[Cell Cycle 6:18, 2232-2235, 15 September 2007]; ©2007 Landes Bioscience

Perspective

Hsp90 and the Fanconi Anemia Pathway

A Molecular Link Between Protein Quality Control and the DNA Damage Response

Takayuki Yamashita*

Tsukasa Oda

Takayuki Sekimoto

Laboratory of Molecular Genetics; Department of Molecular and Cellular Biology; The Institute for Molecular and Cellular Regulation; Gunma University; Maebashi, Gunma, Japan

*Correspondence to: Takayuki Yamashita; Laboratory of Molecular Genetics; The Institute for Molecular and Cellular Regulation; Gunma University; 3-39-15 Showa-machi; Maebashi, Gunma 371-8512 Japan; Tel.: +81.27.220.8830; Fax: +81.27.220.8834; Email: y-taka@showa.gunma-u.ac.jp

Original manuscript submitted: 06/21/07
Manuscript accepted: 06/29/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/article/4653

KEY WORDS

Hsp90, geldanamycin, Fanconi anemia, DNA repair, DNA damage, genomic instability, modifier gene, replicative stress, cancer chemotherapy, stem cell

ABSTRACT

Heat shock protein 90 (Hsp90) is a molecular chaperone that plays an essential role in cell growth and survival. The chaperone exerts these functions by regulating key signaling proteins involved in cell growth/survival and protecting cells from proteotoxic stress. Importantly, Hsp90 inhibitors including geldanamycin analogues show anti-tumor effects. We recently found that Hsp90 promotes stabilization and nuclear localization of the Fanconi anemia (FA) protein FANCA, which is required for activation of the FA pathway. The FA pathway is a multiprotein biochemical pathway involved in genotoxic signaling, defects in which cause genomic instability, hematopoietic stem cell failure and tumor development. Inhibition of Hsp90 impairs the intracellular homeostasis of FANCA, resulting in disruption of the FA pathway. These findings have important implications for rational cancer chemotherapy using Hsp90 inhibitors. We also discuss the possible functions of Hsp90 in FA pathophysiology and stem cell/cancer biology. Based on our findings and other data, we propose that Hsp90 functions as "a guardian of the genome" through the control of DNA repair proteins.

INTRODUCTION

Cellular responses to DNA damage are regulated by multiple distinct but highly interconnected pathways. In recent years, significant progress has been made in our understanding of the molecular mechanisms of genotoxic signaling. In this signaling pathway, DNA damage-induced activation of protein kinases (for example, ATM and ATR) and ubiquitin ligases (for example, Rad18) and consequent phosphorylation and ubiquitination of their substrates play an essential role in the recruitment of various DNA repair proteins present in the nucleus to the chromatin at sites of DNA damage, where they are assembled into multiprotein DNA repair machineries.[1-3] On the other hand, the amplitude of DNA damage responses is profoundly influenced by the nuclear levels of key DNA repair proteins, which are regulated by the balances between protein synthesis and degradation and between nuclear import and export. Prominent examples are seen in familial breast cancer-associated proteins such as BRCA1 and BRCA2. BRCA1 and its binding partner BARD1 shuttle between the cytoplasm and nucleus; and heterodimer complex formation by these proteins promotes their nuclear localization, potentially regulating their functions.[4] A recently identified BRCA2-interacting protein PALB2 is required for the stability and localization of BRCA2, with these two proteins functioning as essential partners.[5] Remarkably, the PALB2 gene was recently found to be responsible for familial susceptibility to breast cancer.[6,7]

MOLECULAR MECHANISMS OF THE FANCONI ANEMIA DNA DAMAGE RESPONSE PATHWAY

Fanconi anemia (FA) is a genetically heterogeneous inherited disorder characterized by congenital anomalies, progressive bone marrow failure and susceptibility to leukemia and solid tumors. Cells from FA patients are hypersensitive to DNA crosslinkers such as mitomycin C, showing chromatid breaks and apoptosis after exposure to such drugs at low concentrations. To date, 13 FA genes have been identified. The protein products encoded by these genes form a complicated biochemical pathway involved in the DNA damage response, which is closely connected with familial breast cancer-associated proteins.[8-11] This pathway, called "the FA pathway" or "the FA/BRCA pathway", includes three major stages

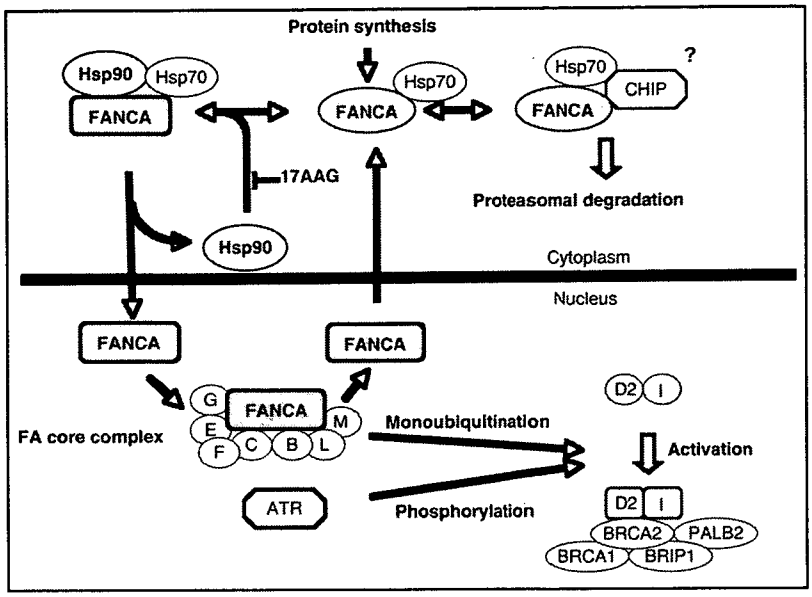

Figure 1. Molecular mechanisms of the FA pathway and the role of Hsp90. FANCA shuttles between the cytoplasm and the nucleus. The Hsp90-based multichaperone complex associates with cytoplasmic FANCA, newly synthesized and exported from the nucleus, promoting its folding into proper conformation required for stabilization and nuclear import. Hsp90 is probably recycled to form a complex with FANCA in the cytoplasm. 17-AAG-mediated inhibition of the chaperone cycle leads to proteasomal degradation of FANCA, possibly through Hsp70-mediated association with a chaperone-dependent ubiquitin-ligase CHIP. In the nucleus, FANCA, FANCB(B), FANCC(C), FANCE(E), FANCF(F), FANCG(G), FANCL(L) and FANCM(M) are assembled into a multisubunit complex (FA core complex) that is required for the downstream activation of the FANCD2(D2)/FANCI(I) complex. See text for other details.

(Fig. 1).First, at least eight FA proteins including FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM are assembled into the FA 'core complex' in the nucleus. FANCL is an E3 ubiquitin-ligase.[8] Second, FANCD2 and its paralogue/binding-partner FANCI are activated by ATR-mediated phosphorylation and the FA core complex-dependent monoubiquitination.[8,9] Third, the active forms of FANCD2 and FANCI are targeted to the chromatin at sites of DNA damage, where they interact with DNA repair proteins including BRCA1 and BRCA2. Interestingly, BRCA2 is identical to FANCD1, and BRCA2-binding protein PALB2 is identical to FANCN.[10,11] Patients with biallelic mutations of these two FA genes (namely, type-D1 and type-N FA patients) characteristically show severe developmental anomalies and pediatric cancers.[8,11] BRCA2 and PALB2 play a critical role in homologous recombination-mediated DNA repair.[5] A BRCA1-associated DNA helicase BRIP1/BACH1 is responsible for type-J FA, but its function in the FA pathway is largely unknown.[8,12] Several studies have suggested that some FA proteins included in the core complex and FANCD2 are involved in the regulation of homology-directed DNA repair.[13,14] However, increasing evidence suggests that these FA proteins function to maintain genome stability through the coordination of multiple DNA repair mechanisms including homologous recombination, non-homologous end-joining and translesion synthesis.[8]

Although there is a notion that the FA core complex is formed in a constitutive manner, several lines of evidence suggests that post-translational control of stability, subcellular localization and protein modification of FA proteins influences function of the FA core complex. For instance, defects in the core complex components, FANCA, FANCC, FANCG and FANCF, seem relatively common in leukemic cells;[15] however, genetic abnormalities of the corresponding FA genes are rarely detected in sporadic cases of leukemia, despite extensive analysis.[16] Thus, aberrant stability control of FA proteins might be involved in the pathogenesis of leukemia. FANCA is primarily localized in the nucleus but shuttles between the cytoplasm and the nucleus.[17,18] FANCA is imported into the nucleus using a bipartite nuclear localization signal (NLS) in its N-terminal region, and is exported from the nucleus through a CRM1-dependent nuclear export machinery.[17,18] FANCG directly binds to the N-terminal region containing the NLS and stabilizes FANCA.[19] Interestingly, overexpression of FANCG not only elevates FANCA protein levels but also prevents nuclear localization of FANCA probably by masking the NLS (Oda T, Yamashita T, unpublished data). In addition, nuclear localization of FANCA seems to be profoundly influenced by its conformation in the C-terminal region, since various patient-derived mutations in this region prevent the nuclear import of FANCA.[20] Furthermore, FANCA is phosphorylated at an unknown serine residue(s), and its phosphorylation level correlates with nuclear localization.[21,22] Finally, impaired activation of the FA pathway due to mislocalization of FANCA was observed in a patient with acquired bone marrow failure.[23] Taken together, these observations suggest that the FA pathway is affected by post-translational regulation of the turnover and trafficking of FANCA. However, the underlying molecular mechanisms remain largely unknown.

We recently reported that the molecular chaperone Hsp90 regulates the FA pathway.[24] During analysis of the FANCA-containing protein complex, we identified Hsp90 and Hsp70, which often cooperate in the form of a multichaperone complex, as possible FANCA-associated proteins. Geldanamycin and its analogues such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) specifically inhibit the chaperone activity of Hsp90 through masking its ATP-binding domain, thus providing a powerful tool with which to study the function of this chaperone. We demonstrated that a cytoplasmic fraction of FANCA interacts with Hsp90 in a 17-AAG-sensitive manner. Furthermore, treatment of cells with 17-AAG induces rapid proteasomal degradation and cytoplasmic retention of FANCA. These data strongly suggest that an Hsp90-based chaperone machinery has a critical role in the stabilization and nuclear import of cytoplasmic FANCA, and that inhibition of Hsp90 induces nuclear depletion of the FA core complex, leading to impaired activation of the FA pathway. Indeed, 17-AAG inhibits DNA damage-induced activation of FANCD2 and enhances DNA crosslinker-induced cytotoxicity, but this drug effect is much less pronounced in FA pathway-defective cells. Furthermore, 17-AAG markedly enhances DNA crosslinker-induced chromosome aberrations. On the other hand, 17-AAG has little effect on ATR activation. Based on these results, we concluded that Hsp90 promotes activation

of the FA pathway through the regulation of FANCA homeostasis.[24] To further confirm this notion, our preliminary data indicate that RNAi-mediated depletion of Hsp90 causes cytoplasmic retention of FANCA (Sekimoto T, Yamashita T, unpublished data).

Hsp90 AND DNA DAMAGE TOLERANCE

Hsp90 and Hsp70 are molecular chaperones that function as part of a protein quality control system.[25,26] They promote not only the maturation of newly synthesized proteins into functional forms, but also the refolding, disaggregation and proteasomal degradation of denatured proteins. In particular, Hsp90 regulates the function and stability of various signaling proteins which play key roles in the regulation of cell growth/survival.[26] These include nuclear hormone receptors, protein kinases (for example, ErbB2 and Raf-1) and transcription factors (for example, p53 and HIF-1α). Importantly, Hsp90-inhibiting geldanamycin derivatives are currently in clinical trials as promising cancer chemotherapeutic agents.[27] The anti-tumor effect of Hsp90 inhibitors is mainly attributed to the proteasomal degradation of cell growth/survival signaling proteins.[26,27] Our findings provide new insights into FA pathophysiology, pharmacological effects of Hsp90 inhibitors and the regulatory mechanisms of cellular DNA damage tolerance.

One interesting hypothesis arising from our findings is that Hsp90 may affect a genotype-phenotype correlation in FA. Extensive mutational analyses in various genetic diseases have revealed that the molecular basis of genotype-phenotype relationship is complicated even in Mendelian inherited disorders. In most of these 'single-gene' diseases, patients with identical mutations in a responsible gene show a broad spectrum of clinical manifestations. Such phenotypic variation is partly explained by modifier genes.[28] Importantly, identification of such modifiers may be useful for developing preventive/therapeutic tools for treating genetic diseases. Although phenotypic variability of FA is partly explained by genetic heterogeneity[11,29] and reversion mosaicism,[30,31] unidentified modifier genes are likely to be involved. Our results raise the possibility that Hsp90 functions as a modifier in FA. To support this notion, Hsp90 is known to mask the phenotypic consequences of mutant proteins by assisting their folding in plants and flies, whereas these cryptic phenotypes are uncovered when Hsp90 functions are compromised by environmental stress.[32] Since FANCA is a polymorphic protein, it is tempting to speculate that Hsp90-assisted folding helps FANCA mutant proteins exert normal or subnormal functions. In this context, environmental stress overloading Hsp90 might affect the clinical phenotypes of FA patients.

