Fancm-deficient mice reveal unique features of Fanconi anemia complementation group M

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The Fanconi anemia (FA) core complex member FANCM remodels synthetic replication forks and recombination intermediates. Thus far, only one FA patient with FANCM mutations has been described, but the relevance of these mutations for the FA phenotype is uncertain. To provide further experimental access to the FA-M complementation group we have generated Fancm-deficient mice by deleting exon 2. FANCM deficiency caused hypogonadism in mice and hypersensitivity to cross-linking agents in mouse embryonic fibroblasts (MEFs), thus phenocopying other FA mouse models. However, Fancm
D2/D2 mice also showed unique features atypical for FA mice, including underrepresentation of female Fancm
D2/D2 mice and decreased overall and tumor-free survival. This increased cancer incidence may be correlated to the role of FANCM in the suppression of spontaneous sister chromatid exchanges as observed in MEFs. In addition, FANCM appeared to have a stimulatory rather than essential role in FANCD2 monoubiquitination. The FA-M mouse model presented here suggests that FANCM functions both inside and outside the FA core complex to maintain genome stability and to prevent tumorigenesis.

INTRODUCTION

Fanconi anemia (FA) is a recessively inherited genetic disorder characterized by developmental abnormalities, progressive bone marrow failure and cancer susceptibility (1). To date, 13 complementation groups have been defined (FA-A, B, C, D1, D2, E, F, G, I, J, L, M, N) and the corresponding genes have been identified (2). At the cellular level, FA is characterized by hypersensitivity to cross-linking agents, a hallmark that is exploited in the clinic as a diagnostic test for FA (3). Therefore, all FA proteins are thought to function in a pathway which mediates cross-linker resistance and maintains genomic stability (4). The FA pathway is commonly divided in an upstream and a downstream part. The upstream part is involved in the monoubiquitination of FANCD2 and FANCI after DNA damage and during S phase (1,5–7). This monoubiquitination is catalyzed by the FA core complex consisting of eight FA proteins, FANCA, -B, -C, -E, -F, -G, -L, -M, and two FA-Associated Proteins, FAAP100 and FAAP24 (8–10). FANCL is the E3 ubiquitin ligase of the FA core complex (6), which is assisted by the E2 enzyme UBE2T (11). Monoubiquitinated FANCD2 and FANCI are targeted to the chromatin where they co-localize with proteins involved in DNA repair, such as RAD51 (12). Subsequently, the FA pathway appears to merge with the BRCA1/BRCA2 pathway, since hypomorphic mutations in BRCA2 have been found in FA-D1 patients, and mutations in PALB2, encoding a partner and stabilizer of BRCA2, have been found in FA-N patients. Moreover, mutations in the gene encoding the BRCA1-interacting protein BRIP1 are found in the FA-J complementation group (13–15). BRCA2/FANCD1, BRIP1/FANCJ and PALB2/FANCN are supposed to act in the downstream part of the FA pathway, since the monoubiquitination of FANCD2 is not affected in patients carrying a defect in these proteins. Although many components of the FA/BRCA pathway have now been identified, the precise role of this pathway in genomic maintenance remains unclear. Further elucidation of

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the FA pathway is in part hampered by the fact that most FA core complex members are orphan proteins that cannot be connected to any known protein family based on sequence homology (16). In this respect FANCM is an exception since it is not only an ortholog of the archaeal DNA repair protein HEF, but also contains two conserved domains: a helicase domain at the N-terminus and a (degenerate) endonuclease domain at the C-terminus (17,18). FANCM is assigned to the helicase superfamily 2 and contains seven helicase-specific motifs (I, Ia II, III, IV, V and VI) that are essential for its helicase function (19). Motif II, the Walker B motif, consists of a DEAD or DEAH box and is necessary for the interaction with Mg$^{2+}$ and thus for ATP hydrolysis. FANCM was shown to have translocase activity and to promote branch migration of Holliday junctions and replication forks (17,20,21) and could provide a link between the FA core complex and DNA cross-link repair.

FANCM was initially identified as FAAP250, a 250 kDa FA-Associated Protein that co-immunoprecipitated with other FA core complex members using an antibody against FANCA (9). The classification of FAAP250 as the FA protein FANCM was based on lack of FAAP250 expression and the presence of biallelic mutations in patient EUFA867. This patient had been excluded from other existing complementation groups based on linkage analysis, cell fusion and cDNA transfection and was therefore assigned to the new reference group FA-M (17). To date only one FA-M patient has been described, from whom only lymphoblasts are available for in vitro studies. In this FA-M reference cell line monoubiquitinated FANCD2 was absent and the levels of FANCA and FANCG were reduced (17). Recently it has become apparent that this patient also carries FANCA mutations, which questioned the actual contribution of FANCM deficiency to the FA phenotype (22).

