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REVIEW

The Fanconi anemia pathway and DNA interstrand cross-link repair

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ABSTRACT

Fanconi anemia (FA) is an autosomal or X-linked recessive disorder characterized by chromosomal instability, bone marrow failure, cancer susceptibility, and a profound sensitivity to agents that produce DNA interstrand cross-link (ICL). To date, 15 genes have been identified that, when mutated, result in FA or an FA-like syndrome. It is believed that cellular resistance to DNA interstrand cross-linking agents requires all 15 FA or FA-like proteins. Here, we review our current understanding of how these FA proteins participate in ICL repair and discuss the molecular mechanisms that regulate the FA pathway to maintain genome stability.

KEYWORDS Fanconi anemia, DNA interstrand cross-link repair, FANCD2-FANCI, mono-ubiquitylation, chromosomal instability

INTRODUCTION

Studying rare genetic disorders with cancer susceptibility has provided valuable insights into the cause and treatment of more common diseases. An example is Fanconi anemia (FA), a rare genetic cancer-susceptibility syndrome. Studies of this disease have not only elucidated the general mechanisms of bone marrow failure, but also increased our knowledge of the molecular genetics and molecular pathogenesis of human cancers. Additionally, the Fanconi anemia pathway is a powerful model system for studying DNA damage signaling and tumorigenesis (Garcia-Higuera et al., 2001a; D'Andrea and Grompe, 2003; Kennedy and D'Andrea, 2005; Thompson et al., 2005; Wang, 2007; de Winter and Joenje, 2009; Rego et al., 2009).

FA was first described by the Swiss pediatrician Guido Fanconi in 1927, featuring congenital malformations, progressive bone marrow failure, cancer predisposition (Alter et al., 2003), and cellular hypersensitivity to DNA interstrand cross-linking agents, such as cisplatin and mitomycin C (MMC) (Sasaki, 1975; German et al., 1987; Auerbach, 1988). FA occurs equally in males and females and is found in all ethnic groups. Though considered primarily a blood disease, FA may affect all systems of the body. FA patients are very likely to develop a variety of cancers at a much earlier age than the general population and are usually smaller than average (Kee and D'Andrea, 2010).

Cloning of FA genes and discovery of their functional interconnections with BRCA genes paved the way to our understanding of the mechanisms of the FA pathway in the maintenance of chromosomal integrity (Venkitaraman, 2004; Mirchandani and D'Andrea, 2006). Now 15 FA or FA-like genes [A, B, C, D1 (BRCA2), D2, E, F, G, I, J (BACH1/BRIP1), L, M, N (PALB2), P (SLX4/BTBD12) and O (RAD51C)] have been identified and these genes lead to over 95% of all known FA patients. Mutations in FA-A, FA-C and FA-G are the most common and account for approximately 85% of patients with FA. FA-D1, FA-D2, FA-E, FA-F and FA-L account for approximately 10%. FA-B, FA-I, FA-J, FA-M, FA-N, FA-P and FA-O result in less than 5%.

OVERVIEW OF THE FANCONI ANEMIA PATHWAY

FA is composed of at least 14 complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L, M, N and P) and one FA-like complementation group (FA-O) (Kee and D'Andrea, 2010; Meindl et al., 2010; Vaz et al., 2010; Crossan et al., 2011; Stoepker et al., 2011; Yamamoto et al., 2011a). Identification of the 15 FA and FA-like genes [FANCA, B, C, D1 (BRCA2), D2, E, F, G, I, J (BACH1/BRIP1), L, M, N (PALB2), P (SLX4/BTCD12) and O (RAD51C)] has led to a significant progress in understanding of this disease. It is believed that all the 15 FA or FA-like proteins cooperate in a common pathway

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required for the cellular resistance to DNA interstrand cross-linking agents, and this pathway is now called “the Fanconi anemia pathway.” (Fig. 1)

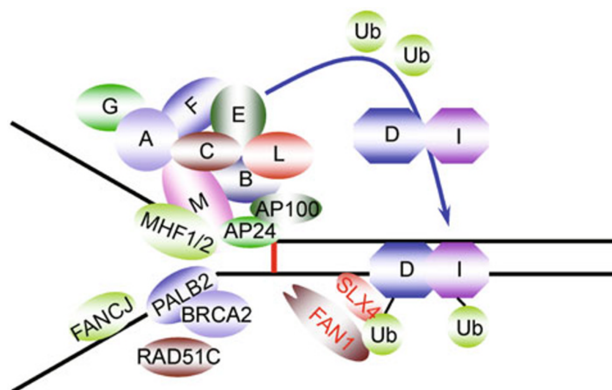

Figure 1. A schematic model for the FA pathway. MHF1-2 and FAAP24 recruit a large multi-subunit ubiquitin E3 ligase, termed the FA core complex, to DNA lesions. The core complex then mono-ubiquitylates FANCD2 and FANCI, mono-ubiquitylated FANCD2-FANCI recruit nuclease FAN1 to damage sites and colocalize with downstream FA proteins (PALB2, BRCA2, FANCJ, RAD51C and SLX4), and facilitate DNA interstrand cross-link repair.

