p53 downregulates the Fanconi anaemia DNA repair pathway

Sara Jaber¹,²,³,⁴,*, Eléonore Toufektchan¹,²,³,⁴,*, Vincent Lejour¹,²,³,⁴, Boris Bardot¹,²,³,⁴ & Franck Toledo¹,²,³,⁴

Germline mutations affecting telomere maintenance or DNA repair may, respectively, cause dyskeratosis congenita or Fanconi anaemia, two clinically related bone marrow failure syndromes. Mice expressing p53Δ31, a mutant p53 lacking the C terminus, model dyskeratosis congenita. Accordingly, the increased p53 activity in p53Δ31/Δ31 fibroblasts correlated with a decreased expression of 4 genes implicated in telomere syndromes. Here we show that these cells exhibit decreased mRNA levels for additional genes contributing to telomere metabolism, but also, surprisingly, for 12 genes mutated in Fanconi anaemia. Furthermore, p53Δ31/Δ31 fibroblasts exhibit a reduced capacity to repair DNA interstrand crosslinks, a typical feature of Fanconi anaemia cells. Importantly, the p53-dependent downregulation of Fanc genes is largely conserved in human cells. Defective DNA repair is known to activate p53, but our results indicate that, conversely, an increased p53 activity may attenuate the Fanconi anaemia DNA repair pathway, defining a positive regulatory feedback loop.

¹ Genetics of Tumour Suppression, Equipe Labellisée Ligue, Institut Curie, Centre de recherche, 26 rue d’Ulm, 75248 Paris Cedex 05, France. ² Sorbonne Universités, UPMC Univ Paris 06, Paris, France. ³ CNRS UMR 3244, Paris, France. ⁴ PSL Research University, Paris, France. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to F.T. (email: franck.toledo@curie.fr).
Inherited bone marrow failure syndromes are a set of clinically related yet heterogeneous disorders in which at least one haematopoietic cell lineage is significantly reduced. Among them, Fanconi anaemia (FA) and dyskeratosis congenita (DC) are caused by germline mutations in key cellular processes, that is, DNA repair and telomere maintenance, respectively.

We recently found that p53 lacking its C-terminal domain, die rapidly after birth with a complete set of features of the telomere syndrome DC, including aplastic anaemia, pulmonary fibrosis, oral leukoplakia, skin hyperpigmentation, nail dystrophy and short telomeres. Loss of the p53 C terminus increases p53 activity in mouse embryonic fibroblasts (MEFs) and in most tested tissues and Dkc1, Rtel1, Ten1 and Terf1). Nutlin, a drug that prevents the Mdm2 ubiquitin ligase from interacting with p53, allowed to confirm that p53 activation leads to the downregulation of these four genes. These data revealed that p53 plays a major role in telomere metabolism.

We previously focused on the potential p53-mediated regulation of genes mutated in DC (Dkc1, Rtel1 and Ten1) or implicated in aplastic anaemia, a milder form of telomere syndrome (Terf1). As a striking evidence for the clinical relevance of our mouse model, patients with severe DC who carry mutations affecting PARN, a negative regulator of p53, were compared, in unstressed WT cells should fall between the means measured in p53+/− and p53−/− cells, and that the means for the three genotypes should be statistically different according to an analysis of variance. Out of the 42 genes, 7 fulfilled these criteria: Blm, Dek, Fancd2, Fen1, Gar1, Recql4 and Timeless (Fig. 1a; Supplementary Fig. 1). Because RECO4 was shown to be downregulated by p53 in human cells, the lower Recql4 mRNA in p53−/− cells were not surprising. The decreased mRNA levels for the six other genes were not anticipated however. To specifically assay for a p53-dependent regulation, we next compared the effects of Nutlin, a drug that activates p53 by preventing its interaction with the ubiquitin ligase Mdm2. Results clearly indicated that p53 activation leads to the downregulation of these genes (Fig. 1b).