Several FA mouse models have been reported to date: two for Fance (23,24), two for Fanca (25,26), two for Faneg (27,28), one for FancI/POG (proliferation of germ cells) (6,29) and one for Fancd2 (30). None of these FA mice displayed the developmental abnormalities that are frequently observed in FA patients, such as missing digits or radii. However, gonadal abnormalities and reduced fertility, which are common in FA patients, have been observed in all FA mouse models. Consistently, ovariates in FA mice contained fewer developing follicles, although the seminiferous tubules in the testes were heterogeneous. Some tubules showed normal spermatogenesis, although others were devoid of germ cells and showed no evidence for active spermatogenesis (23,25,27,31,32). In contrast to FA patients, none of the FA mice developed bone marrow failure or acute myeloid leukemia. Only administration of sublethal doses mitomycin C (MMC) induced hematopoietic failure in FA mice (31). In addition, in some FA mice, microphthalmia and perinatal lethality were observed (30,33). Similar to cells from FA patients, cells derived from FA mouse models were hypersensitive to DNA cross-linking agents, as manifested by reduced survival, increased chromosomal breakage and G2 arrest. Another hallmark of FA core complex defects that was recapitulated in mice was the absence of FANCD2 monoubiquitination (23–28).

We generated a Fancm mouse model to study the effect of Fancm deficiency in vivo and observed that less female Fancm mice were born than expected. Moreover, Fancm mice showed reduced overall and tumor-free survival. The increased cancer incidence may be due to increased genetic instability as observed in FANCM-deficient mouse embryonic fibroblasts (MEFs).

RESULTS

Generation of a Fancm mouse model

Fancm knockout mice were generated by targeted deletion of exon 2, encoding amino acids 158–215 including the DEAD-box. This deletion does not only inactivate the helicase activity of FANCM, but also leads to a frameshift and premature stop in exon 3. A targeting construct was used that integrates a neomycin resistance cassette into intron 2 of the Fancm gene and LoxP sites in introns 1 and 2 flanking exon 2 and the Neo cassette (Fig. 1A). Mice carrying this Fancm mutant allele were obtained (Fig. 1B) and crossed with a Cre recombinase deleter mouse to remove exon 2 and the Neo marker. Since this Fancm mouse model was created by deleting exon 2 we will refer to it as the Fancm$^{\Delta 2}$ model. Fancm-specific cDNA was produced with a primer in exon 10 and subsequently amplified with primers in exon 1 and exon 10. In heterozygous Fancm$^{\Delta 2/+}$ MEFs, amplification of the wild-type allele (Fig. 1C, lane 2, upper band) was more prominent than that of the knock-out allele (lane 2, lower band). Although this was not a quantitative assay, these results suggested that the mRNA corresponding to the knock-out allele was less stable than mRNA transcribed from the wild-type allele. Sequence analysis of the PCR products confirmed the loss of exon 2 from the Fancm$^{\Delta 2}$ allele (data not shown). Quantitative RT-PCR (qPCR) with primers in exon 1 and exon 2 also demonstrated the loss of exon 2 sequence from the Fancm$^{\Delta 2}$ cDNA, since no product was formed and qPCR with primers in exon 21 showed barely detectable levels of Fancm cDNA in a Fanca mutant MEF cell line (Fig. 1D). These data confirm that Fancm expression was lost in our Fancm mouse model, probably due to nonsense-mediated decay of Fancm mRNA. By immunoprecipitation and western blotting with different antisera against human FANC we could demonstrate that Fancm protein was indeed absent in Fancm$^{\Delta 2/+}$ MEFs (Fig. 1E). Furthermore, although FANCM co-immunoprecipitated with FANCA in Fancm$^{\Delta 2/+}$ MEFs, Fancm was absent in a Fanca immunoprecipitate in Fancm$^{\Delta 2/+}$ MEFs. These results showed that no full length FANCM protein was expressed in the Fancm$^{\Delta 2/+}$ mice.

Non-mendelian inheritance of Fancm$^{\Delta 2/\Delta 2}$ alleles in females

The Fancm targeting was performed in the Bruce4 ES cell line and cells were injected into C57/Bl/6J blastocysts. The chimeras were backcrossed to the C57/Bl/6 and subsequently backcrossed to the FVB mouse strain. Fancm$^{\Delta 2/+}$ mice from backcross 2 and 3 were inter-crossed to obtain Fancm$^{\Delta 2/\Delta 2}$ offspring. Male Fancm$^{\Delta 2/\Delta 2}$ mice were born with the expected Mendelian frequency. However, a significant decrease in the number of female Fancm$^{\Delta 2/\Delta 2}$ mice was observed (Table 1).
Up till now, an underrepresentation of female mice has not been reported for FA mouse models. At present, we are unable to explain the decrease in the number of female FancmΔ2/Δ2 mice. Since genetic background might play a critical role we are crossing the FancmΔ2/+ mice into different mouse strains.

