Inactivation of Murine Usp1 Results in Genomic Instability and a Fanconi Anemia Phenotype

Jung Min Kim,1 Kalindi Parmar,1 Min Huang,1 David M. Weinstock,2 Carrie Ann Ruit,1 Jeffrey L. Kutok,3 and Alan D. D’Andrea1,*.  
1Department of Radiation Oncology  
2Department of Medical Oncology  
Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA  
3Department of Pathology, Brigham and Women’s Hospital, 75 Francis Street, Boston, MA 02115, USA  
*Correspondence: alan_dandrea@dfci.harvard.edu  
DOI 10.1016/j.devcel.2009.01.001

SUMMARY

Fanconi anemia (FA) is a human genetic disease characterized by chromosome instability, cancer predisposition, and cellular hypersensitivity to DNA crosslinking agents. The FA pathway regulates the repair of DNA crosslinks. A critical step in this pathway is the monoubiquitination and deubiquitination of FANCD2. Deubiquitination of FANCD2 is mediated by the ubiquitin protease, USP1. Here, we demonstrate that targeted deletion of mouse Usp1 results in elevated perinatal lethality, male infertility, crosslinker hypersensitivity, and an FA phenotype. Usp1−/− mouse embryonic fibroblasts had heightened levels of monoubiquitinated Fancd2 in chromatin. Usp1−/− cells exhibited impaired Fancd2 foci assembly and a defect in homologous recombination repair. Double knockout of Usp1 and Fancd2 resulted in a more severe phenotype than either single knockout. Our results indicate that mouse Usp1 functions downstream in the FA pathway. Deubiquitination is a critical event required for Fancd2 nuclear foci assembly, release from chromatin, and function in DNA repair.

INTRODUCTION

The Fanconi anemia (FA) pathway mediates DNA repair and promotes normal cellular resistance to DNA crosslinking agents (Wang, 2007). The FA pathway is regulated by 13 FA proteins (FANCA, B, C, D1, D2, E, F, G, I, J, L, M, and N) (Grompe and van de Vrugt, 2007). Eight of the FA proteins are assembled in a nuclear ubiquitin E3 ligase complex (FANCA/B/C/E/F/G/L/M), known as the FA core complex, which monoubiquitinates FANC2 and FANCI (Dorsman et al., 2007; Sims et al., 2007; Smogorzewska et al., 2007). The monoubiquitinated FANC2/FANCI complex is targeted to chromatin (Montes De Oca et al., 2004), where it interacts, either directly or indirectly, with additional downstream FA proteins (FANC1, FANCN, and FANCJ). The downstream FA genes are also breast cancer susceptibility genes (Grompe and van de Vrugt, 2007). Recent studies have indicated that the FANC2/FANCI complex is deubiquitinated by the ubiquitin-specific protease, USP1 (Cohn et al., 2007; Huang et al., 2006; Nijman et al., 2005a). USP1 is not an FA gene per se, since no human FA patients harboring mutations in the USP1 gene have been identified. Disruption of the Usp1 gene in chicken cells (DT40) results in crosslinker hypersensitivity, and the chicken Usp1 and Fanc1 genes are epistatic for crosslink repair (Oestergaard et al., 2007).

The human genome encodes over 90 deubiquitinating enzymes (DUBs) (Nijman et al., 2005b). In rare cases, disruption of a ubiquitin E3 ligase results in a similar outcome to disruption of a corresponding DUB (Nijman et al., 2005b), suggesting that coupled ubiquitination and deubiquitination may be essential for the function of some pathways.

In the current study, we disrupted the murine Usp1 gene. Interestingly, Usp1−/− mice had a strong resemblance to FA mice (small size, infertility, mitomycin C [MMC] hypersensitivity, and chromosome instability). In addition, Usp1−/− mice exhibited a higher rate of perinatal lethality and absence of male germ cells. Our results indicate that Usp1 is required for Fancd2 foci assembly and contributes to homologous recombination (HR) repair, suggesting a regulatory role of Usp1 in the FA pathway.

