [8], as are the HR repair components, BRD-1 (BARD1), RAD-51 (RAD51), and XPF-1 (XPF). Similarly, DOG-1 mutants lacking the TLS polymerases, POL eta and POL kappa have significantly more PCR-detectable G-tract deletions than DOG-1 by itself. That the FA pathway and its downstream repair mechanisms are capable of resolving some G-tract-associated secondary structures in the absence of DOG-1 function indicates that the FA pathway is parallel to DOG-1, at least with respect to the maintenance of G-tracts.

A recent study in DT40 cells has expanded the endogenous role of FANCJ. Recently, Sarkies et al. have shown that FANCJ coordinates two independent mechanisms to maintain epigenetic stability near G4 DNA motifs [16]. These mechanisms are dependent on the function of the Y-family polymerase REV1 and the helicases WRNs and BLMs. Similar epigenetic studies have not been performed in C. elegans. However, G-tract instability is significantly increased in DOG-1 mutants animals deficient in the BLM ortholog HIM-6 [7]. Mutants in the C. elegans WRNs ortholog WRN-1 do not exacerbate G-tract deletions, indiecting that if a function in G-tract resolution is conserved in C. elegans, it is dependent on the presence of DOG-1. The C. elegans REV1 ortholog REV-1 has not been studied with respect to G-tract stability.

4. DOG-1 Functions to Reduce ICL-Induced Damage

A diagnostic feature of FA defects is the cross-link sensitivity of cultured cells. The presence of ICLs can result in error-prone repair leading to chromosomal instability (CIN) and cell death. In C. elegans, the absence of DOG-1 also results in sensitivity to ICL-inducing agents such as UVA-activated trimethylpsoralen, nitrogen mustard, and cisplatin, but not to X-rays or UVC [8]. Treatment of DOG-1-deficient animals with ICL agents can result in checkpoint-induced cell cycle arrest and apoptosis of germ cells, as well as chromatin bridges and breaks [8]. In response to ICL treatments, animal’s doubly mutant for DOG-1 and FCD-2 are as equally sensitive as each of the single mutants, potentially placing the helicase function of DOG-1 in the same pathway as FCD-2 [8]. Furthermore, DOG-1 is not required for RAD-51 or FCD-2 foci formation after replication stress or ICL induction, possibly placing DOG-1 downstream of FCD-2. This data correlates with that reported by Bridge et al. [17] who demonstrated that FANCJ mutant DT40 cells are also not defective for FANCJ2 focus formation.

In human cell lines, monoubiquitination of FANCD2 is followed by HR repair. During S phase, ICLs can block replication; consequently, HR and TLS are required to stabilize the fork and restart replication (reviewed in [18]). In C. elegans, HR repair alleviates the loss of DOG-1. DOG-1 does not function directly in DSB repair, however, as it is not sensitive to radiation-induced DSBs [8]. Bridge et al. determined that FANCJ’s role in ICL repair is independent of BRCA1 function by demonstrating rescue of FANCJ phenotypes in DT40 cells with the expression of human FANCJ/BRIP1 lacking its BRCA1-interaction domain [17]. Since DOG-1, like the avian FANCJ, does not contain the BRCA1 interaction domain found in human FANCJ (Figure 2), we infer that the helicase function of DOG-1 is not required for HR-mediated DSB repair following replication block or ICL induction.

The type of repair pathway recruited following replication block is important in maintaining genome stability. In C. elegans [8] and in human and chicken cells [19], FA proteins regulate the decision to repair double strand breaks (DSBs) resulting from replication blocks or ICLs using error-free HR repair rather than error-prone nonhomologous end joining (NHEJ). In the Adamo et al. study [20], it was shown that FA-deficient human cell lines and C. elegans mutants
had chromosomal abnormalities similar to those found in cell lines from cancer and FA patients. However, when the NHEJ component LIG-4 (LIG4) is lacking, the abnormalities do not occur. HR-mediated repair is proposed to be favored due to single-stranded DNA produced by FANCD2 [19]. In C. elegans, this result provides a potential inroad to further dissection of the role of FA in DNA repair and the maintenance of genome stability.