One phenotype shared by all FA mouse models is the appearance of gonadal abnormalities associated with reduced fertility (23–30). We performed histology on the reproductive organs of 3 months old male and female FancmΔ2/Δ2 mice. The testicular
size in Fancm\(^{Δ2/Δ2}\) males was reduced compared with wild-type littersmates (Fig. 2A and B). The seminiferous tubules of the Fancm\(^{Δ2/Δ2}\) males were heterogeneous: some tubules showed an absence of spermatogonia and did not contain sperm (Fig. 2D), although others had normal testicular architecture and sperm production. In addition, Fancm\(^{Δ2/Δ2}\) testes showed hyperplasia of Leydig cells. This pattern was not present in wild-type littersmates (Fig. 2C). We have not thoroughly investigated whether these gonadal abnormalities also affected fertility, but noticed that several male mice had been able to produce offspring. The number of developing follicles in Fancm\(^{Δ2/Δ2}\) ovaries was reduced in comparison to wild-type ovaries and the cortex of Fancm\(^{Δ2/Δ2}\) ovary was depleted of primary follicles (Fig. 2E and F). Due to the low number of Fancm\(^{Δ2/Δ2}\) females, we have not investigated their fertility. These results demonstrate that Fancm\(^{Δ2/Δ2}\) mice phenocopy other FA mouse models with respect to gonadal abnormalities.

**Table 1. Offspring of Fancm\(^{Δ2/Δ2}\) intercrosses**

<table>
<thead>
<tr>
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<th>Male Observed</th>
<th>Expected</th>
<th>Female Observed</th>
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<tr>
<td>Fancm(^{Δ2/Δ2})</td>
<td>18</td>
<td>22</td>
<td>6</td>
<td>22</td>
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<tr>
<td>Fancm(^{Δ+/Δ})</td>
<td>50</td>
<td>43</td>
<td>44</td>
<td>43</td>
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<td>22</td>
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<tr>
<td>Total</td>
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<td>75</td>
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<td>32</td>
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<tr>
<td>P-value</td>
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*Significant deviation from expected Mendelian frequency.

\(\ast\)Based on the number of male animals born.

**Fancm\(^{Δ2/Δ2}\) mice show reduced life span and increased tumor formation**

We did not observe congenital abnormalities in the Fancm\(^{Δ2/Δ2}\) mice. Also the bone marrow of Fancm\(^{Δ2/Δ2}\) mice showed no signs of hypoplasia and the number of hematopoietic stem cells appeared normal (data not shown).

To monitor the effect of FANCM deficiency on survival we followed cohorts of 21 Fancm\(^{Δ2/Δ2}\) (16 males + 5 females), 36 Fancm\(^{Δ2/Δ+}\) (22 males + 14 females) and 21 Fancm\(^{Δ+/Δ}\) (8 males + 13 females) mice for 2 years and plotted the overall survival of this cohort in a Kaplan Meier curve (Fig. 3A). The median survival of Fancm\(^{Δ2/Δ+}\) mice (573 days) was significantly reduced in comparison to Fancm\(^{Δ2/Δ}\) (693 days, \(P\)-value = 0.002) and Fancm\(^{Δ+/Δ}\) (>730 days, \(P\)-value = 0.003) littersmates. The median survival of Fancm\(^{Δ2/Δ2}\) females (491 days) and Fancm\(^{Δ2/Δ+}\) males (573 days) did not significantly differ and also male and female heterozygous littersmates had a similar lifespan. We noted that of the Fancm\(^{Δ2/Δ2}\) mice, 52% (11 out of 21) developed tumors whereas 39% of the Fancm\(^{Δ2/Δ+}\) (14 out of 36) and 14% of the Fancm\(^{Δ+/Δ}\) (3 out of 21) mice developed tumors. Therefore we hypothesized that the Fancm\(^{Δ2/Δ}\) mice have an increased incidence of tumor formation. To follow tumor-free survival we calculated a Kaplan Meier curve that excluded the mice sacrificed because of non-tumor related pathology (Fig. 3B). We observed a statistically significant decrease in tumor-free survival in the Fancm\(^{Δ2/Δ}\) mice compared with the Fancm\(^{Δ2/Δ}\) (\(P\)-value = 0.015) and Fancm\(^{Δ+/Δ}\) (\(P\)-value = 0.001) littersmates. We noted several different tumors in our cohort of which one tumor type, hepatoma, was detected in three Fancm\(^{Δ2/Δ}\) mice as opposed to zero hepatomas in 57 control mice (\(P\)-value = 0.013). A picture of one of these hepatomas is shown in Figure 3C. Apart from an increased tumor incidence we also observed arteritis nodosa in 3 out of 21 Fancm\(^{Δ2/Δ}\) mice compared with 1 in 57 control animals (\(P\)-value = 0.013). This may be indicative of a premature aging phenotype in Fancm\(^{Δ2/Δ}\) mice.