RESULTS

Perinatal Lethality and Growth Retardation in Usp1-Null Mutation

To address the physiological role of Usp1, we performed a targeted gene deletion of Usp1 in the mouse (see Figure S1 available online). Usp1−/− mice were grossly normal and fertile. Murine Usp1 was detected as two distinct protein products of different size in wild-type mouse embryonic fibroblasts (MEFs), and Usp1−/− MEFs displayed approximately 2-fold reduction in Usp1 protein level (Figure S1C). From Usp1−/− intercrosses, Usp1−/− mice were obtained at a low frequency (6%, instead of the expected 25%; Figure 1A). When analyzed during gestation (E13.5–E18.5) or after cesarean delivery at E19.5–E20.5, Usp1−/− embryos, although significantly smaller than their wild-type littermates (Figure 1C and data not shown), were present at the expected Mendelian ratio. Thus, deletion of the Usp1 gene is
**Developmental Cell**

**FA Phenotype in Usp1-Deficient Mice**

![Gross appearances of newborns and 2-week-old wild-type and Usp1 mice.](image)

![Comparison of testis size from 16-week-old wild-type and Usp1 mice.](image)

![Growth curve of wild-type (gray triangles) and Usp1 mice.](image)

Not embryonic lethal; rather, it led to perinatal lethality, since within 1–2 days after birth, up to 80% of the Usp1 mice were dead. Usp1 pups found dead displayed several abnormalities with incomplete penetrance; these included cyanosis, bilateral hydronephrosis, and hemorrhagic edema. When closely monitored at the moment of delivery, most of the Usp1 pups (75%) became progressively cyanotic and died within 2 hr of birth. Thus, cyanosis may be one of the causes of perinatal lethality in newborn Usp1 mice. The surviving Usp1-deficient mice were consistently smaller than their wild-type littermates, at weaning and as adults, suggesting that growth retardation occurred prenatally and persisted into adulthood (Figures 1B and 1C).

**Gonadal Dysfunction in Usp1-Deficient Male Mice**

Usp1 male mice were sterile. The Usp1 testes was strikingly smaller than that of wild-type or Usp1 littermates (Figure 1D and data not shown). Histology of adult testes from Usp1 mice revealed that their seminiferous tubules were markedly atrophic and mostly devoid of spermatogenic cells (e.g., spermatogonia, spermatocytes, spermatids, and spermatooza; Figure 1E). Consistently, the epididymis of Usp1 mice was devoid of spermatozoa (Figure 1E). We examined seminiferous tubules for cellular proliferation by Ki-67 immunostaining (Figure 1F). At 1 day after birth, Ki-67-positive cells were detected in wild-type and Usp1 tubules with similar frequency, and the intensity of Ki-67 staining in Usp1 tubules was even stronger than that of the wild-type tubules. At 3 and 16 weeks, however, cellularity of Usp1 tubules was greatly decreased, and remaining cells were mostly negative for Ki-67 staining (Figure 1F). Immunostaining for cleaved Caspase 3 revealed that Usp1 tubules had a slightly increased frequency of apoptosis 1 day after birth (Figure 1G). However, the majority of Usp1 cells did not express cleaved Caspase 3, suggesting that apoptosis is not the major cause of testicular atrophy in Usp1 mice.

Usp1 female mice showed a reduced fertility as compared with wild-type females. Usp1 ovaries had less oocytes than wild-type ovaries (8.5 ± 0.6 and 2.9 ± 0.3 per section for wild-type and Usp1 12-week-old ovaries, respectively; five females each; Figure 1H).

**MMC Hypersensitivity and Enhanced Fancd2 and PCNA Monoubiquitination in Usp1-Deficient Cells**

There were no significant differences in cell cycle distribution between wild-type and Usp1 primary MEFs (either asynchronous or after release from synchronization in G0 by serum starvation) (Figures S2A and S2B). As predicted from studies with

---

**Figure 1. Perinatal Lethality, Growth Retardation, and Impaired Germ Cell Development in Usp1-Deficient Mice**

(A) Viability of Usp1-deficient mice at different stages of development.
(B) Growth curve of wild-type (gray triangles) and Usp1 (filled triangles) mice.
(C) Gross appearances of newborns and 2-week-old wild-type and Usp1 mice.
(D) Comparison of testis size from 16-week-old wild-type and Usp1 mice.
(E) Sections of wild-type and Usp1 seminiferous tubules and epididymis stained with hematoxylin and eosin. Magnification, 10x.