Among the 15 FA or FA-like proteins, eight are assembled into a nuclear complex (A, B, C, E, F, G, L, and M), termed FA core complex (Kennedy and D’Andrea, 2005; Wang, 2007). A major function of the FA core complex is to mono-ubiquitylate its two substrates, FANCD2 and FANCI. FANCD2 and FANCI form a protein complex called the ID complex (Montes et al., 2005; Dorsman et al., 2007; Smogorzewska et al., 2007). This mono-ubiquitylation event results in the ubiquitin tagged ID complex assembling at the sites of DNA damage in the nucleus where they subsequently colocalize with downstream effectors of FA proteins, including FANCD1 (BRCA2), FANCN (PLAB2), FANCJ (BACH1/BRIP1) and FANCO (RAD51C) (Garcia-Higuera et al., 2001b; Hussain et al., 2004; Wang et al., 2004; Xia et al., 2006; Kumaraswamy and Shiekhattar, 2007; Meindl et al., 2010; Vaz et al., 2010) .

Although mutations in 1 of the 15 FA or FA-like genes account for most cases of the FA disease, there are still some FA patients with unassigned subtypes and identification of additional FA or FA-associated genes will give further insights into the molecular functions and mechanisms of the FA pathway (Moldovan and D’Andrea, 2009).

FA CORE COMPLEX IS A MULTI-SUBUNIT E3 UBIQUITIN LIGASE

Among these gene products, FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM and two FA-associated proteins FAAP24 and FAAP100, as well as the recently identified FANCM-associated histone-fold-containing protein complex, MHF1 and MHF2, constitute the FA core complex, serving as a multi-subunit ubiquitin E3 ligase for FANCD2-FANCI mono-ubiquitylation (Kennedy and D’Andrea, 2005; Wang, 2007). The subcomplex containing FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCL is detected throughout the cell cycle, whereas FANCM, FAAP24, FAAP100, MHF1 and MHF2 appear to be functionally different since they are not constitutively associated with the core complex (Ciccia et al., 2007; Kim et al., 2008; Singh et al., 2010; Yan et al., 2010). Instead, they are required for recognizing stalled replication forks and are indispensable for recruiting the core complex to DNA damage sites (Ciccia et al., 2007; Kim et al., 2008; Singh et al., 2010; Yan et al., 2010). Although FAAP24, FAAP100, MHF1 and MHF2 have shown to be required for efficient mono-ubiquitylation of FANCD2-FANCI and inactivating mutations in any of these gene products destabilize the FA core complex, however, to date, no mutations of FAAP24, FAAP100, MHF1 or MHF2 were found in FA patients (Ciccia et al., 2007; Ling et al., 2007).

Given that all the core components are required for FANCD2-FANCI mono-ubiquitylation and FA pathway activation, each of them must have a certain role in regulating the downstream events in the FA pathway. Several protein-protein interaction studies have provided insights into the molecular architecture of the FA core complex. FANCA and FANCG form a stable subcomplex (Garcia-Higuera et al., 2000), whereas FANCB, FANCL, and FAAP100 are partners in another subcomplex (Medhurst et al., 2006). Subcomplex of FANCC and FANCE interacts with the other subcomplexes and this interaction is stabilized by the FANCF protein (Taniguchi and D’Andrea, 2002; Gordon and Buchwald, 2003). FANCM can form independent subcomplexes with FAAP24 and MHF1-MHF2, respectively (Ciccia et al., 2007; Kim et al., 2008; Singh et al., 2010; Yan et al., 2010). FANCL contains a plant homeodomain (PHD) and functions as an E3 ligase for catalyzing the mono-ubiquitylation of FANCD2-FANCI (Alpi et al., 2008; Hodson et al., 2011). The E2 ubiquitin-conjugating enzyme is approved to be UBE2T, which specifically interacts with the PHD domain of FANCL and is therefore involved in FA ubiquitylation cascade (Meetei et al., 2003; Gurtan et al., 2006; Machida et al., 2006). Like FANCL, disruption of any other core members leads to impaired ubiquitylation of FANCD2 or FANCI; however, whether the other core members only function as structural stabilizers, or are responsible for some events that are critical for FANCD2-FANCI mono-ubiquitylation, are still not well elucidated. Generally, revelation of protein functions is aided by domain analysis of their primary structure. However, unlike FANCL, the other core proteins harbor no recognizable functional domain, which makes it harder to speculate their molecular functions. Interaction experiments have shown the existence of subcomplexes, but it remains unclear whether

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these subcomplexes have anything to do with their functions.