In summary, we observed an increased cancer incidence and associated reduced life span in our Fancm\(^{Δ2/Δ}\) cohort when compared with heterozygous and wild-type littersmates. To investigate whether the increased cancer incidence could be attributed to increased genetic instability, we studied the consequences of Fancm deficiency in MEFs.

**Fancm\(^{Δ2/Δ}\) MEFs show an FA-like phenotype**

A hallmark of FA cells is a pronounced hypersensitivity to cross-linking drugs such as MMC resulting in increased chromosomal breakage, reduced cell viability and a pronounced G2 arrest. We assessed this phenotype in immortalized MEF cell lines that were either deficient or proficient for the FANCM protein. Immortalized Fanca\(^{-/-}\) MEFs were used as an FA control (25). The number of chromosomal breaks and chromosome aberrations was more strongly increased by MMC treatment in Fancm\(^{Δ2/Δ}\) MEFs and Fanca\(^{-/-}\) MEFs than in Fancm\(^{Δ+/Δ}\) MEFs (Fig. 4A).

Another feature of FA cells is a prolonged G2 arrest after exposure to cross-linking agents (34–36). Following incubation with MMC, Fancm\(^{Δ2/Δ}\) MEFs showed a pronounced G2 arrest, which was also apparent in Fanca\(^{-/-}\) MEFs, but was absent in Fancm\(^{Δ+/Δ}\) MEFs (Fig. 4B). The G2 arrest was already noticed after 24 h (Supplementary Material, Fig. S1) and did not correspond to an arrest in mitosis since the cells did not carry an increase in DNA content higher than 4 N. This >4 N population was likely caused by cells that had completed mitosis but failed cytokinesis and had started to synthesize DNA. As shown in Figure 4A, some of the Fancm and Fanca deficient MEFs had escaped from G2 arrest and entered mitosis in the presence of chromosomal aberrations. Since these MEFs were immortalized with EIA they lacked the G1 checkpoint and therefore these 4 N cells could start a new round of DNA replication resulting in a >4 N peak. Correspondingly, this >4 N population was absent in primary MEFs (Supplementary Material, Fig. S2).

Next, we assessed whether FANC1 deficiency resulted in reduced growth after exposure to MMC or cisplatin. As shown in Figure 4C, Fancm\(^{Δ2/Δ}\) MEFs and Fanca\(^{-/-}\) MEFs were hypersensitive to MMC and cisplatin when compared with Fancm\(^{Δ+/Δ}\) MEFs.

Thus far, all these assays confirmed that Fancm\(^{Δ2/Δ}\) MEFs exhibited a cellular phenotype resembling that of Fanca\(^{-/-}\) MEFs and characteristic for FA cells.
The FA core complex acts as an E3-ubiquitin ligase that monoubiquitinates FANCD2 and FANCI. A distinctive feature of cells deficient in any of the FA core complex members is their inability to monoubiquitinate FANCD2 and FANCI during S phase or after replicative stress and DNA damage (4,5,7). FANCM is an integral component of the FA core complex and based on the results obtained with the FA-M reference cell line EUFA867 it was concluded that FANCM was essential for FA core complex formation and FANCD2 monoubiquitination (17). Transient knockdown of FANCM protein using siRNA confirmed the necessity of FANCM for normal FA core complex function (36,37). To investigate whether FANCM is indeed essential for FANCD2 monoubiquitination, lysates from untreated MEFs or MEFs treated with different doses of MMC were probed with a FANCD2 antibody. FANCD2 monoubiquitination is seen as a shift from a short non-monoubiquitinated isoform (FANCD2-S) to a longer monoubiquitinated isoform (FANCD2-L). Western blot analysis revealed the presence of both the short and long isoforms of FANCD2 in the Fancm⁺/⁺ MEFs after MMC treatment (Fig. 5A). In contrast, the FANCD2-L isoform appeared absent in cell lysates from Fanca⁻/⁻ and FancmΔ2/Δ2 MEFs. Upon extended exposure of the western blot, however, the FANCD2-L isoform became visible in lysates from MMC-treated FancmΔ2/Δ2 MEFs, but still remained undetectable in lysates from Fanca⁻/⁻ MEFs. This indicated that in the absence of FANCM, FANCD2 could still be monoubiquitinated in a MMC-dose dependent way, albeit at strongly reduced levels.