(F) Detection of Ki-67 by immunohistochemistry in seminiferous tubules from wild-type and Usp1 mice. Magnification, 40x.
(G) Detection of apoptosis by cleaved Caspase-3 staining in seminiferous tubules from 1-day-old testes. Magnification, 63x.
(H) Usp1 ovaries (lower panel) had reduced numbers of oocytes (arrowhead) compared with wild-type ovaries (upper panel), yet retained the follicle (arrow) from which the oocyte had been released during ovulation. For the quantitative analysis, oocytes were counted from five females of each genotype at 12 weeks, and the number of oocytes was determined for each section (8 sections per each female; total 40 sections). All visible oocytes (including small oocytes and immediately visible oocytes) were counted. Magnification, 5x.
transformed human cell lines (Huang et al., 2006; Nijman et al., 2005a). Usp1−/− MEFs had a compensatory increase in total Fancd2 levels and in its monoubiquitination, as well as proliferating cell nuclear antigen (PCNA) monoubiquitination (Figure 2A). Usp1−/− MEFs exhibited an intermediate increase in Fancd2- and PCNA-monoubiquitination levels, suggesting a haploinsufficiency phenotype (Figure S3).

We next analyzed the response of Usp1−/− MEFs to DNA crosslinking agents. Usp1−/− MEFs showed elevated sensitivity to MMC treatment, whereas the sensitivity of Usp1−/− MEFs to ultraviolet (UV) irradiation was similar to that of wild-type cells (Figure 2B). Furthermore, following MMC treatment, Usp1−/− MEFs exhibited increased chromosomal breaks and radial forms that distinguish FA from other chromosomal breakage syndromes (Figure 2C). Retroviral transduction of wild-type Usp1 partially corrected the MMC-induced chromosome instability of Usp1−/− MEFs (Figure S2D). Since FA cells show an increased accumulation in G2/M following exposure to DNA interstrand crosslinkers, we examined whether Usp1-deficient cells show G2/M accumulation in response to MMC. After a short exposure of MMC, followed by recovery for 24 hr, Usp1−/− MEFs had an increase in G2/M content (57%) compared to that of wild-type MEFs (40%) (Figure S2C). Thus, Usp1−/− MEFs have a specific defect in DNA crosslink repair, similar to FA cells.

per hind limb indicated a significant reduction in Usp1−/− mice, whereas peripheral blood from Usp1−/− mice showed normal values for hemoglobin and white blood cell count (data not shown). Following methylcellulose culture in the presence of increasing concentrations of MMC, Usp1−/− BM cells, like Fancd2−/− BM cells, exhibited hypersensitivity to MMC compared with wild-type cells (Figure 2D). Both Usp1−/− and Fancd2−/− BM cells showed only mild sensitivity to IR compared with wild-type controls. However, Usp1-deficient mice displayed hypersensitivity to total body irradiation (Figure 2E), as has been described for Fancd2-deficient mice (Houghtaling et al., 2003).

**Increased Chromatin Accumulation of Monoubiquitinated Fancd2, but Decreased Nuclear Foci in Usp1-Deficient MEFs**

Monoubiquitinated Fancd2 and FANCI are targeted to chromatin and form DNA repair foci in response to DNA damage (Garcia-Higuera et al., 2001; Smogorzewska et al., 2007). Since Usp1 depletion resulted in increased monoubiquitination of Fancd2, we examined whether this monoubiquitinated Fancd2 is localized to chromatin by using biochemical cell fractionation (Figure 3A). The “S1” fraction contains cytoplasmic and nucleoplasmic proteins; the “S2” fraction contains proteins bound to

**FA Phenotype in Usp1-Deficient Mice**

**Developmental Cell**

FA Phenotype in Usp1-Deficient Mice

Figure 2. Hypersensitivity to DNA Cross-linking Agents in Usp1-Deficient Cells

(A) Increased Fancd2-Ub and PCNA-Ub in Usp1-deficient cells. Wild-type and Usp1−/− MEFs were left untreated, or were treated with MMC (500 ng/ml for 20 hr) or UV (30 J/m², harvested at 3 hr after irradiation). Cell lysates were immunoblotted with indicated antibodies.