Importantly, the finding that p53 downregulates Gar1, which encodes a component of the telomerase complex, strengthened our previous conclusion that p53 plays a significant role in telomere biology. However, Fancd2 appeared as the gene whose expression was most markedly affected by p53 activation (Fig. 1b). This was surprising because, even if primary cells from patients with a FANCd2 mutation may exhibit telomere dysfunction, these patients are diagnosed with FA, a syndrome primarily characterized by defects in DNA repair. This led us to further analyse the p53-dependent regulation of Fancd2. We first verified that the relative decrease in Fancd2 mRNA levels were observed in vivo, in bone marrow cells (BMcs) from p53−/− mice (Fig. 1c). We next tested whether the p53-dependent regulation of Fancd2 detected by quantitative PCR had an impact on Fancd2 protein levels. Lower Fancd2 protein levels were observed in unstressed p53−/− cells compared with unstressed p53+/− or WT cells, and Nutlin treatment led to a decrease in Fancd2 proteins only in WT and p53−/− MEFs, in complete agreement with quantitative PCR data (Fig. 1d; Supplementary Fig. 2).

Expression of telomere-related genes in p53−/− cells. Our initial aim was to test whether, besides the four genes previously identified, p53 could regulate other genes that might contribute to the telomere phenotype of p53−/− mice. We therefore compared, in unstressed p53−/−, wild-type (WT) and p53−/− fibroblasts, mRNA levels for 42 candidate genes reported to be relevant to telomere metabolism. Candidates included genes implicated in telomere syndromes (Acl1/Tpp1, Apollo/Smn1b, C16orf57/Mpn1/Usb1, Naf1, Obfc1/Str1, Parn and Sbds)3,8–10; genes mutated in diseases not primarily associated with telomere biology but for which telomere dysfunction or DC-like features were reported (Dmnt3b, Fancd2 and Recql4); genes encoding proteins of complexes involved in telomere biology, that is, the telomerase (Gar1/Nola1, Ruvbl1 and Ruvbl2), shelterin (Pot1a and Pot1b, Rap1/Terf2ip, and Terf2), CST (Ten1) and CIA (Ciao1, Ipop/Narf1, Mip18 and Mms19) complexes, as well as Cajal bodies (Cajin and Hot1)11, or proteins otherwise proposed to participate in telomere replication or maintenance (Artemis/Snm1c, Blm, Cbs/Erc6, Dek, Dna2, Ercc3/Xpb, Ercc4/Fancp/Xpf, Fance, Fen1, Lmna/Progerin, Nbs1, Pim1, Slek/Fancp, Timless, Tnks1, Tnks1bp1, Ufp1 and Wnrn)6,12–26.

For a gene to be a good candidate, we considered that the mean (from three to four independent experiments) of its mRNA levels in unstressed WT cells should fall between the means measured in p53−/− and p53−/− cells, and that the means for the three genotypes should be statistically different according to an analysis of variance. Out of the 42 genes, 7 fulfilled these criteria: Blm, Dek, Fancd2, Fen1, Gar1, Recql4 and Timeless (Fig. 1a; Supplementary Fig. 1). Because RECO4 was shown to be downregulated by p53 in human cells, the lower Recql4 mRNA in p53−/− cells were not surprising. The decreased mRNA levels for the six other genes were not anticipated however. To specifically assay for a p53-dependent regulation, we next compared the effects of Nutlin, a drug that activates p53 by preventing its interaction with the ubiquitin ligase Mdm2. Results clearly indicated that p53 activation leads to the downregulation of these genes (Fig. 1b).