**Figure 2.** Gonadal abnormalities of FancmΔ2/Δ2 mice. Histological sections of testes (A–D) and ovaries (E, F) from 3 months old mice. (A) Control testis (magnification 2.5×). (B) FancmΔ2/Δ2 testis (2.5×). (C) Control testis (25×). (D) FancmΔ2/Δ2 testis (25×). (E) Control ovary (5×). (F) FancmΔ2/Δ2 ovary (5×).
Ubiquitination was observed in different FANCD2 antibody. Again, residual FANCD2 monoubiquitinated with a human FANCD2 antibody and probed the blot with a mouse (magnification 5×) and Fancm+/− (blue line) mice. Tumor types detected in the individual animals are indicated by colored circles. (C) Representative picture of a hepatoma from a Fancm−/− mouse (magnification 5×).

Figure 3. Kaplan Meier survival curves of FancmΔ2/Δ2 mice and control littermates. (A) Overall survival curves for FancmΔ2/Δ2 (black line), FancmΔ2/+ (purple line) and Fancm+/+ (blue line) mice. Tumor types detected in the individual animals are indicated by colored circles. (B) Tumor-free survival curves for FancmΔ2/Δ2 (black line), FancmΔ2/− (purple line) and Fancm−/− (blue line) mice. (C) Representative picture of a hepatoma from a FancmΔ2/Δ2 mouse.

We also found that in response to HU FANCD2 was monoubiquitinated in FancmΔ2/Δ2 MEFs (Fig. 5B). To verify this unexpected finding we performed an immunoprecipitation with a human FANCD2 antibody and probed the blot with a different FANCD2 antibody. Again, residual FANCD2 monoubiquitination was observed in FancmΔ2/Δ2 MEFs, but not in Fanca−/− MEFs (Fig. 5C). These results suggest that the formation of a functional FA core complex was not critically dependent on FANCM, which was strengthened by the co-precipitation of FANCA and FANCL in FancmΔ2/Δ2 MEFs (Fig. 5D). A further indication for a role of the FA core complex in FANCD2 monoubiquitination in the absence of FANCM was given by chromatin fractionations. As shown in Figure 5E, FANCA was still enriched in the chromatin fraction after MMC treatment in FancmΔ2/Δ2 MEFs, albeit at reduced level in comparison to wild-type MEFs. Furthermore, similar to the situation in wild-type MEFs part of the monoubiquitinated FANCD2 was retained in the chromatin fraction in FancmΔ2/Δ2 MEFs (Fig. 5F). These results indicated that in the absence of FANCM part of the core complex can still localize to the chromatin and monoubiquitinate FANCD2.

DISCUSSION

FANCM is the first FA core complex protein in which conserved domains have been identified that may point to an enzymatic role in DNA repair. Moreover, recent studies showed that FANCM can promote branch migration of Holliday junctions and DNA replication structures (20,21,39), suggesting that FANCM may provide a link between the FA pathway and DNA repair. The Fancm mouse model reported here supports this concept. At first glance, Fancm-deficient mice resembled mice deficient for other FA core complex members: they showed gonadal abnormalities and at the cellular level they displayed increased chromosomal breakage, excessive G2 arrest and crosslinker hypersensitivity. However, four additional atypical features were observed: non-Mendelian segregation of FancmΔ2/Δ2 alleles in females,
decreased overall and tumor-free survival, residual FANCD2 monoubiquitination and increased spontaneous SCEs.

Fancm knockout mice were born at a decreased Mendelian ratio, which was specifically due to an underrepresentation of female Fancm$^{D2/D2}$ mice. In contrast, normal Mendelian ratios were reported for most other FA mouse models (23–28). Perinatal lethality was observed in one of the Fancc mouse models and in the Fancd2 mouse model, both in a C57Bl/6 background, but these studies did not report on differences in gender distribution (30,33). In the Fanc/Pog model, embryonic lethality was seen in a pure 129/Sv background and in the F1 generation of a cross between 129/Sv and C57Bl/6. However, normal Mendelian ratios were observed in F1 offspring from heterozygous crossings between 129/Sv and FVB (29). Female embryonic lethality has been described in mice nullizygous for both Msh2 and p53 in a mixed C57Bl/6 and 129/Sv background, but this was overcome in crosses with BALB/c and SWR/J mice (40,41). In summary, the genetic background affects the viability of knockout mice suggesting the presence of modifier loci that, when combined with an FA defect, result in reduced viability. We are currently crossing the Fancm$^{D2/+}$ mice into the C57Bl/6 strain to investigate whether the underrepresentation of female Fancm$^{D2/D2}$ is modified by the genetic background of the mice.