(B) Survival rates of wild-type and Usp1−/− MEFs to MMC and UV treatment are determined as the percentage of viable cells relative to that for respective untreated cells.

(C) MMC-induced chromosomal aberrations in wild-type (gray bars) and Usp1−/− (filled bars) MEFs following the treatment with MMC for 48 hr. The numbers of chromosomal aberrations (left panel) and radial forms (right panel) per metaphase spread were scored.

(D) Clonogenic survival assay of BM cells from wild-type (open squares), Usp1−/− (gray squares), Usp1−/− (filled squares), and Fancd2−/− (filled triangles) mice. BM cells were treated with increasing doses of MMC (left) or ionizing radiation (IR) (right). After 7–10 days in culture, the numbers of hematopoietic colonies were compared.

(E) Survival of wild-type (n = 10; gray squares) and Usp1−/− (n = 5; filled triangles) mice following 8.25 Gy of whole-body irradiation.

**FA Phenotype in Usp1-Deficient Lymphocytes**

Usp1−/− bone marrow (BM) cells had increased monoubiquitination of Fancd2 and PCNA, as observed in Usp1−/− MEFs (data not shown). The comparison of the number of BM mononuclear cells
wild-type (upper panels) and Usp1-deficient MEFs were defective in both spontaneous (S phase-specific) and DNA damage-inducible Fancd2 foci assembly (Figures 3B and 3C). Fancd2 foci formation was severely impaired in Usp1-deficient MEFs (Figure 3D), despite high levels of monoubiquitinated Fancd2 detected in the chromatin. The absence of Fancd2 foci therefore correlated with the MMC hypersensitivity of Usp1+/− cells. In contrast, Usp1-deficient MEFs exhibited normal levels of γ-H2AX foci and 53BP1 foci in response to MMC treatment (Figure 3E).

**HR Defect in Usp1-Deficient Cells**

HR constitutes a central pathway to mediate repair of MMC-induced DNA damage (Niedzwiedz et al., 2004). Cells deficient for upstream or downstream FA proteins, including Fancd2, are deficient in HR (Litman et al., 2005; Nakanishi et al., 2005; Niedzwiedz et al., 2004; Sims et al., 2007; Smogorzewska et al., 2007; Xia et al., 2006; Yamamoto et al., 2005). The severe defect in Fancd2 foci formation in Usp1+/− MEFs raises the possibility of an HR defect. To determine whether Usp1 is required for HR, we generated Usp1fl/fl MEFs, in which each allele of Usp1 is flanked byloxP sites (Figure 4A). We established four independent clones of Usp1fl/fl MEFs containing a single copy of the HR reporter DR-green fluorescent protein (GFP) (Pierce et al., 2001). These Usp1fl/fl DR-GFP clones were infected with adenovirus expressing Cre recombinase to generate Usp1+/− DR-GFP MEFs. Cre-mediated excision of the loxP-flanked Usp1 gene was confirmed by PCR and immunoblotting (Figure 4B). Following the induction of HR by transfection with an I-SceI expression plasmid, Usp1+/− DR-GFP MEFs showed a 50% reduction in the frequency of GFP+ cells compared with that of Usp1+/− DR-GFP MEFs (Figure 4C). Thus, the deficiency of Usp1 results in a defect in HR repair.

To confirm this effect in human cells, we used DR-U2OS cells, a human osteosarcoma cell line containing an integrated DR-GFP (Xia et al., 2006). siRNA against USP1 reduced the HR frequency in these cells to 45% of control, similar to the siRNA against FANCI (Figure 4D).