Our findings reveal a novel pharmacological effect of Hsp90 inhibitors, which has important implications for the rational design of cancer chemotherapies using Hsp90 inhibitors. DNA interstrand crosslinkers including bifunctional alkylating agents and platinum-based drugs are a major class of anti-tumor agents.[33] Growing evidence suggests that the activity of the FA pathway is an important determinant for tumor sensitivity to these drugs. Epigenetic inactivation of the FANCF gene in several tumor types is associated with their hypersensitivity to DNA crosslinkers.[34] On the other hand, enhanced activity of the FA pathway seems to be responsible for DNA crosslinker resistance.[35] In addition, pharmacological inhibitors of the FA pathway could be used as chemosensitizers of tumor cells to these drugs. For example, curcumin inhibits the FA pathway by unknown mechanisms and sensitizes tumor cells to cisplatin.[36] Similarly, combined use of Hsp90 inhibitors and DNA crosslinkers may have synergistic anti-cancer effects. However, our results raise the possibility that Hsp90 inhibitors cause acquired FA-like disorders as a result of inactivation of the FA pathway, especially when they are used long-term and/or repeatedly.

Hsp90-mediated potentiation of the FA pathway may be important for tumor cell survival in physiological situations. Tumor cells typically show increased expression and activity of Hsp90, which is generally believed to contribute to tumor cells' tolerance to various proteotoxic stressors derived from the microenvironment, including hypoxia and acidosis.[26] On the other hand, tumor cells seem to suffer from continuous replicative stress, derived from their aberrant proliferation, oxidative DNA damage and possibly deprivation of nutrients required for DNA synthesis.[37] These observations suggest that tolerance against replicative stress is probably an important factor for survival advantage of tumor cells. The FA pathway seems to play an integral role in the cellular response to replicative stress through coordinating enzymatic DNA processing, homologous recombination, translesion synthesis and activation of cell cycle checkpoints.[8,38] Thus, we postulate that Hsp90 promotes tumor cell tolerance to replicative stress through activation of the FA pathway, contributing to tumor cell survival. It is plausible that a similar mechanism is involved in the survival of adult stem cells in normal tissues, given that normal stem cells and tumor cells share a number of molecular machineries for their growth/survival.

CONCLUSION

The FA pathway plays an essential role in protecting the genome from genotoxic stress, which is critical for the maintenance of adult stem cells and tumor suppression, especially in hematopoietic tissues. This mechanism is utilized for DNA damage tolerance in tumor cells. We have established that Hsp90-mediated regulation of FANCA has a profound impact on the FA pathway. In addition, Hsp90 regulates various proteins involved in genomic stability, including telomerase, Chk1, topoisomerase II and the NBS1-Mre11-Rad50 complex.[39-42] We propose that Hsp90 functions as a guardian of the genome through the regulation of several DNA damage response pathways, thus playing an important role in the growth and survival of normal stem cells as well as tumor cells.

References

1. Bakkenist CJ, Kastan MB. Initiating cellular stress responses. Cell 2004; 118:9-17.
2. Friedberg EC, Lehmann AR, Fuchs RP. Trading places: How do DNA polymerases switch during translesion DNA synthesis? Mol Cell 2005; 18:499-505.
3. Peterson CL, Cote J. Cellular machineries for chromosomal DNA repair. Genes Dev 2004; 18:602-16.
4. Henderson BR. Regulation of BRCA1, BRCA2 and BARD1 intracellular trafficking. Bioessays 2005; 27:884-93.
5. Xia B, Sheng Q, Nakanishi K, Ohashi A, Wu J, Christ N, Liu X, Jasin M, Couch FJ, Livingston DM. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol Cell 2006; 22:719-29.
6. Rahman N, Seal S, Thompson D, Kelly P, Renwick A, Elliott A, Reid S, Spanova K, Barfoot R, Chagtai T, Jayatilake H, McGuffog L, Hanks S, Evans DG, Eccles D, Breast cancer susceptibility collaboration (UK), Easton DF, Stratton MR. *PALB2*, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 2007; 39:165-7.
7. Erkko H, Xia B, Nikkila J, Schleutker J, Syrjakoski K, Mannermaa A, Kallioniemi A, Pylkas K, Karppinen SM, Rapakko K, Miron A, Sheng Q, Li G, Mattila H, Bell DW, Haber DA, Grip M, Reiman M, Jukkola-Vuorinen A, Mustonen A, Kere J, Aaltonen LA, Kosma VM, Kataja V, Soini Y, Drapkin RI, Livingston DM, Winqvist R. A recurrent mutation in *PALB2* in Finnish cancer families. Nature 2007; 446:316-9.
8. Kennedy RD, D'Andrea AD. The Fanconi Anemia/BRCA pathway: New faces in the crowd. Genes Dev 2005; 19:2925-40.

9. Smogorzewska A, Matsuoka S, Vinciguerra P, McDonald IIIrd ER, Hurov KE, Luo J, Ballif BA, Gygi SP, Hofmann K, D'Andrea AD, Elledge SJ. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. Cell 2007; 129:289-301.
10. Xia B, Dorsman JC, Ameziane N, de Vries Y, Rooimans MA, Sheng Q, Pals G, Errami A, Gluckman E, Llera J, Wang W, Livingston DM, Joenje H, de Winter JP. Fanconi anemia is associated with a defect in the BRCA2 partner *PALB2*. Nat Genet 2007; 39:159-61.
11. Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, Kalb R, Neveling K, Kelly P, Seal S, Freund M, Wurm M, Batish SD, Lach FP, Yetgin S, Neitzel H, Ariffin H, Tischkowitz M, Mathew CG, Auerbach AD, Rahman N. Biallelic mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet 2007; 39:162-4.
12. Cantor SB, Andreassen PR. Assessing the link between BACH1 and BRCA1 in the FA pathway. Cell Cycle 2006; 5:164-7.
13. Yamamoto K, Ishiai M, Matsushita N, Arakawa H, Lamerdin JE, Buerstedde JM, Tanimoto M, Harada M, Thompson LH, Takata M. Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. Mol Cell Biol 2003; 23:5421-30.
14. Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, Wang ZQ, Jasin M. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci USA 2005; 102:1110-5.
15. Xie Y, de Winter JP, Waisfisz Q, Nieuwint AW, Scheper RJ, Arwert F, Hoatlin ME, Ossenkoppele GJ, Schuurhuis GJ, Joenje H. Aberrant Fanconi anaemia protein profiles in acute myeloid leukaemia cells. Br J Haematol 2000; 111:1057-64.
16. Tischkowitz MD, Morgan NV, Grimwade D, Eddy C, Ball S, Vorechovsky I, Langabeer S, Stoger R, Hodgson SV, Mathew CG. Deletion and reduced expression of the Fanconi anemia *FANCA* gene in sporadic acute myeloid leukemia. Leukemia 2004; 18:420-5.
17. Lightfoot J, Alon N, Bosnoyan-Collins L, Buchwald M. Characterization of regions functional in the nuclear localization of the Fanconi anemia group A protein. Hum Mol Genet 1999; 8:1007-15.
18. Ferrer M, Rodriguez JA, Spierings EA, de Winter JP, Giaccone G, Kruyt FA. Identification of multiple nuclear export sequences in Fanconi anemia group A protein that contribute to CRM1-dependent nuclear export. Hum Mol Genet 2005; 14:1271-81.
19. Garcia-Higuera I, Kuang Y, Denham J, D'Andrea AD. The Fanconi anemia proteins FANCA and FANCG stabilize each other and promote the nuclear accumulation of the Fanconi anemia complex. Blood 2000; 96:3224-30.
20. Adachi D, Oda T, Yagasaki H, Nakasato K, Taniguchi T, D'Andrea AD, Asano S, Yamashita T. Heterogeneous activation of the Fanconi anemia pathway by patient-derived FANCA mutants. Hum Mol Genet 2002; 11:3125-34.
21. Yamashita T, Kupfer GM, Naf D, Suliman A, Joenje H, Asano S, D'Andrea AD. The Fanconi anemia pathway requires FAA phosphorylation and FAA/FAC nuclear accumulation. Proc Natl Acad Sci USA 1998; 95:13085-90.
22. Yagasaki H, Adachi D, Oda T, Garcia-Higuera I, Tetteh N, D'Andrea AD, Futaki M, Asano S, Yamashita T. A cytoplasmic serine protein kinase binds and may regulate the Fanconi anemia protein FANCA. Blood 2001; 98:3650-7.
23. Lensch MW, Tischkowitz M, Christianson TA, Reifsteck CA, Speckhart SA, Jakobs PM, O'Dwyer ME, Olson SB, Le Beau MM, Hodgson SV, Mathew CG, Larson RA, Bagby Jr GC. Acquired FANCA dysfunction and cytogenetic instability in adult acute myelogenous leukemia. Blood 2003; 102:7-16.
24. Oda T, Hayano T, Miyaso H, Takahashi N, Yamashita T. Hsp90 regulates the Fanconi anemia DNA damage response pathway. Blood 2007; 109:5016-26.
25. Young JC, Agashe VR, Siegers K, Hartl FU. Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol 2004; 5:781-91.
26. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. Nat Rev Cancer 2005; 5:761-72.
27. Sharp S, Workman P. Inhibitors of the HSP90 molecular chaperone: Current status. Adv Cancer Res 2006; 95:323-48.
28. Nadeau JH. Modifier genes and protective alleles in humans and mice. Curr Opin Genet Dev 2003; 13:290-5.
29. Wagner JE, Tolar J, Levran O, Scholl T, Deffenbaugh A, Satagopan J, Ben-Porat L, Mah K, Batish SD, Kutler DI, MacMillan ML, Hanenberg H, Auerbach AD. Germline mutations in BRCA2: Shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. Blood 2004; 103:3226-9.
30. Soulier J, Leblanc T, Larghero J, Dastot H, Shimamura A, Guardiola P, Esperou H, Ferry C, Jubert C, Feugeas JP, Henri A, Toubert A, Socie G, Baruchel A, Sigaux F, D'Andrea AD, Gluckman E. Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway. Blood 2005; 105:1329-36.
31. Hamanoue S, Yagasaki H, Tsuruta T, Oda T, Yabe H, Yabe M, Yamashita T. Myeloid lineage-selective growth of revertant cells in Fanconi anaemia. Br J Haematol 2006; 132:630-5.
32. Sangster TA, Lindquist S, Queitsch C. Under cover: Causes, effects and implications of Hsp90-mediated genetic capacitance. Bioessays 2004; 26:348-62.
33. McHugh PJ, Spanswick VJ, Hartley JA. Repair of DNA interstrand crosslinks: Molecular mechanisms and clinical relevance. Lancet Oncol 2001; 2:483-90.
34. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: Recent progress. Blood 2006; 107:4223-33.
35. Chen Q, Van der Sluis PC, Boulware D, Hazlehurst LA, Dalton WS. The FA/BRCA pathway is involved in melphalan-induced DNA interstrand cross-link repair and accounts for melphalan resistance in multiple myeloma cells. Blood 2005; 106:698-705.
36. Chirnomas D, Taniguchi T, de la Vega M, Vaidya AP, Vasserman M, Hartman AR, Kennedy R, Foster R, Mahoney J, Seiden MV, D'Andrea AD. Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway. Mol Cancer Ther 2006; 5:952-61.
37. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio Jr RA, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, 39. Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005; 434:907-13.
38. Zhu W, Dutta A. Activation of Fanconi anemia pathway in cells with re-replicated DNA. Cell Cycle 2006; 5:2306-9.
39. Holt SE, Aisner DL, Baur J, Tesmer VM, Dy M, Ouellette M, Trager JB, Morin GB, Toft DO, Shay JW, Wright WE, White MA. Functional requirement of p23 and Hsp90 in telomerase complexes. Genes Dev 1999; 13:817-26.
40. Arlander SJ, Eapen AK, Vroman BT, McDonald RJ, Toft DO, Karnitz LM. Hsp90 inhibition depletes Chk1 and sensitizes tumor cells to replication stress. J Biol Chem 2003; 278:52572-7.
41. Barker CR, McNamara AV, Rackstraw SA, Nelson DE, White MR, Watson AJ, Jenkins JR. Inhibition of Hsp90 acts synergistically with topoisomerase II poisons to increase the apoptotic killing of cells due to an increase in topoisomerase II mediated DNA damage. Nucleic Acids Res 2006; 34:1148-57.
42. Dote H, Burgan WE, Camphausen K, Tofilon PJ. Inhibition of Hsp90 compromises the DNA damage response to radiation. Cancer Res 2006; 66:9211-20.

A requirement of FancL and FancD2 monoubiquitination in DNA repair

Sohsuke Seki[1,2,a], Mioko Ohzeki[1,3,a], Akiko Uchida[1,4], Seiki Hirano[1], Nobuko Matsushita[1], Hiroyuki Kitao[1,b], Tsukasa Oda[5], Takayuki Yamashita[5], Naoki Kashihara[3], Akio Tsubahara[2], Minoru Takata[1,b,*] and Masamichi Ishiai[1]

[1]*Department of Immunology and Molecular Genetics, and*
[2]*Department of Rehabilitation Medicine, and*
[3]*Department of Nephrology, Kawasaki Medical School, Kurashiki, Okayama 701-0192, Japan*
[4]*Department of Hematology, Oncology and Respiratory Medicine, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Okayama 700-8558, Japan*
[5]*Laboratory of Molecular Genetics, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma 371-8512, Japan*

The rare hereditary disorder Fanconi anemia (FA) can be caused by mutations in components of the FA core complex (FancA/B/C/E/F/G/L/M), a key regulator FancD2, the breast cancer susceptibility protein BRCA2/FancD1, or the newly identified FancJ/BRIP1 helicase. By performing yeast two-hybrid (Y2H) screens using N-terminal chicken (ch) FancD2 as a bait, we have identified chFancL, the likely ubiquitin E3 ligase subunit of the FA core complex. We also found that ectopically expressed FancD2 and FancL co-immunoprecipitated in 293T cells, and this interaction was dependent on the PHD domain of FancL. *FANCL*-disrupted chicken DT40 cells displayed defects in both FancD2 monoubiquitination and focus formation. Importantly, cell lines lacking the *FANCL* or *FANCD2* genes, or carrying a "knock-in" mutation of the FancD2 monoubiquitination site (where the Lys 563 residue is changed to Arg), displayed quantitatively identical defects in the repair of I-*Sce*I-induced chromosomal breaks by homologous recombination (HR). These data establish the role of *FANCL* and FancD2 monoubiquitination in HR repair.