FANCD2-FANCI MONO-UBIQUITYLATION IS THE CENTRAL EVENT IN THE FA PATHWAY

Protein ubiquitylation is emerging as an important form of covalent modification that regulates various biological processes including DNA damage checkpoints and DNA repair pathways (Ramaekers and Wouters, 2011). During S-phase or in response to DNA damage, FANCD2 and FANCI become mono-ubiquitylated (Montes et al., 2005; Dorsman et al., 2007; Smogorzewska et al., 2007). This modification results in the translocation of these two proteins to chromatin within cells where they assemble into DNA repair foci. In these foci, this ID complex interacts with downstream FA proteins (FANCD1, FANCN, FANCJ, FANCP and FANCO) (Hussain et al., 2004; Wang et al., 2004; Xia et al., 2006; Sy et al., 2009; Zhang et al., 2009) and proteins of other DNA repair pathways (e.g. RAD51, PCNA and REV1) (Niedzwiedz et al., 2004; Nojima et al., 2005; Guo et al., 2006; Howlett et al., 2009; Geng et al., 2010; Long et al., 2011), suggesting possible crosstalk between the FA pathway, homologous recombination (HR), and translesion synthesis (TLS).

Recent studies have provided mechanistic insights into how the E1-E2-E3 cascade mediates the mono-ubiquitylation of FANCD2 and FANCI. A yeast two-hybrid screen with FANCL as a bait leads to the discovery of UBE2T, the E2 enzyme in the FANCD2 and FANCI mono-ubiquitylation pathway (Machida et al., 2006). FANCL recruits UBE2T via its PHD domain, whereas its WD40 repeats bind and stabilize the FA complex (Gurtan et al., 2006). FANCE binds directly to FANCD2 (Pace et al., 2002), suggesting that it mediates the interaction of the FA ubiquitin ligase complex with its substrates. However, we still do not know exactly how the E2 enzyme UBE2T, the FA ubiquitin ligase complex, and the substrates (FANCD2 and FANCI) are brought together in living cells, as they are recruited independently to chromatin (Alpi et al., 2007; Alpi et al., 2008).

Besides the FA core complex, ataxia telangiectasia and Rad3-related kinase ATR is also required for efficient FANCD2 mono-ubiquitylation (Friedel et al., 2009). In response to ICL, FANCD2 is phosphorylated in an ATR-dependent manner and this phosphorylation takes place on threonine 691 and serine 717 sites (Ho et al., 2006). Phosphorylation of FANCD2 on these two sites thereby promotes FANCD2 mono-ubiquitylation and enhances cellular resistance to DNA cross-linking agents (Ho et al., 2006). Phosphorylation of these two sites is also required for establishment of the intra-S-phase checkpoint response (Ho et al., 2006). Like FANCD2, phosphorylation of FANCI has also been shown to be necessary for the mono-ubiquitylation and localization of both FANCI and FANCD2 to DNA damage sites (Ishiai et al., 2008a). Since FANCI carrying phosphomimic mutations showed constitutive activation of the FA pathway even in the absence of DNA damage (Ishiai et al., 2008b), it has been proposed that the phosphorylation of FANCI may function as a molecular switch to turn on the FA pathway (Ishiai et al., 2008a). Thus, understanding the mechanisms of how this switch may work and how phosphorylation of FANCI triggers the mono-ubiquitylation of FANCD2 will provide novel insights into how the FA pathway is regulated.