Figure 4. Cellular phenotype of Fancm$^{D2/D2}$ MEFs. (A) Fancm$^{D2/D2}$ MEFs and Fanca$^{−/−}$ MEFs showed markedly elevated chromosomal breakage after MMC treatment (50 nM for 48 h, white bars) compared with wild-type MEFs. (B) Fancm$^{D2/D2}$ MEFs showed a marked accumulation in the G2 phase of the cell cycle after MMC treatment (50 nM for 72 h) similar to the response of Fanca$^{−/−}$ MEFs, whereas a G2 arrest was not apparent in control Fancm$^{+/+}$ MEFs. (C) Fancm$^{D2/D2}$ MEFs (squares) and Fanca$^{−/−}$ MEFs (triangles) showed reduced colony survival after continuous exposure to MMC or cisplatin when compared with Fancm$^{+/+}$ MEFs (diamonds).
An important difference between the Fancm\(^{Δ2/Δ2}\) mice and other FA defective mice (Fanca, Fancc, Fancd2, Fancg and POG/Fancl) is the reduced life span and increased cancer incidence in the homozygous Fancm mice compared with littermates. For two FA mouse models, the Fancc and Fancd2 deficient mice, long term survival cohorts have been published. In the Fancc defective mice no tumors were observed after 600 days and only in combination with loss of tumor suppressor Trp53 (Trp53\(^{+/-}\) or Trp53\(^{-/-}\)) Fancc loss resulted in a shortened latency of tumorigenesis (42). For the Fancd2 deficient mice a statistically significant increase in the incidence of both adenomas and carcinomas was reported (30), but in a follow-up study of the same group this difference was not considered significant (43). Again, only the combination of Trp53\(^{+/-}\) with Fancd2 loss resulted in a significant decrease in tumor-free survival (43). The decreased overall and tumor-free survival observed in the Fancm\(^{Δ2/Δ2}\) mice therefore appears unique for this FA mouse model and suggests that FANCM has additional functions independent of the FA core complex in the prevention of tumor formation.

This additional function may be reflected by the elevated spontaneous SCEs in primary Fancm\(^{Δ2/Δ2}\) MEFs. Like in Fancm deficient MEFs, disruption of FANCM in the chicken B cell line DT40 resulted in an increased background level of SCEs (18,38). This phenotype is shared with cells from Bloom’s Syndrome patients, which are deficient for BLM, an anti-recombinogenic RecQ helicase. The BLM protein acts as a tumor-suppressor gene by suppressing recombination and thereby reducing loss of heterozygosity (LOH) through mitotic recombination (44). The helicase domain of FANCM is shared with yeast orthologs MPH1 (Saccharomyces cerevisiae) and FML1 (Schizosaccharomyces pombe), that play a regulatory role in homologous recombination repair by replication fork reversal and D-loop disruption (45–47). Possibly human and mouse FANCM have a similar role. The basal increase in SCE frequency may indicate that FANCM prevents the formation of DSBs after replication fork stalling and may be a reflection of the increased damage at the replication fork. It has been shown that the FANCM protein can remodel replication fork substrates (20,21). Possibly in the absence of FANCM stalled replication forks are not correctly processed leading to collapsed replication forks and DSBs. These DSBs may be repaired via various repair pathways, some of which result in SCEs (19). Analogous to cells deficient for the BLM protein this elevated frequency of spontaneous SCEs may result in an increased rate of LOH and could explain the increased cancer incidence observed in the Fancm\(^{Δ2/Δ2}\) mice. However, FANCM seems to have no function in the outcome
of the recombination process as supported by the lack of an additional significant increase in SCE frequency upon MNNG treatment, which induces DSBs, when compared with control MEFs.

Residual FANCD2 monoubiquitination was detectable in Fancm^{Δ2/Δ2} MEFs. This indicates that in the absence of FANCM a functional FA core complex can still be formed, a conclusion supported by the co-precipitation of FANCA and FANCL in Fancm^{Δ2/Δ2} MEFs and enrichment of FANCA in the chromatin fraction. Possibly, FANCM has a role in recruiting the FA core complex to the site of damage and positions this complex in the vicinity of FANCD2 for efficient monoubiquitination, as suggested by siRNA experiments in HeLa cells (36). These findings seem to be in contrast with data published on the FA-M lymphoblasts (EUFA867).

In these cells, immunoblotting showed no evidence for FANCD2 monoubiquitination, reduced levels of FANCA and FANCG and defective nuclear localization of FANCA and FANCL. This discrepancy between the human FA-M lymphoblasts and murine Fancm^{Δ2/Δ2} MEFs can however be explained by the presence of biallelic FANCA mutations in the EUFA867 cells, which implies that the human reference cell line is deficient for both FANCM and FANCA (22). Therefore, our knockout mice and MEFs represent the first mammalian model system in which the role of FANCM can be investigated in a defined genetic background with littermate controls.

In summary, we have shown that FANCM deficiency is associated with an FA phenotype in mice and MEFs. The system also revealed several unique features related to FANCM deficiency, including decreased overall survival, increased cancer incidence and female perinatal lethality in mice, and residual FANCD2 monoubiquitination and increased SCEs in MEFs. These novel phenotypes show that FANCM has an important role in the prevention of tumorigenesis and suggest that FANCM partially acts independent of the FA core complex to maintain genome stability.