Introduction

Fanconi anemia (FA) is a rare hereditary disorder characterized by bone marrow failure, compromised genome stability and an increased incidence of cancer (D'Andrea 2003; Venkitaraman 2004). Complementation analysis revealed at least 12 causative genes, and 11 of them (FancA/B/C/D1/D2/E/F/G/J/L/M) have been cloned (Thompson 2005). Cells lacking these genes display increased levels of chromosome breakage, particularly following induction of DNA interstrand cross-links (ICL) by drugs such as mitomycin C (MMC) or cisplatin (Sasaki & Tonomura 1973; Sasaki 1975). This property has been used as a diagnostic hallmark for FA, and suggests an important role for FA proteins in repairing ICLs. The molecular mechanism of ICL repair (McHugh *et al.* 2000; Dronkert & Kanaar 2001) is still poorly understood; however, it likely requires the intimate interplay of a number of distinct repair pathways and molecules (Nojima *et al.* 2005), including nucleotide excision repair, homologous recombination (HR) repair, translesion DNA synthesis (TLS), SNM1A (Sensitivity to Nitrogen Mustard 1A) and SNM1B (Ishiai *et al.* 2004) and FA proteins.

Recent studies have indicated that the FA pathway promotes DNA repair function through both HR and TLS pathways (Yamamoto *et al.* 2003; Niedzwiedz *et al.* 2004; Hirano *et al.* 2005; Nakanishi *et al.* 2005). However, how FA proteins exert their function is still unclear. Eight FA proteins constitute the nuclear FA core complex (FancA/B/C/E/F/G/L/M). Only two of them, FancL and FancM, have conserved sequence motifs that suggest an obvious catalytic function. FancL contains a

Communicated by: Keiji Tanaka
**Correspondence*: E-mail: minorut@hiroshima-u.ac.jp
[a]These authors contributed equally to this work.
[b]*Present address*: Department of Human Genetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8553 Japan.

DOI: 10.1111/j.1365-2443.2007.01054.x

PHD-type zinc-finger (PHD finger) domain (Meetei *et al.* 2003a), while helicase and nuclease domains are found in the FancM protein (Meetei *et al.* 2005; Mosedale *et al.* 2005), which is homologous to Archea Hef (Nishino *et al.* 2005). Upon DNA damage or replication fork stalling, the FancD2 protein is modified by monoubiquitination on a specific Lys residue (Lys 561 in human (h) protein). Following monoubiquitination, which is likely mediated by FancL (Meetei *et al.* 2003a), FancD2 is targeted to chromatin and accumulates at or close to sites of damaged DNA, forming nuclear foci that can be detected by immunofluorescence. Consistent with the functional studies (Nakanishi *et al.* 2005; Yamamoto *et al.* 2005), FancD2 foci co-localize with HR proteins, such as Rad51, BRCA1 (Taniguchi *et al.* 2002a) and BRCA2 (Wang *et al.* 2004), or with the TLS factor Rev1 (Niedzwiedz *et al.* 2004) following ICL induction. This monoubiquitination seems both necessary and sufficient for FancD2 activation as shown by ectopic expression of either mutated FancD2 (Garcia-Higuera *et al.* 2001; Taniguchi *et al.* 2002b) or a FancD2-ubiquitin fusion protein (Matsushita *et al.* 2005). Any mutation that has been identified in the core complex components appears to compromise the E3 ligase activity as well as structural integrity of the complex, resulting in common down-stream defects in monoubiquitination and focus formation of FancD2. In addition, the disrupted core complex may lose its own effector function, which is still undefined but might be carried out by components such as FancM (Matsushita *et al.* 2005; Meetei *et al.* 2005; Mosedale *et al.* 2005).

Identification of the other two FA genes, FancD1/BRCA2 (Howlett *et al.* 2002) and FancJ/BRIP1 (Bridge *et al.* 2005; Levitus *et al.* 2005; Levran *et al.* 2005; Litman *et al.* 2005), has forged a strong link between FA and familial breast cancer. While mutations in a single allele of BRCA2 predispose carriers to breast or ovarian cancer, biallelic hypomorphic mutations are found in a subset of FA patients (Howlett *et al.* 2002). Such patients are clinically rather distinct, for they often develop early onset leukaemia and brain tumors, which are rarely seen in individuals from the other complementation groups (Hirsch *et al.* 2003; Wagner *et al.* 2004). FancJ/BRIP1 was originally identified through interaction with another breast cancer suppressor, BRCA1 (Cantor *et al.* 2001). The role of BRCA2 is well characterized as a major regulator of Rad51, a central molecule in HR that mediates homology searching and strand transfer (Venkitaraman 2002). On the other hand, BRIP1 protein has a helicase activity, which preferentially unwinds a forked duplex structure that may mimic a stalled replication fork (Gupta *et al.* 2005). Of importance, in both *fancd1* and *fancj* cell lines, monoubiquitination of FancD2 occurs normally, placing these molecules down-stream of, or parallel to, the FA core complex–FancD2 pathway (Howlett *et al.* 2002; Levitus *et al.* 2004).

In this study, we have identified chicken FancL as a FancD2-interacting protein by yeast two-hybrid (Y2H) screening. We have established and characterized a *FANCL*-deficient mutant DT40 cell line, and confirmed a role for FancL in HR. To rigorously test whether the physiological function of FancD2 totally relies on its monoubiquitination, we generated DT40 cells carrying a monoubiquitination site "knock-in" mutation (Lys 563 changed to Arg) at the endogenous *FANCD2* locus, and compared phenotypes among *FANCD2*-null mutant (designated *fancd2-null*), the "knock-in" K563R mutant (*fancd2-K563R*) and *FANCL*-deficient (*fancl*) cells. Our results establish a requirement for FancL and FancD2 monoubiquitination in the repair of DSBs mediated by HR.

Results

Identification of FancL as a FancD2-interacting protein by Y2H screening

Previous studies showed that FancD2 plays crucial roles in the DNA damage response; however, the precise molecular mechanism by which FancD2 is activated is largely unknown. To analyze this, we employed the Y2H screening to search for proteins that interact with FancD2, using an N-terminal chFancD2 fragment (chFancD2 2-722) (Fig. 2A). Full length chFancD2 was not suitable for screening because of autoactivation of the reporters (data not shown). We isolated 15 positive clones. DNA sequencing of the interacting clones revealed that one of them encoded full-length chPHF9, later identified as FancL (Meetei *et al.* 2003a). Chicken PHF9/FancL encodes a protein with 373 amino acids, and the percentage amino acid sequence identity with hFancL is 68%. Human FancL has three potential WD40 repeats and a PHD finger motif (Meetei *et al.* 2003a), and these were all well conserved in chFancL (NCBI/EMBL/DDBJ accession number: AB214907 for chFancL). A part of the amino acid sequence has been described in Matsushita *et al.* (2005) as supplement Fig. S4A.

To confirm the interaction detected by Y2H, we transfected 293T cells with vectors which express FLAG-tagged chFancD2 and GFP-tagged chFancL. In anti-FLAG immunoprecipitates prepared from cell lysates of the transfected 293T cells, anti-GFP Western blotting could successfully detect the presence of GFP-chFancL (Fig. 1). Furthermore, we detected Y2H interaction between N-terminal fragment of hFancD2 (1-719 amino acids) and hFancL (Table 1), indicating that FancD2–FancL interaction is evolutionarily conserved.

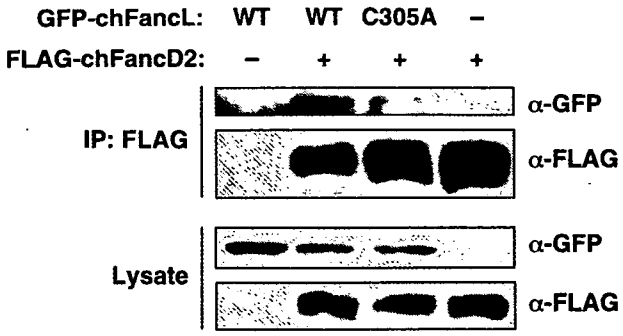

Figure 1 Co-immunoprecipiation of FLAG-chFancD2 with GFP-chFancL. 293T cells were co-transfected with FLAG-chFancD2 and GFP-chFancL, and lysates were prepared 24 h after transfection. WT or C305A, wild-type or the PHD domain mutant (C305A) of chicken FancL, respectively. After anti-FLAG immunoprecipitates or whole cell lysates were separated by SDS-PAGE, GFP-chFancL was detected by Western blotting using anti-GFP antibody.

We extended the Y2H assay to determine which regions were important for the interaction between chFancD2 and chFancL. First, we focused on chFancD2. To cover the entire chFancD2 protein (1439 amino acids), we made three bait plasmids each carrying different chFancD2 fragments (residues 2-429, 162-1082 and 1083-1439), but none of these could interact with full-length chFancL using the Y2H assay (Fig. 2A). Hence, the original bait used for screening (chFancD2 2-722) was divided into three pieces; namely 2-177, 162-429 and 430-722, and these were similarly tested for Y2H interaction with chFancL. Again, none of the fragments could bind to chFancL (data not shown). These data suggest either that a large portion within chFancD2 2-722 is required for interaction with chFancL or that the interaction domain is formed from two or more non-contiguous regions of the polypeptide. Note that the bait chFancD2 2-722 contains the Lys 563 monoubiquitination site. However, the similar bait carrying K563R mutation (chFancD2 KR 1-758) was still able to interact with chFancL (data not shown).

Next, we tried to narrow down the interacting portion in chFancL. To test whether the PHD domain is important for FancL–FancD2 interaction, we changed the two conserved amino acid residues to Ala (chFancL C305A, which was described in Matsushita *et al.* (2005), and W339A). In addition, the Pro 330 and the Gln 348 residues, which are conserved from human to fish, were each mutated to Lys and Thr (P330K and Q348T), the corresponding residues found in the *Drosophila* FancL. The C305A mutation reduced the chFancD2–chFancL interaction in both Y2H (Fig. 2C) and co-immunoprecipitation (Fig. 1) assays. This could be due to destruction of the whole protein structure, since the equivalent C307A mutation in hFancL impaired the interaction with a few other FA proteins (Table 1, see below). However, the other PHD domain mutations (P330K and Q348T), but not W339A, also affected the interaction (Fig. 2B,C) indicating that the PHD finger domain is required for interaction with chFancD2. We also divided the full length chFancL cDNA into four overlapping fragments as shown in Fig. 2B, and tested Y2H interaction. None of them interacted significantly with the chFancD2 2-722 bait, indicating that both PHD domain (305-374 region) and N-terminal 1-143 region are required for the interaction. The inverse combination of the bait–prey was not feasible, since the bait contained the full-length chFancL autoactivated reporters (data not shown).

Table 1 Interactions between human FancL, various FA genes by Y2H

	Bait: human FA genes						
Prey	A	B	C	D2	E	F	G
hFancL	+*	–	–	+	–	+	N.T.†
hFancLC307A	–	–	–	–	–	–	N.T.
vector	–	–	–	–	–	–	N.T.

+, weak growth and pale blue colonies; –, no growth and white colonies.
*Activation of *LEU2* and *β-galactosidase* (*lacZ*) reporter genes as shown in Fig. 2.
†N.T., not testable because of the autoactivation of reporters.

Since hFancL was isolated as a FancA-associated protein, it should be a part of the FA core complex (Meetei *et al.* 2003a). We examined whether FancL directly binds to other components of the FA core complex by Y2H. The bait plasmids were prepared by subcloning a variety of human FA core complex genes, because fewer chicken FA genes were available. Using hFancL as a prey, we found that, besides hFancD2, only hFancA and hFancF displayed significant binding with hFancL (Table 1). These interactions were disrupted by the C307A mutation (equivalent to chFancL C305A). Consistently, a previous report showed that co-immunoprecipitation between hFancA and hFancL was reduced by the C307A mutation (Meetei *et al.* 2003a).