FANCD2 DEUBIQUITYLATION IS REQUIRED FOR EFFICIENT ICL REPAIR

Ubiquitylation is a reversible regulatory modification that can control protein functions. The deubiquitylation of FANCD2 has been shown to be equally important to its mono-ubiquitylation (Nijman et al., 2005; Cohn et al., 2007; Oestergaard et al., 2007). FANCD2 is deubiquitylated by USP1, a ubiquitin-specific cysteine protease that also hydrolyzes monoubiquitin from proliferating cell nuclear antigen (PCNA) (Huang et al., 2006; Brown et al., 2009). USP1 localizes to chromatin and interacts with FANCD2. The stability and enzymatic activity of USP1 is strongly stimulated by UAF1, the USP1 associated factor 1 (Cohn et al., 2007). UAF1 contains several WD40 repeats predicted to form a complete propeller structure that is important for the formation of an active protein heterodimer (Cohn et al., 2007). As expected, depletion of USP1 or UAF1 results in increased levels of chromatin-bound mono-ubiquitylated FANCD2 (Nijman et al., 2005; Cohn et al., 2007; Oestergaard et al., 2007); however, persistence of FANCD2 mono-ubiquitylation sensitizes DT40 and bone marrow cells to DNA cross-linking agents (Oestergaard et al., 2007; Murai et al., 2011). Although the precise mechanism involved in this regulation is still unknown, several possibilities have been indicated. First, deubiquitylation of FANCD2 may be required to release FANCD2 from DNA repair complex(es), allowing subsequent repair steps to take place in order to complete the ICL repair process (Nijman et al., 2005; Oestergaard et al., 2007; Kim et al., 2009). Second, persistence of FANCD2 mono-ubiquitylation may be toxic to cells, and causes a significant increase in MMC sensitivity (Nijman et al., 2005; Oestergaard et al., 2007; Kim et al., 2009).

FA PROTEINS ACT WITH MULTIPLE DNA REPAIR PATHWAYS TO PROMOTE ICL REPAIR

ICL are the most toxic lesions incurred during normal metabolism or cancer chemotherapy. ICL covalently tether both strands of a DNA duplex, thereby blocking DNA strand separation and preventing DNA metabolism, such as transcription and replication (Thompson et al., 2005; Kee and D'Andrea, 2010). Therefore, the repair of DNA ICL presents a unique challenge to cells and a single DNA repair

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pathway seems difficult to deal with such a formidable structure. Genetic analysis of *Saccharomyces cerevisiae* and mammalian DNA repair mutants has shown that various proteins implicated in FA, nucleotide excision repair (NER), HR and TLS involve in the detection and repair of ICL (De Silva et al., 2000; Dronkert and Kanaar, 2001; Grossmann et al., 2001; Wang et al., 2001; Niedzwiedz et al., 2004; Saffran et al., 2004; Niedernhofer et al., 2005; Lehoczký et al., 2007; Wang, 2007).

Once two adjacent replication forks converge at an DNA ICL, the crosslink is unhooked via dual incisions by structure-specific endonucleases such as MUS81/EME1 (Hanada et al., 2006), ERCC1/XPF (Bhagwat et al., 2009) and/or the newly identified FAN1 endonuclease (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010), which eventually leads to uncouple one sister chromatid from the other. The ssDNA gaps resulting from ICL unhooking are then filled by translesion DNA polymerases such as REV1, polymerase ζ and/or polymerase η (Waters et al., 2009; de Groote et al., 2011). The bypassed, unhooked crosslink resembles single nucleotide lesions and can be repaired by NER proteins (Cole, 1973; Evans et al., 1997; Kuraoka et al., 2000) or by the DNA glycosylase NEIL1 (Bandaru et al., 2002). Finally, homologous recombination allows the re-establishment of the replication fork and plays a crucial role in the restoration of the damaged DNA. In support, cells deficient in Rad51, RAD51 paralogs or other proteins involved in homologous recombination display hypersensitivity to DNA interstrand cross-linking agents (Meindl et al., 2010; Vaz et al., 2010; Long et al., 2011).

LATEST ADVANCES IN THE FA PATHWAY

Although the mono-ubiquitylation of FANCD2-FANCI licenses ICL repair (Knipscheer et al., 2009), the events downstream of ubiquitylation remain enigmatic. The emerging model of ICL repair in mammals implies that the mono-ubiquitylated FANCI-FANCD2 complex might promote the recognition and subsequent removal of DNA lesions in reactions that generally involve the nucleolytic cleavage of DNA strands through coordination with some known or yet-to-be-identified nucleases, yet the putative FANCD2-FANCI dependent nucleases have remained unclear. In this regard, the identification and characterization of FAN1 (Fanconi anemia-associated nuclease 1) (also known as KIAA1018) by four independent groups brings fundamental new information to the table (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). FAN1 was identified using three different approaches, including bioinformatics searching for nucleases containing ubiquitin-binding zinc finger (UBZ) domain, a mass spectrometry-based proteomic approach for deciphering protein-protein interaction and a genome-wide small hairpin RNA screen for sensitivity to cross-linking agents MMC. FAN1 contains an UBZ domain at its N terminus and a conserved VRR-nuclease domain at its C terminus. When ICL occurs, FANCD2 and FANCI are modified by a single ubiquitin moiety and compromise a complex at sites of DNA lesions. Subsequently, FAN1 is recruited to ICL through an interaction between its UBZ domain and the ubiquitylated ID complex (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). Depletion of FAN1 leads to increased cellular sensitivity to DNA cross-linking agent MMC (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). This ICL hypersensitivity can be rescued by wild-type FAN1 but not by mutants harboring point mutations at the nuclease or UBZ domain, indicating that both the nuclease activity and the UBZ domain of FAN1 are required for FAN1 function in ICL repair (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). Nevertheless, the exact function of FAN1 in ICL remains to be further clarified and the identification of the physiological substrate(s) of FAN1 during ICL will be the key to understanding the role of this nuclease.