The defect in HR observed in Usp1-deficient cells could occur because the cellular pool of Fancd2 is primarily ubiquitinated and bound to chromatin, leaving inadequate cellular levels of free Fancd2. If so, forced expression of Fancd2 could reverse the defect. To clarify this, Usp1+/− DR-GFP MEFs were stably transfected with empty retroviral vector, or the retroviral vector encoding wild-type Usp1, Usp1C90S (i.e., catalytically inactive Usp1), or Fancd2, and these cells were subsequently infected with Cre-expressing adenovirus (Figures 4D and 4E). Expression of the heterologous proteins in the absence of endogenous Usp1 was confirmed by immunoblotting (Figure 4D). Immunoblotting for Fancd2 revealed a partially reverted L:S ratio (i.e., FANCD2-L [monoubiquitinated FANCD2] to FANCD2-S [unubiquitinated Fancd2] ratio) in Usp1+/− MEFs expressing wild-type Usp1, but not Usp1C90S. Consistent with this pattern, wild-type Usp1, but not Usp1C90S, corrected the HR defect of Usp1-deficient MEFs (Figure 4E). Interestingly, expression of excess Fancd2 did not restore HR activity, indicating that the HR defect in Usp1-deficient cells is due to low cellular levels of free Fancd2.
Double Knockout of Usp1 and Fancd2 Results in a More Severe Phenotype

We next generated Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> double-knockout mice by crossing Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> mice. Interestingly, Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> females showed more severe ovarian atrophy than either group of single-knockout mice (Figure 4F). Western blots showed that DNA damage signaling in response to MMC or UV irradiation is not significantly different from that of either of the single mutants or wild-type controls (Figure S5). However, an increase in baseline phosphorylation of Chk2 was observed in Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> fibroblasts, suggesting that spontaneous DNA damage may accumulate in Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> mice (Figure S5). Furthermore, BM cells from Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> mice were more sensitive to MMC than either group of single-knockout mice (Figure 4G). Although it has been reported that mice deficient in Fanc proteins tend to display more severe phenotype in C57B6 background (Agoulnik et al., 2002; Chen et al., 1996; Houghtaling et al., 2003; Wong et al., 2003), our Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> mice in C57BL6 × 129Sv mixed genetic background consistently showed enhanced defects compared with either their single-knockout littermates or Usp1<sup>-/-</sup> mice in C57B6 background (Figure 4G and data not shown). Therefore, it is unlikely that the severe phenotype observed for Usp1<sup>-/-</sup> Fancd2<sup>-/-</sup> mice is solely due to the difference in genetic background. Taken together, these data suggest that Usp1 may regulate additional DNA repair pathways in addition to the FA pathway, perhaps by controlling the ubiquitination state of substrates other than Fancd2 or Fanci.

**DISCUSSION**

We have demonstrated that Usp1-deficient mice have a similar phenotype to that of other FA mouse models. FA-deficient mice are generally small, exhibit reduced fertility, and have heightened crosslinker sensitivity of their primary cells (Agoulnik et al., 2002; Chen et al., 1996; Cheung et al., 2004; Houghtaling et al., 2003; Koomen et al., 2002; McAllister et al., 2002; Pelas et al., 1991; Whitney et al., 1996; Wong et al., 2003;
Yang et al., 2001). Usp1-deficient mice are small and exhibit male infertility, chromosomal instability, and cellular hypersensitivity to crosslinking agents. Moreover, the Usp1-deficient mice exhibit a more severe phenotype than most FA mouse models, with approximately 80% perinatal lethality, testicular atrophy, and depletion of male germ cells. Such a similarity in phenotypes of mice deficient in Usp1 and Fanc genes further argues for their genetic (epistatic) relationships. At the same time, the elevated perinatal lethality and heightened cellular levels of PCNA-Ub in Usp1−/− cells, which are not observed in FA cells, suggest that Usp1 may have other functions (and perhaps other substrates) in somatic cells, which may account, at least in part, for the absence of USP1 deficiency in humans.