FANCL-deficient cells show defects in FancD2 activation and repair of DNA damage

To characterize the physiological function of FancL, we generated *FANCL* gene disruptants in the chicken B cell

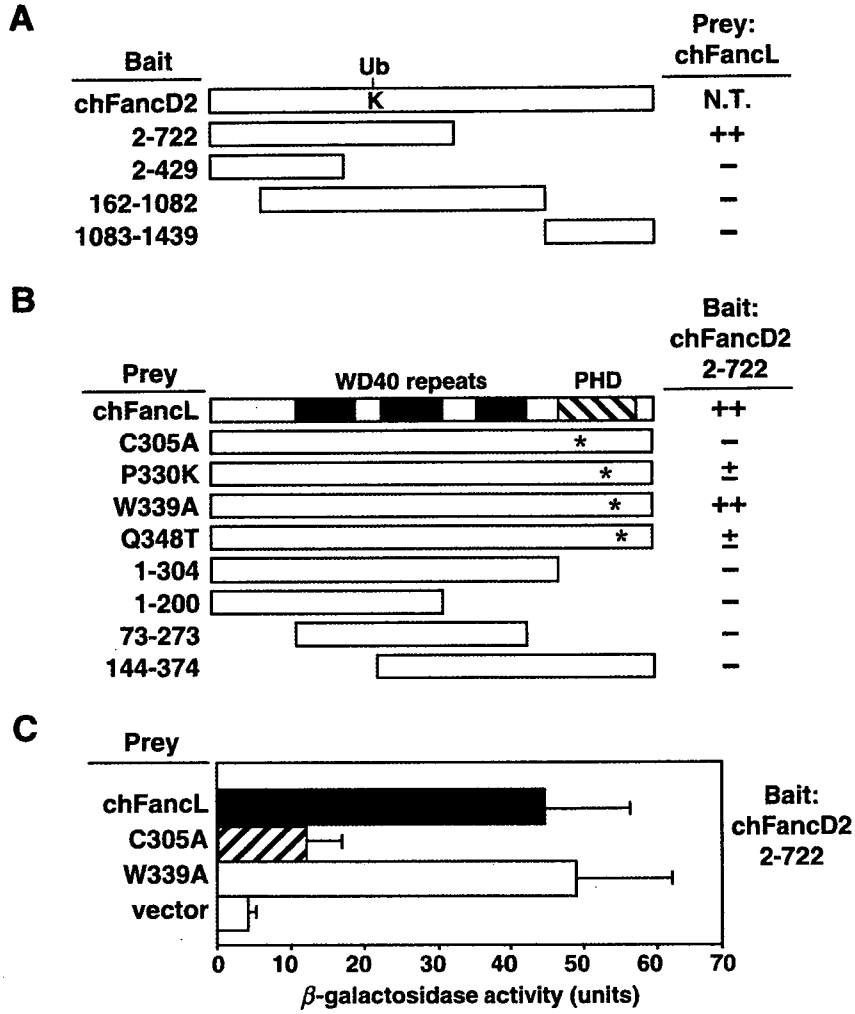

Figure 2 Interaction between chFancD2 and chFancL by Y2H analysis. Yeast cells were co-transformed with the bait plasmid containing a chFancD2 fragment and the prey plasmid containing a chFancL fragment. Activation of *LEU2* and *lacZ* reporter genes is shown in a semiquantitative way: ++, strong growth and blue colonies; ±, very weak growth and pale blue colonies; –, no growth and white colonies; N.T., not testable because of the autoactivation of reporters. (A) chFancD2 fragments used as a bait plasmid for assay with full-length chFancL prey plasmid as indicated. (B) chFancL fragments used as prey plasmid for assay with the chFancD2 2-722 bait plasmid. (C) β-galactosidase activity was determined using a liquid assay and calculated in Miller units. Yeast with the chFancD2 2-722 bait plasmid and the prey containing chFancL or its mutant (C305A or W339A) were used. Empty prey plasmid was included as a control. The data shown are means ± standard deviation (SD) of three independent transfectants.

line DT40. Based on the cDNA sequence, we PCR-amplified genomic DNA fragments of *chFANCL* and designed the targeting construct (Fig. 3A). The gene targeting was achieved by serial transfections with the construct, and gene disruption was verified by Southern (Fig. 3B) and Northern blot analyzes (Fig. 3C).

We first looked at the monoubiquitination of FancD2 in *fancl* cells. As shown in Fig. 3D, monoubiquitinated FancD2 (L-form) was markedly increased in intensity in response to MMC treatment in wild-type cells. In contrast, the L-form was not detected in *fancl* cells even after MMC treatment (Fig. 3D). This defect was restored by the expression of GFP-chFancL (Fig. 3D). Consistently, MMC-induced FancD2 focus formation was markedly decreased by *FANCL* disruption (see Fig. 5A, middle panel).

The DNA repair capacity of *fancl* cells was assessed using colony survival assays following exposure to DNA damaging agents. *fancl* cells were only slightly more sensitive to X-ray than were the wild-type controls (Fig. 3E), but were extremely sensitive to the DNA cross-linkers, cisplatin (as previously reported in Matsushita *et al.* 2005) and MMC (Fig. 3F). Again, the defects were complemented by the expression of GFP-chFancL in *fancl* cells (Fig. 3E,F, and Supplement Fig. S4C in Matsushita *et al.* 2005).

To test whether *fancl* cells have HR defects, we examined the frequencies of gene targeting events that occur at two independent genomic loci. Wild-type and *fancl* cells were transfected with gene targeting vectors in parallel, and targeting events were examined by Southern blot analysis. *FANCL*-deficient cells had a drastically reduced gene targeting efficiency compared to wild-type cells, and this defect was partially complemented by expression of GFP-chFancL (Table 2). These results indicate that *fancl* cells display phenotypes that are observed in other DT40 FA mutants, and that the severity of the defects is very similar to that seen in *fancd2-null* mutant cells (Yamamoto *et al.* 2005).

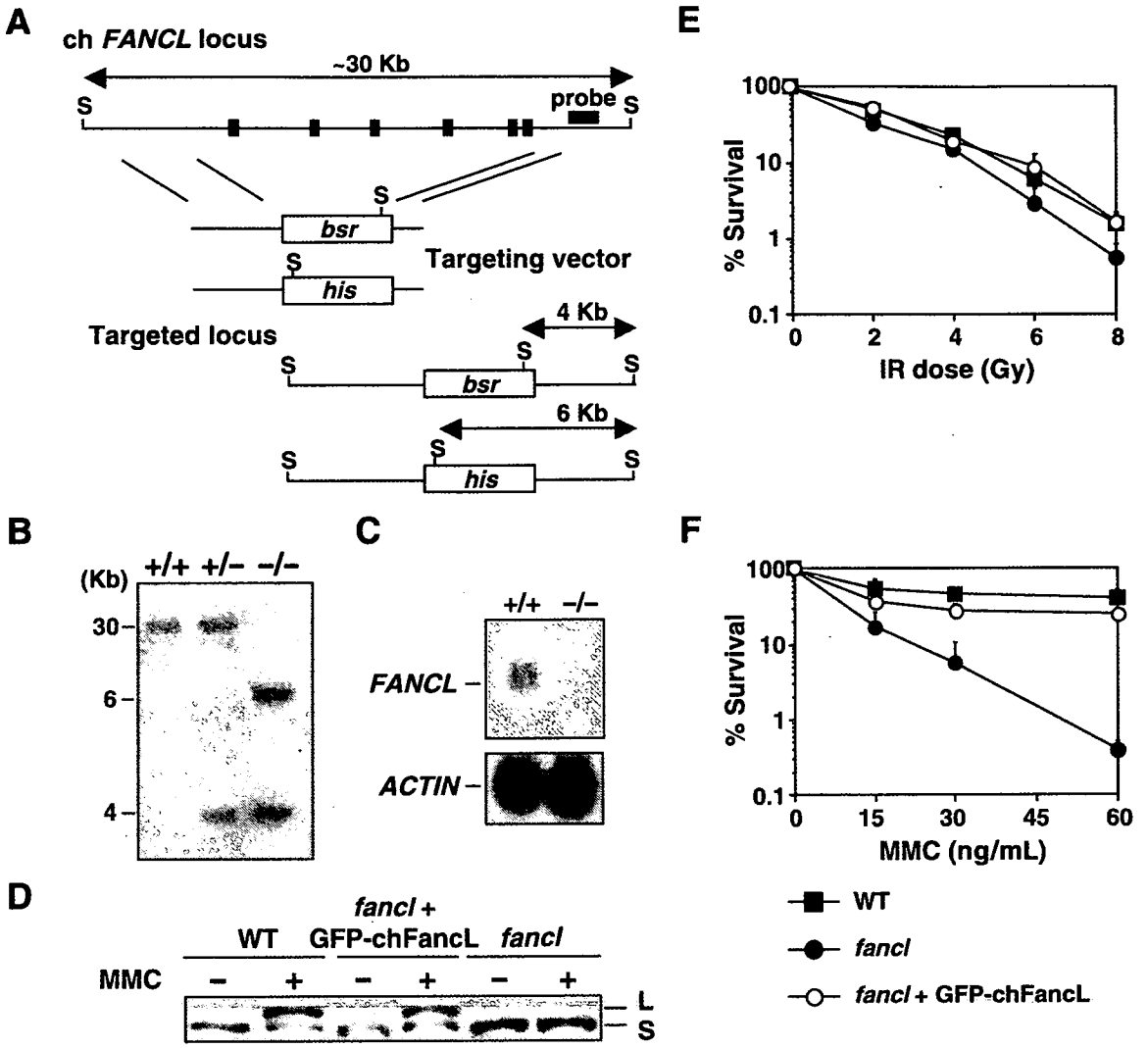

Figure 3 Targeted disruption of *chFANCL* loci in DT40 cells. (A) Schematic representation of partial *chFANCL* locus, the gene targeting constructs and the configuration of targeted alleles. The black box indicates the positions of exons that were disrupted. S, *Sac*I site. (B) Southern blot analysis of *Sac*I-digested genomic DNA from cells with indicated genotypes by using flanking probes as shown in panel A. (C) Northern blot analysis of total RNA from wild-type and *fancl* cells with a chFancL cDNA probe. (D) Western blot analysis of whole cell lysate prepared from wild-type, *fancl* and complemented *fancl* cells with GFP-chFancL expression vector using anti-chFancD2 sera. Cells were treated with MMC (500 ng/mL) for 6 h. L or S denotes the FancD2-L and FancD2-S forms, respectively. (E and F) Sensitivities of wild-type and *fancl* DT40 cells to DNA-damaging agents. The fraction of the surviving colonies following treatments with X-ray (E) or MMC (F) is shown. The data shown are means ± SD of at least three separate experiments.

Generation of FancD2 monoubiquitination site (K563R) mutant cells

In DT40 cells, it is quite straightforward to introduce a specific mutation into an endogenous locus by "knock-in" gene targeting. To examine the physiological significance of monoubiquitination of FancD2, we generated a *FANCD2* mutation in which the critical Lys residue was changed to Arg (*fancd2-K563R*). As summarized in (Fig. 4A,B), we first deleted three exons containing the monoubiquitination site in one allele by the *FANCD2-bsr* vector. Then the other allele was "knocked-in" with K563R mutation using *D2-K563R-his* vector. Finally, the *bsr* and *his* resistance gene cassettes were excised by GFP-Cre expression as described in Experimental procedures. Genotypes generated in each step in these procedures were verified by Southern blotting (Fig. 4C), and the mutation in *fancd2-K563R* cells was confirmed by DNA

Table 2 Targeted integration efficiencies in *fancl* cells

Genotype	Targeting vector	
	Ovalbumin-puro	*Xrcc2-puro*
wild-type	24/52 (46.2%)	7/22 (31.8%)
fancl	5/78 (6.4%)	1/65 (1.5%)
fancl + *GFP-chFANCL*	4/26 (15.4%)	2/20 (10.0%)

Shown are numbers of targeted colonies per total number of colonies analyzed by Southern blotting. The percentage of targeted integration events is given in parentheses.

sequencing of both PCR-amplified genomic DNA and transcripts (data not shown). Indeed, Western blotting showed that FancD2 L-form was not detected in *fancd2-K563R* cells before or after MMC treatment (Fig. 4D).

Direct comparison of *fancl*, *fancd2-null* and *fancd2-K563R* cells

We compared the phenotypes of *fancl*, *fancd2-null* and *fancd2-K563R* cells. First, we examined focus formation and chromatin targeting of FancD2 in *fancl* and *fancd2-K563R* cells. While wild-type cells displayed robust formation of FancD2 foci following MMC treatment, similarly treated *fancl* and *fancd2-K563R* cells could not form any significant levels of FancD2 foci (Fig. 5A). In wild-type cells, the FancD2 L-form was found to be targeted to chromatin after MMC treatment; however, both S- and L-forms in *fancl* and *fancd2-K563R* cells were not significantly localized in chromatin fraction (Fig. 5B). These results were consistent with the notion that chFancD2 was monoubiquitinated at K563 site, which was most likely catalyzed by chFancL. We next carried out cell growth analysis in parallel, and found that *fancl*, *fancd2-null* and *fancd2-K563R* cells displayed a similarly reduced growth rate compared to wild-type cells (Fig. 6A). We also found that the plating efficiency of these mutants was comparable (~30%), but was reduced relative to that of wild-type cells (nearly 100%).

It is known that FA mutant cells have defects in HR-mediated repair of DSBs (Yamamoto *et al.* 2003, 2005; Nakanishi *et al.* 2005). To directly test whether *fancl*, *fancd2-null* and *fancd2-K563R* mutant cells displayed differences in HR repair capacity, we carried out an assay for HR-mediated repair of an I-*Sce*I-induced DSB. Wild-type, *fancl*, *fancd2-null* and *fancd2-K563R* mutant cells that have the artificial recombination substrate SCneo integrated at the *OVALBUMIN* locus were used for this assay. A chromosomal DSB was introduced in one of the two tandem non-functional *neo* genes by transient introduction of an I-*Sce*I-encoding plasmid. A functional *neo* gene can be reconstituted if the DSB is repaired by HR using another partial *neo* gene as a template. Thus, HR-directed DSB repair capacity could be measured by counting the number of G418-resistant colonies (Yamamoto *et al.* 2003). The reduction in HR efficiency was 26, 24 and 30-fold in *fancd2-null*, *fancd2 K563* and *fancl* mutants, respectively, compared to the wild-type control (Fig. 6B). There were no statistically significant differences amongst the mutant cells. These results suggest that FancD2 monoubiquitination is essential for the activity of FancD2 in HR-mediated repair, and that the FancD2 K563R mutant protein has essentially no residual activity for the repair of chromosomal DSBs by HR.