More recently, RAD51C, one of the RAD51 paralogs that is essential for RAD51-mediated HR, was identified as a causative gene biallelically mutated in a family with "FA-like" phenotypes through analysis of a pedigree with characteristic congenital anomalies (Vaz et al., 2010). An accompanying study further reported monoallelic mutations in the same gene associated with increased breast and ovarian cancer risk (Meindl et al., 2010). These findings strengthen the close genetic relationship between FA and breast cancer-associated pathways (Garcia-Higuera et al., 2001b; Venkitaraman, 2004; Xia et al., 2006; Wang, 2007). However, while these studies indicated that RAD51C could be the 14th FA complementation group (FANCO), the assignment was tentative, since there was only a single family reported so far, and the only surviving patient (observed at age of 10 years) did not show the hematological abnormalities or malignancy commonly observed in FA patients (Vaz et al., 2010). Thus, the functional relationship between RAD51C and FA proteins needs to be further investigated.

The newest addition to the protein members of Fanconi anemia is FANCP (also known as SLX4 or BTBD12) (Crossan et al., 2011; Kim et al., 2011; Stoepker et al., 2011; Yamamoto et al., 2011b). SLX4/BTBD12 has been previously implicated as a scaffold that interacts with multiple proteins including nucleases XPF-ERCC1, MUS81-EME1 and SLX1 (Fekairi et al., 2009; Muñoz et al., 2009; Svendsen et al., 2009). Biallelic SLX4/BTBD12 mutations were uncovered in a total of six individuals from four unrelated kindreds of distinct geographical origin (Crossan et al., 2011; Kim et al., 2011; Stoepker et al., 2011; Yamamoto et al., 2011b). The clinical phenotypes of these six individuals were typical of that of classic FA and included congenital abnormalities and pediatric hematopoietic dysfunction. The cellular ICL hypersensitivity of these patient cells can be partially rescued by

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expression of the wild-type human SLX4/BTBD12 protein (Stoepker et al., 2011). Thus, biallelic mutations in the SLX4/BTBD12 gene underlie FA complementation group P (FANCP). Interestingly, like FAN1, FANCP/SLX4/BTBD12 also can be recruited to ICL through an interaction between its UBZ domain and the ubiquitylated ID complex (Yamamoto et al., 2011b). Moreover, *in vitro* experiments show that FANCP/SLX4/BTBD12 is able to enhance activity of its associated nucleases (Crossan et al., 2011; Kim et al., 2011; Stoepker et al., 2011; Yamamoto et al., 2011b). However, exactly at which step FANCP/SLX4/BTBD12 acts to promote ICL repair remains unclear.

SUMMARY AND PERSPECTIVES

Ever since the first identification of the FANCC gene, there have been tremendous advances in the molecular understanding of the FA disorder. Identification of FAN1, SLX4 and RAD51C as downstream members of the ICL repairosome of mono-ubiquitylated FANCD2-FANCI has brought us one step closer to understanding the elusive physiologic role of the ID complex modification (Fig. 1). Nevertheless, there are still significant gaps in this signaling pathway. For example, the links between various FA proteins and homologous recombination enzymes and translesion polymerases remain largely elusive. Meanwhile, the elucidation of how FANCD2/FANCI, FAN1, FANCP/SLX4/BTBD12, RAD51C and the other downstream FA proteins coordinate the whole ICL repair process represents a major challenge for future directions.

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ABBREVIATIONS

ATR, ataxia telangiectasia and Rad3-related kinase; FA, Fanconi anemia; FAN1, Fanconi anemia-associated nuclease 1; HR, homologous recombination; ICL, DNA interstrand cross-link; NER, nucleotide excision repair; TLS, translesion synthesis; UAF1, the USP1 associated factor 1; USP1, ubiquitin-specific cysteine protease

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