The relationship between TLS and HR repair in C. elegans has been teased apart somewhat by the characterization of two genes, polq-1 (POLQ) and hel-308 (HELQ) [21]. POLQ-1 has a helicase domain at the N-terminus and a polymerase domain at the C-terminus and has been implicated in recombination-independent and TLS-dependent ICL repair (reviewed in [22]). The helicase HEL-308, on the other hand, is proposed to function in HR along with the FA pathway in ICL repair. In C. elegans, there are two genetically distinct pathways, a BRC-1-POLQ-1 pathway and an FA (FCD-2, DOG-1)-HR-HEL-308 pathway. At least one of these pathways must be functional for animals survival as mutants in hel-308 results in synthetic lethality when combined with brc-1 mutants (reviewed in [9]). These results separate the helicase function of DOG-1 from the BRC-1/BRCA-1 repair pathway and further distinguish the role of DOG-1 as independent of HR repair. Initially these results may appear paradoxical. FCD-2 is not required for G-tract stability and the double mutant dog-1; fcd-2 increased G-tract deletions 3-fold [8], placing DOG-1 upstream of the FA pathway. However, in the case of ICL sensitivity, the double mutant is not more sensitive. One interpretation of these data is that DOG-1 is epistatic to the FA pathway. Both findings are consistent with DOG-1 attempting unsuccessfully to remove the cross-linked structure.

How does this inform our understanding of DOG-1’s helicase function and the relationship between G-rich secondary structures and ICLs? There is ample evidence that DOG-1 is unique in its role to maintain G-rich DNA that can form G4-like secondary structures [7, 8, 11]. Additionally, it has been demonstrated that purified FANCJ efficiently unwinds a variety of G4 structures dependent upon intrinsic FANCJ ATP hydrolysis and the availability of a 5’ ssDNA tail [22]. None of the other helicases that are able to unwind G4 structures can compensate for the loss of DOG-1. This is supported by the fact that in C. elegans DOG-1 has a unique phenotype and that in other systems only FANCJ has been shown to prevent breaks in G-rich DNA. These structures are, however, not covalently linked. There is no evidence that the DOG-1/FANCJ helicase can resolve covalently linked ICLs. So what is the connection?

We propose the following model as a resolution of this apparent paradox (Figure 3). G4 structures are known to form in a variety of circumstances as proposed by Wu et al. [22], which could include within a single strand of DNA, between DNA strands and between strands on separate chromosomes. The latter resulting in chromosomal translocations if not repaired correctly. In the absence of crosslinking agents, these secondary structures can form and uniform depending upon the availability of DOG-1. In the C. elegans genome, there are nearly 400 poly-G regions distributed

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**Figure 3:** A model for DOG-1 function in genome stability and ICL response. The left panel illustrates DOG-1’s role in G-tract maintenance. G4 formation on the lagging strand is resolved by the helicase function of DOG-1 and replication proceeds efficiently. In the absence of DOG-1 HR mediated by the FA pathway resolves a subset of stalled forks. Repair utilizing the mutagenic NHEJ repair mechanism results in deletions. The right panel describes a possible model for DOG-1 ICL sensitivity. In the presence of DOG-1, G4 structures may be resolved and not available as substrate for ICL stabilization. In the absence of DOG-1 G4 structures are available as substrate for ICL stabilization leading to an in increase in fork stalling, which is interpreted as an ICL sensitivity phenotype.
along each of the chromosomes and this pattern of distribution is conserved in a related nematode [23] providing a rich source of substrate for DOG-1. In the presence of a crosslinking agent, many of which have affinity for G’s, secondary structures formed by these G-rich regions might be targets for covalent crosslinking. Here we suggest that once the secondary structures are detected by FA pathway components the first responder is DOG-1. The pathway detector may not distinguish between a noncovalent secondary structure and a crosslink. If the structure is not covalently linked, DOG-1 resolves it. If it is covalently linked, and not resolved by DOG-1, FA pathway-directed TLS and HR repair the lesion. In the absence of DOG-1, there is likely to be an increase in stabilized G-rich structures that may be beyond the ability of the FA pathway to respond to, giving the appearance of a crosslink sensitive phenotype. Further experiments will be needed to move towards a more complete understanding of the crosstalk among FA proteins.

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