Next, we examined the cross-linking sensitivity of these mutant cells using a colony formation assay in the presence of cisplatin (Fig. 6C) or following brief exposure to MMC (Fig. 6D). *Fancd2-null* and *fancd2-K563R* mutants showed almost the same level of sensitivity to these cross-linking agents, indicating that the cross-link repair function of FancD2 depends on its monoubiquitination. In contrast, *fancl* cells were slightly, but significantly, more sensitive than either *fancd2-null* or *fancd2-K563R* cells.

Discussion

To date, only FancE has been reported to directly interact with FancD2 among the FA core complex components (Medhurst *et al.* 2001; Pace *et al.* 2002; Gordon & Buchwald 2003). In this study, we have identified chicken PHF9/FancL as a FancD2-interacting protein by Y2H screening, and confirmed that by co-immunoprecipitation between ectopically expressed proteins. We suggest that the interaction between endogenous proteins should be weak and transient, and thus could be undetectable, since purified FA core complex containing FancL does not have FancD2 protein as its stable component (Meetei *et al.* 2003b). Nonetheless, our observation reinforces the notion that FancL monoubiquitinates FancD2 in a direct manner, although this has not been clearly established by *in vitro* reconstitution experiments.

We also found that any partial deletion of FancL or some of the tested point mutations in the PHD domain abrogated the interaction (Fig. 2), indicating that at least the PHD domain and the N-terminal 1-143 region are required to interact with chFancD2. hFancL C307A mutation also disrupted interactions with hFancA and hFancF (Table 1). As we have previously described, the equivalent chicken C305A mutation abrogated the capacity of the GFP-chFancL protein to complement

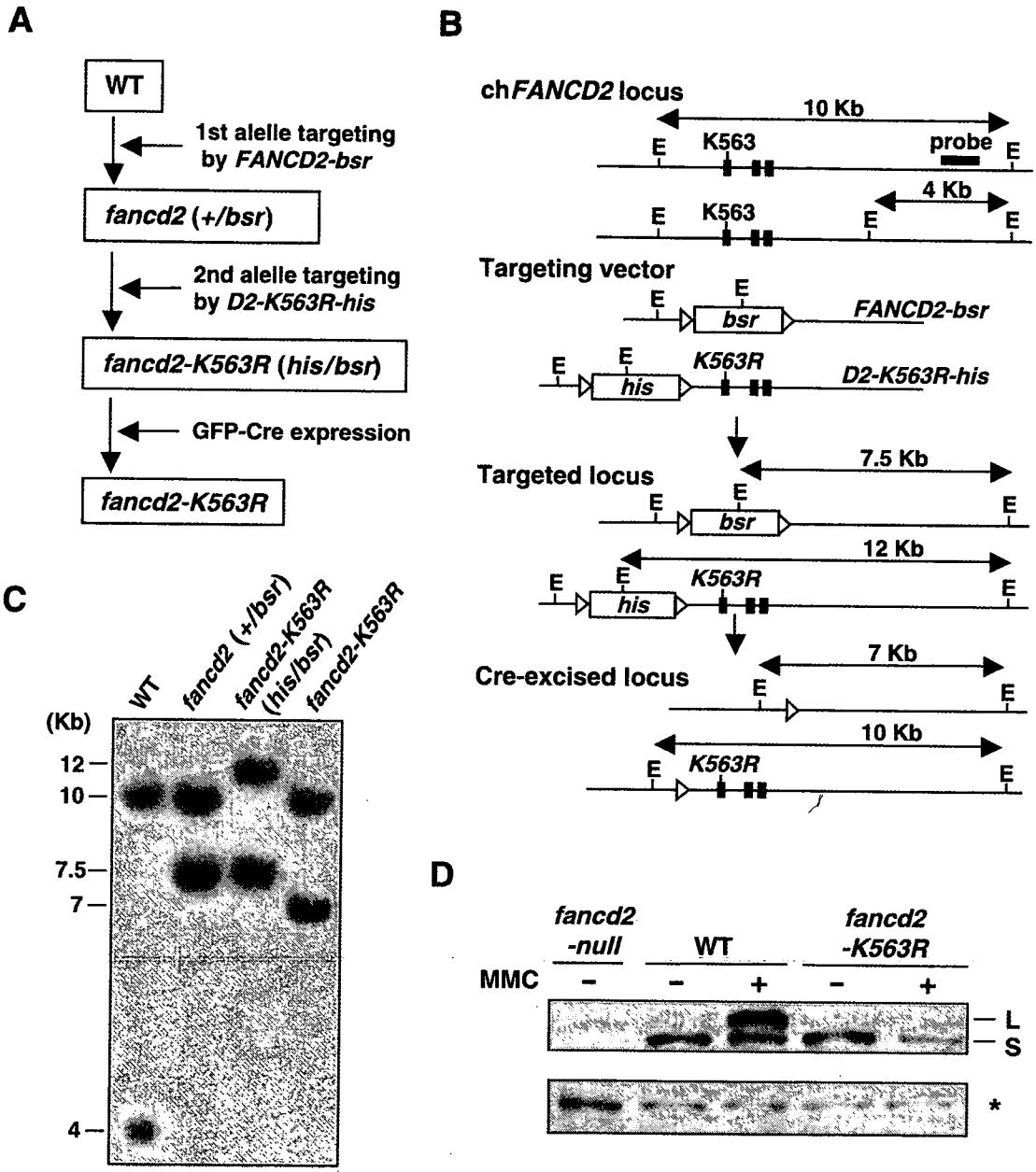

Figure 4 Generation of *fancd2-K563R* "knock-in" cells. (A) A strategy for making *fancd2-K563R* cells as described in Experimental procedures. The drug-resistant gene cassettes, which contain a loxP site at both ends, were removed by GFP-Cre expression and subsequent clone-sorting. (B) Schematic representation of partial chFancD2 locus, the gene targeting constructs and the configuration of targeted alleles. The black box indicates the positions of exons. The white arrowhead indicates the loxP site. E, *Eco*RI site. (C) Southern blot analysis of *Eco*RI-digested genomic DNA from cells with indicated genotypes by using flanking probes as shown in panel B. (D) Western blot analysis of whole cell lysate prepared from wild-type and *fancd2-K563R* cells with or without MMC treatment, as in Fig. 3D. Asterisk indicates nonspecific band as loading control of the samples.

cisplatin sensitivity as well as chromatin localization when expressed in *fancl* cells (Matsushita *et al.* 2005). Consistent with these results, a previous study reported that the human mutation (C307A) disrupted co-immunoprecipitation with hFancA, the ability to complement *fancl* patient's cells with FancD2 monoubiquitination and *in vitro* auto-ubiquitination activity (Meetei *et al.* 2003a). Thus this mutation likely disrupts multiple aspects of FancL function,

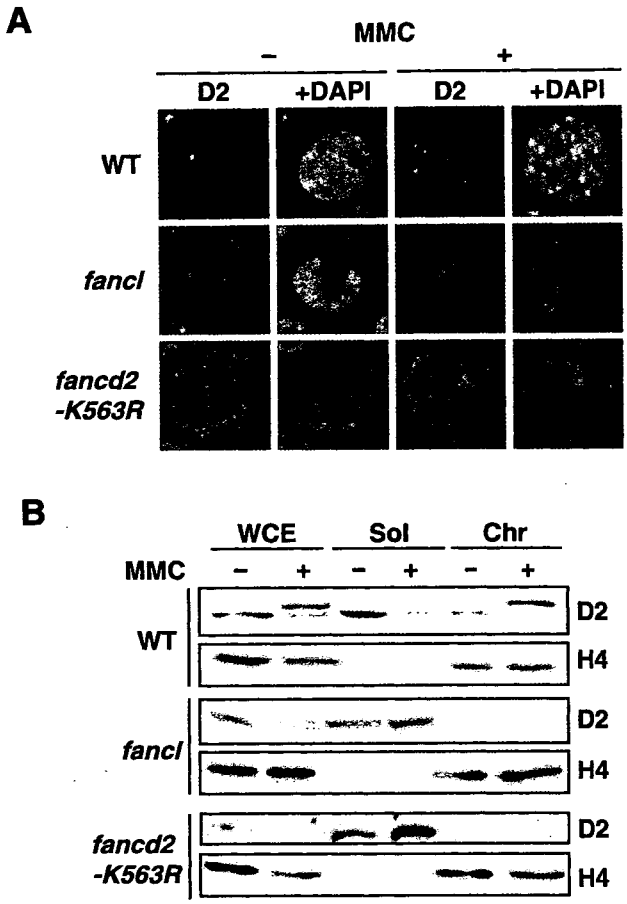

Figure 5 Characterization of *fancd2-K563R* cells. (A) FancD2 focus formation after MMC (500 ng/mL, 6 h) treatment. Cytospin slides were prepared, stained with anti-chFancD2 antibody followed by FITC-conjugated secondary antibody (Invitrogen) and DAPI, and observed under TCS-SP2 confocal laser-scanning microscope (Leica Microsystems, Wetzler, Germany). (B) Chromatin targeting of FancD2 protein. Cells were treated with MMC (500 ng/mL, 6 h) or left untreated, and then fractionated. Each fraction was separated by SDS-PAGE, and Western blotting was carried out using anti-chFancD2 or anti-Histone H4 (Upstate, Lake Placid, NY). WCE, whole cell extract; Sol, soluble fraction; Chr, chromatin fraction. Each lane of WCE, Sol, or Chr fractions contain proteins extracted from 100 thousand, one million, or two million cells, respectively.

including maintenance of the integrity of the core complex as well as ubiquitin E3 ligase activity. It would be interesting to test other mutations in a similar manner to dissect functions of FancL protein.

We have provided evidence of roles for FancL in ICL repair and HR. These functions were not unexpected, given the proposed role of FancL in the monoubiquitination and activation of FancD2. Several studies have implicated the monouibiquitination as a critical modification for activation of FancD2 for DNA repair. For example, mutant FancD2 protein lacking the monoubiquitination site abolished these functions when expressed in *FANCD2*-deficient PD20 cells (Garcia-Higuera *et al.* 2001; Taniguchi *et al.* 2002b). However, such ectopic expression does not necessarily provide definitive proof for a physiological role of the monoubiquitination, since this may lead to overproduction of FancD2. Furthermore, the endogenous *cis* element could be crucial for regulation of *FANCD2*, since defective immunoglobulin gene conversion in *fancd2* cells could not be complemented by CMV promoter-driven expression of FancD2 (Yamamoto *et al.* 2005). Similarly, the defects in targeted integration frequencies could not be fully complemented in both *fancd2* (Yamamoto *et al.* 2005) and *fancl* (this study) by expression of the corresponding expression vectors.

To more rigorously test the role of monoubiquitination (as well as FancL) for FancD2 activation, we generated *fancd2-K563R* cells and compared these cells with *fancd2-null* and *fancl* mutants. We found that *fancd2-K563R* and *fancd2-null* mutants displayed essentially the same degree of defects in cell growth, HR-mediated chromosomal DSB repair, and cisplatin and MMC sensitivity, indicating that absence of monoubiquitination functionally equates to absence of the protein. Thus, the monoubiquitination is critically important for FancD2 to function, at least in the assays analyzed here.

In cell growth and HR-directed DSB repair assays, the *fancl* and *fancd2* mutants showed a similar degree of impairment. In contrast, *fancl* cells showed consistently more severe cross-linking agent sensitivity compared to the other two *fancd2* mutants. Since two independently derived *fancl* clones (data not shown) were both more sensitive to cross-linkers than were the *fancd2* mutants, it seems unlikely that these results were due to simple clonal variation. This raises the following two possibilities. First, it is possible that FancL may ubiquitinate another substrate that modulates ICL repair, but not HR. Absence of FancL affects the function of this putative substrate as well as that of FancD2, and therefore the level of cisplatin sensitivity in *fancl* cells is the combined effects of the failure of two modifications. Alternatively, or additionally, *FANCL* mutation may disrupt the core complex, which will have the effect of disrupting the DNA repair activity mediated by the core complex. Based on our data using FancD2-fusion proteins, we have proposed that the core complex has its own role in DNA repair (Matsushita *et al.* 2005). FancM is an obvious candidate for an effector molecule of the core complex because of its potential DNA modifying activity. For example, *FANCL* mutation