MATERIALS AND METHODS

ES cell targeting and generation of Fancm^{Δ2/Δ2} mice

Targeting and production of mice were performed at Ozgene (Australia). The Fancm targeting vector was linearized and electroporated into Bruce4 ES cells. After selection with G418, genomic DNA from surviving clones was digested with ScaI and NotI and screened by Southern blotting with a 5′ probe and 3′ probe, respectively (primers for 5′ probe: TTGAAATGGTCCAGTGTCACA and GATAGCCAGTGGTGTGTATCC and primers for 3′ probe: TGGGGTGTTTCTGCTCTGACC and ATGTCTGCCATTCTCTGCG). Correctly targeted ES cell clones were injected into C57BL/6.6 blastocysts. Chimicric mice were crossed with a B6 deleter mice expressing Cre recombinase, to recombine the LoxP sites and remove exon 2 and the selection marker. The Fancm^{Δ2/Δ2} mice were then backcrossed to the FVB strain. Homozygotes were produced by matings of BC2 and BC3 heterozygous mice.

Generation of cDNA, sequence analysis and qPCR

Total RNA was isolated from Fancm^{+/+}, Fancm^{Δ2/+} and Fancm^{Δ2/Δ2} MEFs using RNA-Bee Total RNA Isolation Reagent (Campro Scientific). cDNA was prepared by reverse transcription using a primer in exon 10 (primer in exon 10: 5′-CCACCCGCCCTCGAACTG-3′). From this template, a product was amplified by PCR using primers in exon 1 (primer in exon 1: 5′-TTCATTGCCGGCTGGTCA-3′) and exon 10. This PCR product was cloned into the pGEM™-T Easy vector (Promega). Vector primer T7 was used for sequencing. For qPCR, 1 μg of total RNA was used to prepare cDNA by reverse transcription using oligodN6 random primers (Roche Diagnostics). Subsequently, the cDNA was used as a template for qPCR in the presence of SYBR-green (Applied Biosystems) to label the product.
Fluorescence detection was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All measurements were performed in triplicate and the relative amounts of cDNA were compared with actin as a reference for total cDNA. Subsequently, the Fancm<sup>−/+</sup> cDNA concentration was used for normalization. For qPCR of exon 1–2 we used 5′-GCTGAAATGACAGGTTCAACTC-3′ as forward and 5′-CATTTACATGACCTGCGG-3′ as reverse primer, for qPCR of exon 21 we used 5′-TGCTTCACACCATGTACTGG-3′ as forward and 5′-AGCCACATGCAGGAGTGAAGT-3′ as reverse primer.

Histological analysis

Mice were sacrificed and isolated organs were fixed in formalin. The testes and ovaries of mutant and wild-type mice were fixed in EAF fixative (ethanol–acetic acid–formol saline). Fixed organs were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Kaplan Meier survival curves

Overall and tumor-free survival curves were generated using SPSS software (Version 15), statistical significance between genotypes was determined using built-in analysis for survival curves consisting of a log rank test yielding a P-value.

Isolation, culturing and transduction of MEFs

MEFs were derived from 13-day-old embryos and cultured in GMEM (Invitrogen-GIBCO) supplemented with 10% fetal calf serum, 1 mM non-essential amino acids (Invitrogen-GIBCO), 10 mM sodium pyruvate (Invitrogen-GIBCO), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen-GIBCO) and 0.1 mM β-mercaptoethanol (Merck) at 37°C, under a humidified atmosphere of air plus 5% CO₂. MEFs were immortalized by viral infection with E1A. Ecotropic retroviral supernatants were produced by transfecting Phoenix cells by calcium phosphate co-precipitation. Thirty-six hours post-transfection, retroviral supernatants were filtered through a 0.45 µm filter (MCE membrane, Millipore) and used to infect MEFs. MEFs were transduced in the presence of 4 µg/ml polybrene.

MMC-induced chromosomal breakage analysis

MEFs immortalized with E1A were cultured for 48 h in the absence or presence of 50 nM MMC. For each cell culture, 50 metaphases were analyzed for chromatid-type chromosomal abnormalities. To quantify chromosomal abnormalities the interchange aberrations were converted into break events. All scoring was performed blind to eliminate counting bias (48).

Growth inhibition assays

E1A immortalized MEFs were seeded in 96-well microplates on day 0 in Dulbecco’s modified Eagle’s medium with glutamate (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) and sodium pyruvate (1 mM) and supplied with 2-fold serial drug dilutions on day 1. On day 7, the cells were fixed by adding trichloroacetic acid (Merck) to a final concentration of 5% (v/v). After 1 h at 4°C, plates were washed five times with demi water, dried and stained for 30 min with 50 µl sulforhodamine B (0.4%, w/v) (Sigma). Following three wash steps with 1% acetic acid, 200 µl 10 mM Tris was added to dissolve the staining. Absorbance at 540 nm was measured using a Tecan infinite m200 plate reader (Tecan). After correction for medium-only and no-drug controls, data points were fitted using the general formula for a sigmoid curve.