The mechanism by which USP1 depletion leads to MMC hypersensitivity is unknown, and several models are possible. First, loss of USP1 results in elevated FANC-D2-Ub and depletion of the unubiquitinated form of FANC-D2 (FANC-D2-S). Loss of FANC-D2-S may impair the ability of a cell to respond to additional crosslink damage. Accordingly, the normal function of USP1 may be to recycle free FANC-D2-S (and free Ubiquitin) in the cell. However, forced expression of Fancd2 in Usp1−/− MEFs did not correct the HR defect (Figures 4A–4C). Alternatively, excessive chromatin accumulation of Fancd2-Ub in Usp1−/− cells may recruit an additional repair factor to chromatin, reducing its availability to participate at sites of damage. Second, elevated Fancd2-Ub levels in the Usp1-deficient MEFs may be toxic to cells, and promote MMC sensitivity. However, double knockout of Usp1 and Fancd2 in mice did not improve the MMC sensitivity, suggesting that high levels of FANC-D2 ubiquitination do not account for the cellular phenotype of Usp1−/− mice (Figures 4F and G). Third, coupled ubiquitination and deubiquitination may be necessary for proper FA pathway function, suggesting that USP1 acts at a critical later step in the FA pathway itself (Figure S6). For instance, FANC-D2 monoubiquitination by the FA core complex promotes chromatin loading and assembly of DNA repair complexes. Usp1-mediated deubiquitination of FANC-D2-Ub may catalyze additional later events in the DNA repair process. Consistent with this model, Usp1-deficient cells have elevated FANC-D2-Ub in chromatin, but impaired foci formation of Fancd2.

Usp1 depletion also results in prolonged, elevated levels of PCNA-Ub. Depletion of USP1 in human cell lines, by siRNA, results in elevated PCNA-Ub levels and elevated mutagenesis (Huang et al., 2006), secondary to increased recruitment of error-prone polymerases, such as DNA polymerase η. One might expect, therefore, that Usp1-deficient mice will exhibit an increased point mutation frequency and, perhaps, an increased cancer incidence.

**EXPERIMENTAL PROCEDURES**

**Generation of Usp1 Knockout Mice**

Usp1 conditional knockout mice (in C57BL/6J genetic background) were generated by Ingenko under a consortium agreement. A targeting vector was engineered to disrupt the Usp1 gene in C57BL/6J-derived embryonic stem cells. Exon 3 was targeted for replacement by exon 3 flanked by LoxP sites and neo cassette flanked by Flp recombination target sites. Elia-Cre-transgenic female mice (JAX stock no. 003724) were used to generate heterozygous Usp1−/+:Cre mutant mice. The Cre transgene was removed by backcrossing to wild-type C57BL/6J mice.

**Supplemental Data**

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.developmentalcell.com/supplemental/S1534-5807(09)00003-3/.

**ACKNOWLEDGMENTS**

We thank Maria Jasin and Hisao Masai for DR-U2OS and for Cre recombinase-expressing adenovirus, respectively. We thank Lisa Moreau for chromosomal breakage analysis, Kenneth Law for immunohistochemistry, Eunmi Park for help in characterization of newborn mice, and Tony Huang for helpful discussions. We thank Patricia Stuckert, Mary Kathryn DeLoach, and Kaya Zhu for technical assistance. This study was supported by NIH grants RO1DK43889, RO1HL52725, PO1DK50654, and U19AI087751.

Received: August 28, 2008

Revised: November 15, 2008

Accepted: January 6, 2009

Published: February 16, 2009

**REFERENCES**


**HR Assay**

For HR assay with MEFs, the DR-GFP reporter, hypoxanthine-guanine phosphoribosyl transferase-DR-GFP-hygromycin (Nakanishi et al., 2005), was integrated into the genome of Usp1fl/fl MEFs. Clones with a single copy of DR-GFP were identified by Southern blotting. These clones were then used for infection with either an “empty” pBABE-puro retrovirus or the pBABE-puro retrovirus into which wild-type Usp1, Usp1fl/fl, or Fancd2 CDNA was inserted. Retrovirus-infected cells were selected in puromycin before infection with adenovirus expressing Cre recombinase. Usp1fl/fl DR-GFP MEFs were transfected with I-Sce expression plasmid (pCBASce) in triplicate wells of 24-well plates using Lipofectamine 2000. The parallel transfection with pEGFP-C1 was used to normalize transfection efficiency. At 48 hr after transfection, cells were trypsinized and single-cell suspensions were analyzed by flow cytometry.