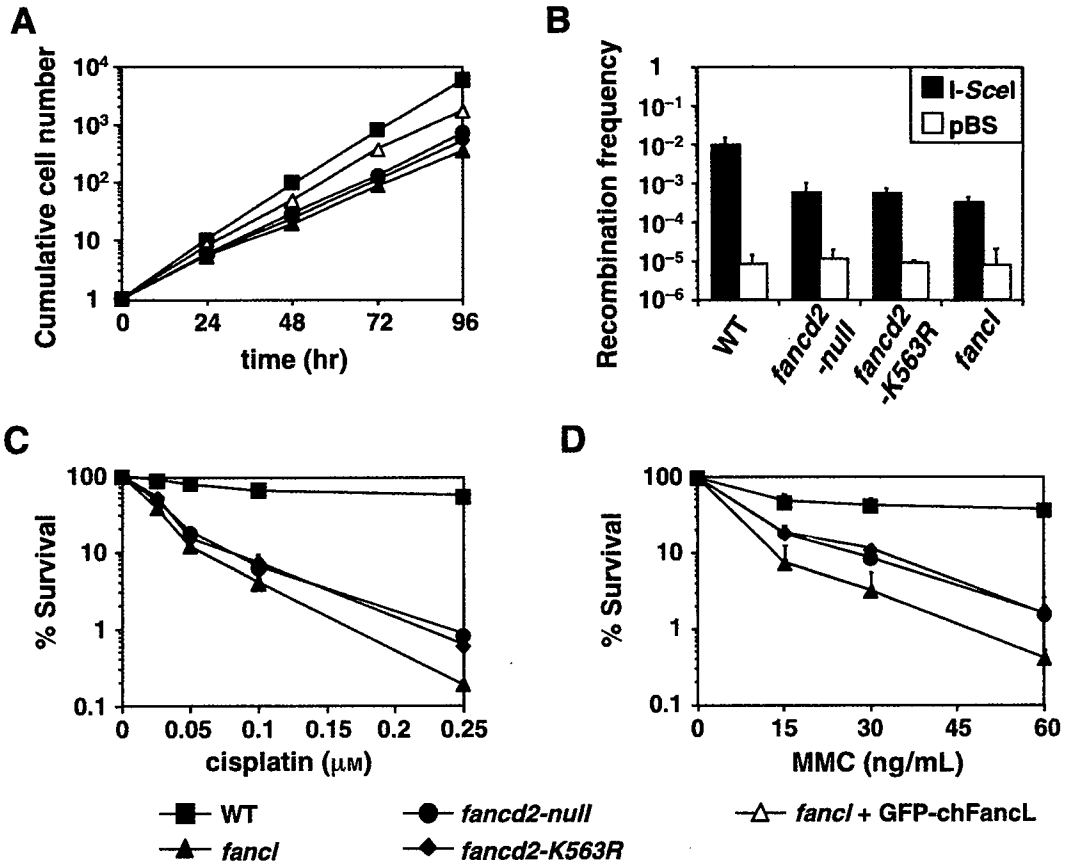

Figure 6 Parallel analysis of wild-type, *fancd2-null*, *fancd2-K563R* and *fancl* cells. (A) Growth curves of cells with indicated genotypes. Cell numbers were monitored by flow cytometry using a fixed number of plastic beads as a standard. (B) Measurement of recombination frequencies by an I-*Sce*I-induced DSB repair system. Cells carrying the recombination substrate were transiently transfected with the indicated plasmids and the transfectants were selected in the presence of G418. The data shown are means ± SD of at least three independent experiments. For I-*Sce*I-induced recombination frequency, statistical significance was detected between wild-type vs. *fancd2-null*, *fancd2-K563R* or *fancl* cells, respectively (Bonferroni/Dunn test, P-value < 0.0001 in all three comparisons). There was no statistical significant difference among *fancd2-null*, *fancd2-K563R* and *fancl* cells. (C and D) Colony survival assay in the presence of cisplatin (C) or following a 1 h exposure to MMC (D) of cells with indicated genotypes. The data shown are means ± SD of at least three separate experiments.

may affect mobilization of FancM into sites of cross-link damage. Consistently, *fancm* mutant DT40 cells do not have a defect in the I-*Sce*I-based HR repair assay (Mosedale *et al.* 2005).

In summary, we isolated chicken FancL as an interacting partner of FancD2, and confirmed the role of FancL in HR repair. Generation of *fancd2-K563R* cells, and direct comparison with *fancd2-null* cells, provided more definitive proof for a crucial role of monoubiquitination in FancD2 activation. Our data also suggest that there is a FancD2-independent role for FancL in ICL repair, but not in HR. The functional relationships between FancD2 and the core complex are still poorly defined and clearly deserve further investigation.

Experimental procedures

Y2H assay

The Y2H assay was performed using the Matchmaker LexA two-hybrid system (BD Clontech, Palo Alto, CA, USA) following the manufacturer's protocol (Ishiai *et al.* 2004). For screening of FancD2-interacting proteins, an N-terminal fragment of chFancD2 (amino acid residues 2-722) was cloned in-frame with the LexA DNA binding domain in the bait plasmid, pEG202. EGY48 yeast cells containing both the bait and the pSH18-34 reporter plasmids were transformed with a pJG4.5-DT40 cDNA library (kindly provided by Dr Ryo Goitsuka, Science University of Tokyo, Chiba, Japan). Using a dual reporter expression system (*lacZ* and *LEU2*), double positive clones was selected from 9×10^5 transfectants. Plasmid

DNA was recovered, and the cDNA inserts were PCR-amplified and sequenced. To detect the interaction between chFancD2 and chFancL, various chFancD2 and chFancL fragments were cloned into the bait or prey plasmid, respectively. Point mutation was introduced using QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA). *hFANCA*, *hFANCB*, *hFANCE* and *hFANCG* cDNAs or the *hFANCC* cDNA were kindly provided by Dr Hans Joenje (VU University Medical Center, Amsterdam, the Netherlands) or Dr Manuel Buchwald (Hospital for Sick Children, Toronto, Canada), respectively. *hFANCL* cDNA was obtained from Biological Resource Center, National Institute of Technology and Evaluation (Kisarazu, Chiba, Japan). *hFANCF* (full length) or *hFANCD2* (1-719 amino acids) cDNAs were isolated by RT-PCR and verified by sequencing. Various human FA genes were cloned into the bait plasmids, while *hFANCL* was cloned into the prey plasmid. The interactions of two proteins were evaluated by growth on synthetic assay media lacking leucine and by blue/white colony coloration on synthetic media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Nacalai Tesque Inc., Kyoto, Japan). For quantitative analysis, the β-galactosidase activity (Miller unit) was measured by liquid culture assay using *o*-nitrophenyl-β-D-galactopyranoside (Nacalai Tesque Inc.) as the substrate.

Cell culture and expression plasmids

Chicken DT40 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% chicken serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin and streptomycin in a 5% CO_2 incubator at 39.5 °C. Generation of *fancd2* (hereafter designated *fancd2-null*) DT40 cells were previously described (Yamamoto *et al.* 2005). Cell growth analysis was done as described (Yamamoto *et al.* 2003). Construction of GFP-chFancL expression plasmid was previously described (Matsushita *et al.* 2005). An expression vector for FLAG-chFancD2 was constructed by inserting full-length *chFANCD2* cDNA in frame into pFLAG-C1, which was made by replacing the EGFP segment of pEGFP-C1 (Clontech) with synthetic FLAG oligonucleotides. The GFP-Cre expression plasmid pBS598 was kindly provided by Dr Brian Sauer (Stowers Institute for Medical Research, Kansas City, MO, USA) (Gagneten *et al.* 1997). Stable or transient transfections were done by electroporation as described (Yamamoto *et al.* 2003).

Co-immunoprecipitation experiments

293T cells were transiently transfected with GFP-chFancL and FLAG-chFancD2 expression vectors, and were harvested 24 h later. Cells were lyzed in lysis buffer (1% NP-40, 10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 1 mM PMSF) supplemented with proteinase inhibitor cocktail (Complete EDTA-free Tablet, Roche Diagnostics, Basel, Switzerland). FLAG-chFancD2 was immunoprecipitated using agarose-beads conjugated with monoclonal anti-FLAG antibody M2 (Sigma, St. Louis, MO, USA). The immunoprecipitates or whole cell extracts were separated with 6% (for FancD2) or 7.5% (for FancL) SDS-PAGE, and then transferred to a membrane and detected with anti-FLAG or anti-GFP (Clontech) antibodies.

Generation of *FANCL*-deficient or *FANCD2*-K563R "knock-in" mutant DT40 cells

The genomic fragment of *chFANCL* or *chFANCD2* was isolated by PCR amplification from DT40 genomic DNA. The *FANCL*-targeting vector was designed by replacing a ~20 kb genomic fragment containing six exons that correspond to chFancL amino acids 105-272, with a *bsr*- or *his*-resistant gene cassette. For making the *fancd2-K563R* "knock-in" vector, K563R mutation was introduced using a QuikChange Kit (Stratagene), and a *his*-resistance cassette was inserted in the intron (see Fig. 4B). DT40 cells were targeted sequentially by the *FancD2-bsr* targeting vector (Yamamoto *et al.* 2005) followed by the *fancd2-K563R-his* vector. The GFP-Cre expression plasmid was transiently transfected by electroporation to remove the *bsr*- and *his*-cassettes, which are flanked by *loxP* sequences. Twenty-four hours after transfection, GFP-positive cells were clone-sorted using FACSAria (Becton Dickinson). Removal of the cassettes was ensured by Southern blot analysis with *chFANCD2* probe.

Measurement of sensitivity of cells to DNA damaging agents

Colony formation was assayed in medium containing 1.4% methylcellulose. Cells were irradiated with X-rays (Linear Accelerator; Mitsubishi Electric, Inc., Tokyo, Japan), or exposed for 1 h to MMC (Kyowa Hakkou, Tokyo, Japan) or continuously to cisplatin (Nihon Kayaku, Tokyo, Japan) as previously described (Yamamoto *et al.* 2003). After the cells were cultured for 7 (for wild-type) to 14 days (for mutant cells), visible colonies were counted.

Measurement of HR-mediated repair of DSBs by I-*Sce*I expression

Analysis of HR-mediated repair of I-*Sce*I-induced DSBs was done as described (Yamamoto *et al.* 2003). The recombination substrate, SCneo (kindly provided by Dr Maria Jasin, Sloan-Kettering Institute, NY, USA), was targeted into the *OVALBUMIN* locus in wild-type and FA mutant cells. I-*Sce*I expression vector, or the control plasmid, pBluescript, was transiently transfected by electroporation, and cells were selected in 96-well culture plates containing 2 mg/mL G418 (Nacalai Tesque Inc.). Surviving colonies were counted after 14 days.

General techniques for DT40 cell analysis

Northern blot analysis, Western blotting, measurement of targeted integration frequencies, subnuclear focus formation assay, preparation of subcellular fractionation into the soluble and chromatin were done as previously described (Yamamoto *et al.* 2003; Ishiai *et al.* 2004; Matsushita *et al.* 2005).

Acknowledgements

We would like to thank Ms Emi Uchida, Keiko Namikoshi and Masayo Kimura for expert and extensive technical assistance; Drs

Ian D. Hickson and Peter J. McHugh (Weatherall Institute, Oxford University, UK) and Dr Jean-Yves Masson (Laval University Cancer Research Center, QuÈbec, Canada) for critical reading of the manuscript; Dr Kenshi Komatsu (Radiation Biology Center, Kyoto University, Japan) for anti-chicken FancD2 antibody; Dr Ryo Goitsuka for pJG4.5-DT40 cDNA library used in Y2H screening; Drs Hans Joenje and Manuel Buchwald for human FA cDNAs; Dr Brian Sauer for GFP-Cre plasmid pBS598; Dr Maria Jasin for SCneo and I-*Sce*I expression plasmids; Dr Yoshinari Imajo and Mr Jyuichi Kubota for irradiating cells with Linear Accelerator; Ms Kazuko Hikasa and Ms Kyoko Takahashi for secretarial assistance. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (M.I. and M.T.). Financial supports were also provided by the Naito Foundation, the Sagawa Foundation for Promotion of Cancer Research (M.T.), the Ryobi Teien Memorial Foundation (M.I.) and Kawasaki Medical School (Project Research Grant 15-201A, 16-205T, 16-206T, 17-210T, 17-216T and 18-202Y).

References

Bridge, W.L., Vandenberg, C.J., Franklin, R.J. & Hiom, K. (2005) The BRIP1 helicase functions independently of BRCA1 in the Fanconi anemia pathway for DNA crosslink repair. *Nature Genet.* **37**, 953–957.

Cantor, S.B., Bell, D.W., Ganesan, S., Kass, E.M., Drapskin, R., Grossman, S., Wahrer, D.C., Sgroi, D.C., Lane, W.S., Haber, D.A. & Livingstone, D.M. (2001) BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell* **105**, 149–160.

D'Andrea, A.D. (2003) The Fanconi road to cancer. *Genes Dev.* **17**, 1933–1936.

Dronkert, M.L. & Kanaar, R. (2001) Repair of DNA interstrand cross-links. *Mutat. Res.* **486**, 217–247.

Gagneten, S., Le, Y., Miller, J. & Sauer, B. (1997) Brief expression of a GFP cre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. *Nucleic Acids Res.* **25**, 3326–3331.

Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. & D'Andrea, A.D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell* **7**, 249–262.

Gordon, S.M. & Buchwald, M. (2003) Fanconi anemia protein complex: mapping protein interactions in the yeast 2- and 3-hybrid systems. *Blood* **102**, 136–141.

Gupta, R., Sharma, S., Sommers, J.A., Jin, Z., Cantor, S.B. & Brosh, R.M. Jr (2005) Analysis of the DNA substrate specificity of the human BACH1 helicase associated with breast cancer. *J. Biol. Chem.* **280**, 25450–25460.

Hirano, S., Yamamoto, K., Ishiai, M., Yamazoe, M., Seki, M., Matsushita, N., Ohzeki, M., Yamashita, Y.M., Arakawa, H., Buerstedde, J.-M., Enomoto, T., Takeda, S., Thompson, L.H. & Takata, M. (2005) Functional relationships of FANCC to homologous recombination, translesion synthesis, and BLM. *EMBO J.* **24**, 418–427.

Hirsch, B., Shimamura, A., Moreau, L., Baldinger, S., Hag-Alshiekh, M., Bostrom, B., Sencer, S. & D'Andrea, A.D. (2003) Biallelic BRCA2/FANCD1 mutations: association with spontaneous chromosomal instability and solid tumors of childhood. *Blood* **103**, 2554–2559.

Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., de Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E.A. & D'Andrea, A.D. (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* **297**, 606–609.

Ishiai, M., Kimura, M., Namikoshi, K., Yamazoe, M., Yamamoto, K., Arakawa, H., Agematsu, K., Matsushita, N., Takeda, S., Buerstedde, J.-M. & Takata, M. (2004) DNA cross-link repair protein SNM1A interacts with PIAS1 in nuclear focus formation. *Mol. Cell. Biol.* **24**, 10733–10741.

Levitus, M., Rooimans, M.A., Steltenpool, J., Cool, N.F., Oostra, A.B., Mathew, C.G., Hoatlin, M.E., Walsfisz, Q., Arwert, F., de Winter, J.P. & Joenje, H. (2004) Heterogeneity in Fanconi anemia: evidence for two new genetic subtypes. *Blood* **103**, 2498–2503.

Levitus, M., Waisfisz, Q., Godthelp, B.C., *et al.* (2005) The DNA helicase BRIP1 is defective in Fanconi anemia complementation group. *J. Nat. Genet.* **37**, 934–935.

Levran, O., Attwooll, C., Henry, R.T., *et al.* (2005) The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. *Nat. Genet.* **37**, 931–933.

Litman, R., Peng, M., Jin, Z., Zhang, F., Zhang, J., Powell, S., Andreassen, P.R. & Cantor, S.B. (2005) BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. *Cancer Cell* **8**, 255–265.

Matsushita, N., Kitao, H., Ishiai, M., Nagashima, N., Hirano, S., Okawa, K., Ohta, T., Yu, D.S., McHugh, P.J., Hickson, I.D., Venkitaraman, A.R., Kurumizaka, H. & Takata, M. (2005) A FancD2-monoubiquitin fusion reveals hidden functions of Fanconi anemia core complex in DNA repair. *Mol. Cell* **19**, 841–847.

McHugh, P.J., Sones, W.R. & Hartley, J.A. (2000) Repair of intermediate structures produced at DNA interstrand cross-links in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **20**, 3425–3433.

Medhurst, A.L., Huber, P.A., Waisfisz, Q., de Winter, J.P. & Mathew, C.G. (2001) Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway. *Hum. Mol. Genet.* **10**, 423–429.

Meetei, A.R., de Winter, J.P., Medhurst, A.L., Walisch, M., Waisfisz, Q., van de Vrugt, H.J., Oostra, A.B., Yan, Z., Ling, C., Bishop, C.E., Hoatlin, M.E., Joenje, H. & Wang, W. (2003a) A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat. Genet.* **35**, 165–170.

Meetei, A.R., Medhurst, A.L., Ling, C., Xue, Y., Singh, T.R., Bier, P., Steltenpool, J., Stone, S., Dokal, I., Mathew, C.G., Hoatlin, M., Joenje, H., de Winter, J.P. & Wang, W. (2005) A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. *Nat. Genet.* **37**, 958–963.

Meetei, A.R., Sechi, S., Wallisch, M., Yang, D., Young, M.K., Joenje, H., Hoatlin, M.E. & Wang, W. (2003b) A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. *Mol. Cell. Biol.* **23**, 3417–3426.

Mosedale, G., Niedzwiedz, W., Alpi, A., Perrina, F., Pereira-Leal, J.B., Johnson, M., Langevin, F., Pace, P. & Patel, K.J. (2005) The vertebrate Hef ortholog is a component of the Fanconi

anemia tumor-suppressor pathway. *Nat. Struct. Mol. Biol.* **12**, 763–771.

Nakanishi, K., Yang, Y.-G., Pierce, A.J., Taniguchi, T., Digweed, M., D'Andrea, A.D., Wang, Z.-Q. & Jasin, M. (2005) Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc. Natl. Acad. Sci. USA* **102**, 1110–1115.

Niedzwiedz, W., Mosedale, G., Johnson, M., Ong, C.Y., Pace, P. & Patel, K.J. (2004) The Fanconi anaemia gene FANCC promotes homologous recombination and error-prone DNA repair. *Mol. Cell* **15**, 607–620.

Nishino, T., Komori, K., Ishino, Y. & Morikawa, K. (2005) Structural and functional analyses of an archaeal XPF/Rad1/Mus81 nuclease: asymmetric DNA binding and cleavage mechanisms. *Structure (Camb)* **13**, 1183–1192.

Nojima, K., Hochegger, H., Saberi, A., *et al.* (2005) Multiple repair pathways mediate tolerance to chemotherapeutic cross-linking agents in vertebrate cells. *Cancer Res.* **65**, 11704–117011.

Pace, P., Johnson, M., Tan, W.M., Mosedale, G., Sng, C., Hoatlin, M., de Winter, J., Joenje, H., Gergely, F. & Patel, K.J. (2002) FANCE: the link between Fanconi anaemia complex assembly and activity. *EMBO J.* **21**, 3414–3423.

Sasaki, M.S. (1975) Is Fanconi's anaemia defective in a process essential to the repair of DNA cross links? *Nature* **257**, 501–503.

Sasaki, M.S. & Tonomura, A. (1973) A high susceptibility of Fanconi's anemia to chromosome breakage by DNA cross-linking agents. *Cancer Res.* **33**, 1829–1836.

Taniguchi, T., Garcia-Higuera, I., Andreassen, P.R., Gregory, R.C., Grompe, M. & D'Andrea, A.D. (2002a) S-phase–specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood* **100**, 2414–2420.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P.R., Gregory, R.C., Kim, S.T., Lane, W.S., Kastan, M.B. & D'Andrea, A.D. (2002b) Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. *Cell* **109**, 459–472.

Thompson, L.H. (2005) Unraveling the Fanconi anemia-DNA repair connection. *Nat. Genet.* **37**, 921–922.

Venkitaraman, A.R. (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**, 171–182.

Venkitaraman, A.R. (2004) Tracing the network connecting brca and Fanconi anaemia proteins. *Nat. Rev. Cancer* **4**, 266–276.

Wagner, J.E., Tolar, J., Levran, O., Scholl, T., Deffenbaugh, A., Satagopan, J., Ben-Porat, L., Mah, K., Batish, S.D., Kutler, D.I., MacMillan, M.L., Hanenberg, H. & Auerbach, A.D. (2004) Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. *Blood* **103**, 3226–3229.

Wang, X., Andreassen, P.R. & D'Andrea, A.D. (2004) Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol. Cell. Biol.* **24**, 5850–5862.

Yamamoto, K., Hirano, S., Ishiai, M., Morishima, K., Kitao, H., Namikoshi, K., Kimura, M., Matsushita, N., Arakawa, H., Buerstedde, J.-M., Komatsu, K., Thompson, L.H. & Takata, M. (2005) Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. *Mol. Cell. Biol.* **25**, 34–43.

Yamamoto, K., Ishiai, M., Matsushita, N., Arakawa, H., Lamerdin, J.E., Buerstedde, J.-M., Tanimoto, M., Harada, M., Thompson, L.H. & Takata, M. (2003) Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. *Mol. Cell. Biol.* **23**, 5421–5430.

Received: 16 September 2006
Accepted: 30 November 2006

International Journal of
HEMATOLOGY

In Vitro Effect of Fludarabine, Cyclophosphamide, and Cytosine Arabinoside on Chromosome Breakage in Fanconi Anemia Patients: Relevance to Stem Cell Transplantation

Miharu Yabe,[a] Hiromasa Yabe,[b] Satoshi Hamanoue,[c] Hiroyasu Inoue,[d] Masae Matsumoto,[d] Takashi Koike,[e] Hiroyuki Ishiguro,[e] Tsuyoshi Morimoto,[e] Satoshi Arakawa,[f] Toshio Ohshima,[f] Atsuko Masukawa,[f] Hayato Miyachi,[a] Takayuki Yamashita,[g] Shunichi Kato[b]

[a]Department of Laboratory Medicine, Tokai University School of Medicine, Isehara, Japan; [b]Department of Cell Transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan; [c]Division of Hematopoietic Stem Cell Transplantation and Hematology, National Cancer Center Hospital, Tokyo, Japan; [d]Division of Pediatrics, Kanagawa Children's Hospital, Yokohama, Japan; [e]Specialized Clinical Science, Pediatrics, Tokai University School of Medicine, Isehara, Japan; [f]Central Laboratory, Tokai University School of Medicine, Isehara, Japan; [g]Laboratory of Molecular Genetics, The Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan

Received September 28, 2006; received in revised form December 27, 2006; accepted January 16, 2007

Abstract

Designing stem cell transplantation (SCT) conditioning regimens for Fanconi anemia (FA) has proved difficult because of hypersensitivity to the DNA cross-linking agents. We performed chromosome fragility tests with 56 FA patients and with 50 non-FA patients with severe aplastic anemia or myelodysplastic syndrome. We evaluated peripheral blood lymphocyte specimens cultured for 72 hours and treated with mitomycin C, diepoxybutane (DEB), cyclophosphamide (CY) metabolites, cytosine arabinoside (Ara-C), and fludarabine (Flu) metabolite (9-β-D-arabinofuranosyl-2-fluoroadenine [2-F-Ara-A]). The DEB and CY metabolite tests were highly sensitive and specific for FA ($P < 10^{-4}$ for both tests), and the number of aberrations per cell for DEB correlated with that for the CY metabolite test ($P < 10^{-4}$) but did not correlate with the number of aberrations per cell for the Ara-C and 2-F-Ara-A tests. The difference in breakage frequencies between FA and non-FA patients for cultures treated with 2-F-Ara-A was not statistically significant. Most of the breakages observed in cells treated with 2-F-Ara-A-and Ara-C were chromatid breaks. It may be possible to determine the appropriate CY dose in the preconditioning regimen for SCT in FA patients on the basis of the in vitro effects on fragility, and Flu or Ara-C may be a safer drug than high-dose CY for conditioning in FA patients.

Int J Hematol. 2007;85:354-361. doi: 10.1532/IJH97.06191

Key words: Fanconi anemia; Chromosome fragility test; Fludarabine; Preconditioning; Stem cell transplantation

1. Introduction

Fanconi anemia (FA) is a rare hereditary disease associated with congenital abnormalities, progressive bone marrow failure, and a predisposition to malignancy [1]. The diagnosis of FA is usually confirmed by the hypersensitivity to DNA cross-linking agents such as diepoxybutane (DEB), allowing clinicians to make a diagnosis of FA in the absence of clinically detectable congenital anomalies [2]. To date, complementation studies have demonstrated 12 separate FA complementation groups (A through G, including D1, D2, I, J, l, and M). Hematopoietic stem cell transplantation (SCT) is the most effective therapy for the correction of stem cell defects; however, the early results for SCT have been poor because of the prolonged toxicity of cyclophosphamide (CY) and the development of severe acute graft-versus-host disease (GVHD) [3]. These results have led to the use of low-dose CY with thoracoabdominal irradiation (TAI) [4]. Although a probability of 5-year survival of

Correspondence and reprint requests: Miharu Yabe, MD, Department of Laboratory Medicine, Tokai University School of Medicine, Shimokasuya 143, Isehara, Kanagawa 259-1193, Japan; 81-463-93-1121; fax: 81-463-93-8607 (e-mail: miharu@is.icc.u-tokai.ac.jp).

Table 1.
Characteristics of the Patients*

	FA (n = 56), n	Non-FA (n = 50), n
Sex		
Male	32	26
Female	24	24
Family history of FA	27	0
Malformation syndrome		
No anomalies	4	39
Short stature and/or skin	10	11
Limited (<3 sites)	31	0
Extensive (≥3 sites)	11	0
Hematologic abnormalities		
Bone marrow failure	36	37
Clonal abnormalities	3	4
MDS/AML	17	9
Complementation groups		
Group A	18	0
Group G	9	0
Group C	8	0

*FA indicates Fanconi anemia; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia.

greater than 70% can be achieved with this approach if an HLA-matched sibling donor is available, it is notable that graft failure and acute GVHD are still the main causes of death [5,6]. The uniform use of low-dose CY (20 mg/kg) for SCT may not be necessary in all FA patients, because the fragility of chromosomes in FA patients is heterogeneous, owing to individual variation [2]. MacMillan et al reported that the presence of DEB-insensitive cells is associated with an increased risk of graft failure [7]. They also reported that the use of higher-dose total body irradiation did not improve engraftment. We have reported that individualized preparative regimens based on each patient's sensitivity to CY metabolites improve the outcomes of SCT for FA patients with an HLA-matched sibling donor [8]. For FA patients lacking an HLA-matched sibling donor, the use of HLA-matched unrelated donors has been proposed, but the 3-year probability of survival was only 33% in a study conducted by a European group for conditioning with low-dose CY, TAI, and antithymocyte globulin [9]. A limited number of FA patients who received a fludarabine (Flu)-based regimen reportedly achieved durable engraftment in alternative-donor transplantation without severe toxicity or acute GVHD [10]. In the present study, which included 56 FA and 50 non-FA patients, we used the chromosome fragility test to evaluate sensitivity to CY metabolites, cytosine arabinoside (Ara-C), and Flu metabolite (9-β-D-arabinofuranosyl-2-fluoroadenine [2-F-Ara-A]) in order to achieve a less toxic conditioning regime and a lower risk of rejection in SCT for FA patients.