G<sub>2</sub> arrest

Cells were seeded in Petri dishes at 2.5 × 10<sup>5</sup> cells and allowed to attach overnight. Cells were exposed to either 50 or 100 nM MMC and were grown for 24, 48 or 72 h. Cells were trypsinized and fixed in 70% ethanol at 4°C for at least 1 h. Subsequently, cells were washed in PBS and nuclei were stained with propidium iodide (4 µg/ml) and treated with RNase A (2 µg/ml) in 200 µl PBS, incubated at 37°C for 15 min, and analyzed in a flowcytometer using FACS ‘Cell Quest’ software and ‘Summit’ software.

Immunoblot and immunoprecipitation analysis

For preparation of whole-cell extracts, cells were lysed for 30 min on ice in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 0.1%SDS supplemented with 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml). For immunoblot analysis equivalents of 200 000 cells were loaded on a 3–8% Tris-Acetate NuPAGE gradient gel (Invitrogen) and proteins were separated by gel-electrophoresis at 25 mA during 3.5 h, according to the manufacturer’s protocol. Proteins were transferred to Immobilon-P-Transfer membranes (Millipore) and the membranes were blocked with 5% dry milk in PBS-Tween (0.1%). The membranes were incubated with the indicated antibodies. After washing with PBS-Tween (0.1%), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (DakoCytomation). Proteins were visualized with ECL. For immunoprecipitation reactions, cells were lysed for 30 min on ice in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP40 supplemented with 1 tablet complete protease inhibitor cocktail (Roche) per 50 ml), and incubated with either guinea pig antibody against human FANCD2 (amino acids 55–292), guinea pig antiserum against murine FANCA (49) or guinea pig antiserum against human FANCM (49) and 50% protein A beads (Pharmacia), overnight at 4°C, under gentle agitation. Prior to immunoblot analysis, lysates were washed five times in lysis buffer after which immunoblot analysis was performed as described above. Antibodies used for immunoblot analysis were rabbit polyclonal antiserum against murine FANCĐ2 (gift from Dr A.D. D’Andrea) (50), rabbit polyclonal antiserum against human FANCĐ (gift from Dr K.J. Patel), rabbit polyclonal antiserum against murine FANCA (6,51), rabbit polyclonal antiserum against human FANCL and rabbit polyclonal antiserum against human FANCM (gifts from Dr W. Wang) (6).
Chromatin fractionations

The chromatin fractionation was essentially performed as in (52). Briefly, cells were collected by trypsinization and pelleted. The pellet was resuspended and incubated for 10 min in ice-cold buffer containing 10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.2% Triton X-100, 0.1% triton, 0.5 mM DTT, and complete protease inhibitor cocktail (Roche). The suspension was homogenized and the supernatant containing the cytoplasmic fraction was collected after 15-min centrifugation at 4000g at 4°C. The chromatin/membrane-containing pellet was resuspended in cold PBS supplemented with 600 mM NaCl, 1% N-octyl glucoside, and 125 units of DNAse, incubated for 30 min in an ultrasonic bath; and centrifuged for 15 min at 18 000g at 4°C. Chromatin/membrane proteins were collected with the supernatant. 1.3 mg of protein of each fraction were separated on a 3–8% Tris-Acetate NuPAGE gradient gel (Invitrogen). As control for the fractionation procedure antibodies against γ-tubulin (cytoplasmic, Sigma), topoisomerase I (nuclear) and Lamin B (chromatin, Santa Cruz) were used.

Sister chromatid exchanges

To experimentally induce SCEs we chose to mimic a replication-associated DSB with MNNG (Serva, Heidelberg, Germany). The alkylating agent MNNG produces single-strand gaps in the first replication cycle that, if left unrepaired, due to the presence of O6-methylguanine-DNA methyltransferase inhibitor O6-benzylguanine, results in a replication-associated DSB (53). Cycling cells were labeled for 48 h with 8 μg/ml 5-bromodeoxyuridine (BrdU). Exposure time to MNNG (6 μM) and O6-benzylguanine (20 μM) was 48 h. The metaphase spread were essentially prepared as described (54). Briefly, slides were stained with bisBenzimide H33258 (Sigma B-2883), illuminated with UV for 1 h, heated for 90 min in 2 × SSC at 65°C, followed by staining in 10% Giemsa solution (Merck 1.09204). Twenty metaphases were analyzed for the presence of SCEs.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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