2. Materials and Methods

2.1. Patients

From 1986 to 2005, we analyzed data from 56 patients with FA and 50 patients who were about to undergo allogeneic bone marrow transplantation (BMT) for aplastic anemia, myelodysplastic syndrome, or acute myeloid leukemia. The diagnosis of FA was confirmed in all cases by testing for chromosomal breakage induced by DEB, mitomycin C (MMC), and CY metabolites in peripheral blood lymphocytes; in some patients, a complementary group was determined, as has been described previously [11-13]. Characteristics of the patients are summarized in Table 1. The extent of malformation syndrome was assessed according to the number of involved anatomic sites (head, limbs, kidneys, gastrointestinal tract, urogenital tract, and cardiovascular system). Extensive malformation syndrome was diagnosed if at least 3 sites were involved, including the involvement of at least 1 deep organ (kidney, gastrointestinal or urogenital tract, or cardiovascular system); other site combinations were diagnosed as limited malformation syndrome [9].

2.2. Definition of Hematologic Abnormalities

The onset of bone marrow failure was defined as the time one of the following laboratory parameter values used in the International Fanconi Anemia Registry (IFAR) study was observed: a platelet count $<100 \times 10^9$/L, a hemoglobin level <10 g/dL, or an absolute neutrophil count $<1 \times 10^9$/L [2].

A diagnosis of myelodysplastic syndrome or acute myeloid leukemia was made according to the French-American-British classification.

2.3. Cytogenetic Studies

Chromosome preparations and studies of 72-hour cultures of phytohemagglutinin-stimulated peripheral blood lymphocytes were performed according to standard procedures.

One culture remained untreated. MMC (Kyowa, Tokyo, Japan) and DEB (Sigma-Aldrich, Milwaukee, WI, USA) were added to a final concentration of 0.01 μg/mL and 0.1 μg/mL, respectively, at 24 and 48 hours before harvest. The method used for the DEB study was the same as that used in the IFAR study [2]. CY is metabolized in the liver, so the sera containing activated forms of CY were obtained from other non-FA BMT patients with severe aplastic anemia. Blood was taken 1 hour after the start of the first CY dose (50 mg/kg) from the patients who had provided informed consent. When hydrolyzed in the acid state, CY was allowed to produce normustard-like agents, which are CY metabolites with alkylating activity, and was converted by 4-(p-nitrobenzyl)pyridine. The amount of color development was determined spectrophotometrically at 540 nm. The concentrations of CY metabolites ranged from 4.0 to 28.2 μg/mL, and these sera were stored in a freezer at –20°C. A single serum sample with a final CY metabolite concentration of 0.4 μg/mL or 0.8 μg/mL was added to the culture for 48 hours of treatment. The peak blood concentration of Ara-C ranged from 1×10^{-4} M to 1×10^{-5} M after the patients had received treatment with high-dose Ara-C (2-3 g/m^2) [14]. Ara-C was added to the cultures to a final concentration of 1×10^{-6} M for 6, 24, or 48 hours. Flu is an antitumor purine analogue, of which 2-F-Ara-A is a major metabolite [15]. The peak blood concentration of 2-F-Ara-A ranged

Table 2.
Chromosome Fragility Tests Performed with Chemical Agents in Fanconi Anemia (FA) and Non-FA Patients*

Agent	Concentration	Exposure Time, h	FA Patients, n	Non-FA Patients, n
Spontaneous			56	50
Mitomycin C	0.01 μg/mL	24	30	24
Diepoxybutane	0.1 μg/mL	48	44	45
CY metabolites	0.4 μg/mL	48	56	48
	0.8 μg/mL	48	47	13
Ara-C	1×10^{-6} M	6	14	3
	1×10^{-6} M	24	30	7
	1×10^{-6} M	48	25	7
2-F-Ara-A	2×10^{-6} M	48	33	12
	5×10^{-6} M	48	26	12

*CY indicates cyclophosphamide; Ara-C, cytosine arabinoside; 2-F-Ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine.

from 2×10^{-6} M to 5×10^{-6} M [16]. 2-F-Ara-A was added to the cultures to a final concentration of 2×10^{-6} M or 5×10^{-6} M, and the cultures were incubated for 48 hours. In vitro testing of these agents' toxicity against peripheral blood lymphocytes obtained from healthy volunteers and FA patients were carried out on 48-hour cultures with a methylthiazolyldiphenyl-tetrazolium (MTT) assay. The 30% lethal doses (LD_{30}) in vitro for these 3 drugs (CY metabolites, Ara-C, and 2-F-Ara-A) were calculated from optical densities. When the concentrations of these drugs were higher than the LD_{30}, we were able to obtain a few metaphases for chromosomal analysis. The LD_{30} values for Ara-C and 2-F-Ara-A in healthy volunteers and FA patients were approximately 1×10^{-6} M and 2×10^{-6} M, respectively. LD_{30} values for CY metabolites in FA patients were approximately 0.8 μg/mL, but the values in healthy volunteers were greater than 0.8 μg/mL.

Chromosomal analysis was performed on 50 to 100 Giemsa-stained metaphase spreads from each sample, and 100 Giemsa-stained metaphase spreads from the untreated preparation were also analyzed. Each cell was scored for the chromosome number and the types of structural abnormalities. Achromatic areas less than a chromatid in width were scored as gaps, and exchange configurations, translocations, and dicentric and ring chromosomes were scored as rearrangements. Gaps were excluded from the calculation of chromosome breakage frequencies, and rearrangements were scored as 2 breaks [2]. Structural chromosome aberrations were specified as follows: percentage of aberrant metaphases, number of breaks per cell, number of aberrations per aberrant cell, and types of aberrations. Details of the chromosome fragility test are shown in Table 2.

2.4. Statistical Analysis

Computations were carried out with the GraphPad Prism software package (GraftPad Software, San Diego, CA, USA). The Welch *t* test or Mann-Whitney analyses were used to compare the various groups.

3. Results

3.1. Clinical Manifestations and Complementation Groups of FA Patients

Table 1 shows the numbers of FA and non-FA patients with congenital malformation and a family history of FA. Twenty-five percent of the FA patients had no anomalies aside from a short stature and/or skin pigmentation, and 2 patients showed neither a family history of FA nor malformation. Among the non-FA patients, 22% were identified because they exhibited a short stature and/or skin pigmentation associated with FA.

Of the 56 FA patients, FA gene mutation was detected in samples from 35 patients. Eighteen patients belonged to the FA-A complementation group, 9 belong to the FA-G group, and 8 belong to the FA-C group (Table 1).

3.2. Cytogenetic Testing

Table 3 summarizes the results of cytogenetic testing. We examined peripheral blood lymphocytes from patients with and without FA for their sensitivity to MMC, DEB, CY metabolites, Ara-C, and 2-F-Ara-A. The independent variable was the group (FA and non-FA), and the predictors were the number of breaks per cell, the percentage of cells with breaks, and the number of breaks per aberrant cell. The cultures of 7 of the 30 FA patients analyzed by the MMC test had fewer than 0.1 breaks/cell, and these FA patients could not be discriminated from non-FA patients. On the other hand, the breakage frequency in the DEB test for the FA group was greater than 0.2 breaks/cell, whereas the frequency for the non-FA group fell to less than 0.2 breaks/cell. There was thus no overlap between the FA and non-FA groups in the results of the DEB test. Similarly, the CY metabolite test also clearly separated the 2 groups at a frequency of 0.1 breaks/cell (Figure 1). The numbers of breaks per cell in cultures treated with CY metabolites or Ara-C was heterogeneous for FA patients (range, 0.12-4.75 and 0.1-3.73 breaks/cell, respectively), but the frequencies of breakage in

Table 3.
Sensitivity of Fanconi Anemia (FA) and Non-FA Lymphocytes from Individual Patients to Chemical Agents*

Agent	Diagnosis	No. of Patients	Breaks/Cell, n	Aberrant Cells, %	Aberrations/Aberrant Cell, n
Spontaneous	FA	56	0.06 ± 0.09 (0-0.49)	7.92 ± 8.76 (0-52.0)	1.01 ± 0.19 (1-1.56)
	Non-FA	50	0.00 ± 0.01 (0-0.02)	1.00 ± 1.30 (0-4.0)	1.00 ± 0.00 (1-1.0)
MMC (0.01 μg/mL)	FA	30	0.31 ± 0.38 (0.08-1.56)	20.1 ± 16.0 (3-69.0)	1.37 ± 0.51 (1-2.10)
	Non-FA	24	0.05 ± 0.07 (0-0.31)	6.3 ± 6.0 (0-22.5)	1.04 ± 0.17 (1-1.72)
DEB (0.1 μg/mL)	FA	44	2.28 ± 1.77 (0.21-8.54)	66.5 ± 22.7 (11-100.0)	3.00 ± 1.59 (1-8.54)
	Non-FA	45	0.02 ± 0.03 (0-0.12)	3.1 ± 2.9 (0-12.0)	1.02 ± 0.08 (1-1.33)
CY (0.4 μg/mL)	FA	56	1.17 ± 0.93 (0.12-4.75)	52.7 ± 23.1 (9-93.8)	1.96 ± 0.86 (1-5.06)
	Non-FA	48	0.02 ± 0.02 (0-0.09)	2.9 ± 2.6 (0-10.0)	1.00 ± 0.00 (1-1.0)
Ara-C (1×10^{-6} M)	FA	30	0.90 ± 0.78 (0.1-3.73)	55.7 ± 22.3 (18-92.3)	1.64 ± 1.02 (1-6.20)
	Non-FA	7	0.39 ± 0.34 (0.05-0.86)	28.1 ± 20.2 (12-64.0)	1.29 ± 0.15 (1-1.68)
Flu (2×10^{-6} M)	FA	33	0.14 ± 0.12 (0.01-0.59)	24.3 ± 16.7 (6.0-52.0)	1.01 ± 0.00 (1-1.23)
	Non-FA	12	0.08 ± 0.12 (0-0.44)	9.9 ± 10.7 (2.0-37.0)	1.02 ± 0.06 (1-1.19)

*Chromosomal analysis was performed after a 48-hour treatment with diepoxybutane (DEB), cyclophosphamide metabolites (CY), or fludarabine metabolite (Flu) (9-β-D-arabinofuranosyl-2-fluoroadenine [2-F-Ara-A]), and after a 24-hour treatment with mitomycin C (MMC) or cytosine arabinoside (Ara-C). Values are presented as the mean ± SD (range).

cultures treated with 2-F-Ara-A were very low for FA patients (range, 0.01-0.59 breaks/cell). The difference between the FA and non-FA patients in breakage frequency for cultures treated with 2-F-Ara-A was not statistically significant. The dose-response graph for chromosomal aberrations induced by CY metabolites shows significant elevations in break frequency with increasing concentration in the cultures from 47 FA patients but not in the cultures from 13 non-FA patients (Figure 2A). 2-F-Ara-A induced a significant dose-dependent elevation in break frequency in cell cultures from both 26 FA patients and 11 non-FA patients (Figure 2B).

Lymphocytes from 3 non-FA patients that were treated with Ara-C for 6 hours before harvesting exhibited an increased breakage rate, but not when the lymphocytes were treated for 24 or 48 hours, as previously described. This result may have been due to repair of the induced damage. The lymphocytes of 9 FA patients behaved differently (Figure 3). Cases of nonrecovery of FA lymphocytes after Ara-C treatment were observed, and the lymphocytes from some FA patients did exhibit increased breakage when they were treated with Ara-C for 24 and 48 hours.

3.3. Chromosome Breakage Types for Patients with Structural Abnormalities

The types of aberration induced by Ara-C or 2-F-Ara-A in the lymphocytes from 24 FA patients were different from those induced by CY metabolites. Most of the breakages induced by Ara-C or 2-F-Ara-A were breaks or gaps, and a few chromatid translocations, such as exchanges, were also found. In contrast, more than 50% of the breakages induced by CY metabolites were chromatid translocations (Figure 4).

3.4. Correlation between the Results of Cytogenetic Testing with DEB and CY Metabolites

Lymphocytes from 44 FA patients underwent both CY metabolite and DEB tests in our laboratory. There was a linear correlation between the percentages of aberrant metaphases in lymphocytes treated with CY metabolites and the percentages in lymphocytes treated with DEB ($r = 0.84$) (Figure 5).

4. Discussion

The diagnosis and treatment of FA is complicated by the great variability in disease severity. Monitoring disease progression to determine the appropriate therapy, such as

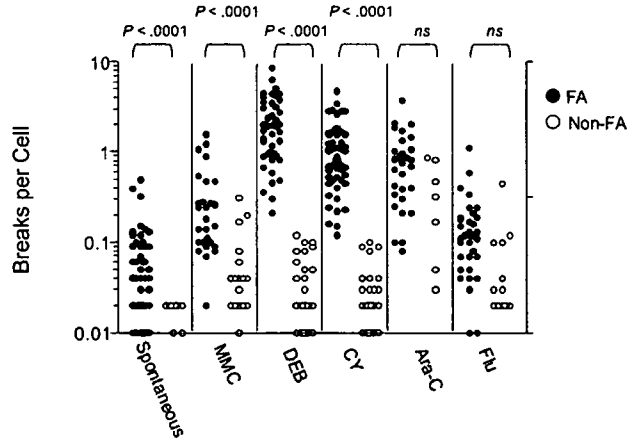

Figure 1. Patterns of sensitivity to chemical agents in Fanconi anemia (FA) and non-FA patients. Spontaneous indicates spontaneous breakage; MMC, mitomycin C–induced breakage (0.01 μg/mL); DEB, diepoxybutane-induced breakage (0.1 μg/mL); CY, cyclophosphamide metabolite–induced breakage (0.4 μg/mL); Ara-C, cytosine arabinoside–induced breakage (1×10^{-6} M); Flu, 9-β-D-arabinofuranosyl-2-fluoroadenine (2-F-Ara-A)-induced breakage (2×10^{-6} M); ns, not statistically significant.