p53 activation leads to increased E2F4 binding at Fancd2. The p53-mediated downregulation of many genes requires the cdk inhibitor p21, and occurs through the recruitment, upon p53 activation, of E2F4 repressive complexes at their promoters. Consistently, this mechanism would account for the telomerase complex, strengthened our previous conclusion that p53 plays a significant role in telomere biology. However, Fancd2 appeared as the gene whose expression was most markedly affected by p53 activation (Fig. 1b). This was surprising because, even if primary cells from patients with a FANCd2 mutation may exhibit telomere dysfunction, these patients are diagnosed with FA, a syndrome primarily characterized by defects in DNA repair. This led us to further analyse the p53-dependent regulation of Fancd2. We first verified that the relative decrease in Fancd2 mRNA levels were observed in vivo, in bone marrow cells (BMcs) from p53−/− mice (Fig. 1c). We next tested whether the p53-dependent regulation of Fancd2 detected by quantitative PCR had an impact on Fancd2 protein levels. Lower Fancd2 protein levels were observed in unstressed p53−/− cells compared with unstressed p53+/− or WT cells, and Nutlin treatment led to a decrease in Fancd2 proteins only in WT and p53−/− MEFs, in complete agreement with quantitative PCR data (Fig. 1d; Supplementary Fig. 2).
expression of Fancd2 is known to vary during the cell cycle, the differences in Fancd2 mRNA levels observed between WT and p53^−/−^ MEFs would not simply result from differences in G1/S ratios. Rather, our results indicate that p53 activation promotes the recruitment of E2F4 at the Fancd2 gene, and that E2F4 plays a major role in the repression of Fancd2.

In the experiments above, p53 activation resulted from a treatment with Nutlin, a molecule that acts as a specific Mdm2 inhibitor. We next tested whether similar results could be obtained in response to DNA damage, by evaluating the effects of doxorubicin, a clastogenic anticancer agent. Doxorubicin treatment led to decreased Fancd2 mRNA and protein levels in WT and p53^−/−^ MEFs, but not p53^−/−^ MEFs (Supplementary Fig. 5a,b). Furthermore, we observed increased E2F4 binding at the Fancd2 promoter in doxorubicin-treated WT cells, compared with untreated Wt or doxorubicin-treated p53^−/−^ cells (Supplementary Fig. 5c). Thus, both Nutlin and doxorubicin lead to p53 activation and consecutive Fancd2 downregulation.

Interestingly, the Blm and Fen1 genes, also downregulated by p53 (Fig. 1b), respectively, encode an helicase that associates with Fanc proteins in a multi-enzyme complex, and an endonuclease stimulated by a Fanc protein. Furthermore, Rtel1, one of the four telomere-related genes we previously found regulated by p53, encodes a Fanc-like helicase. This led us to further evaluate the impact of p53 activation on the FA DNA repair pathway.

**p53 downregulates many Fanc genes.** Because the expression levels of four FA genes had been tested in our previous experiments—Fancd2, Fancd2, Fancp/Sld4 and Fenq1/Ercc4 (Fig. 1; Supplementary Fig. 1), we next compared, in unstressed p53^−/−^, WT and p53^−/−^ MEFs, mRNA levels for the 15 remaining FA genes. Strikingly, 11 were less expressed in p53^−/−^ cells (Fig. 3a). Again, Nutlin was used to confirm the p53-mediated downregulation of these genes (Fig. 3b). As for
Fancd2, this p53-mediated downregulation required p21 (Supplementary Fig. 6), and p53 activation correlated with an increased binding of E2F4 near the transcription start site of each of these Fanc genes (Fig. 3c). We next used the sequence of six functional CDE/CHRs to define a positional enrichment matrix, which was then used to search in silico for candidate CDE/CHRs near the E2F4-binding sites identified in ChIP assays. Using this approach, candidate CDE/CHR motifs were identified for 9 out of the 11 tested Fanc genes, with the best candidate motifs for Fanci and Fancr (Fig. 4a; Supplementary Fig. 7). These data led us to further analyse the p53-mediated regulation of Fanci and Fancr. We first verified that the relative decreases in Fanci and Fancr mRNA levels were observed in vivo, in BMCs from p53<sup>+/−/−</sup> mice (Supplementary Fig. 8). We then found that p53 activation leads to decreased Fanci and Fancr protein levels ex vivo (Fig. 4b; Supplementary Fig. 9). Luciferase assays next showed that mutating the CDE site in each candidate CDE/CHR abolished the Nutlin-dependent repression of the Fanci and Fancr promoters (Fig. 4a,c).

We also observed that a 24-h long treatment with doxorubicin led to decreased Fanci and Fancr mRNA, and protein levels in WT and p53<sup>+/−/−</sup> MEFs, but not p53<sup>−/−</sup> MEFs (Supplementary Fig. 10a). Furthermore, the nine other Fanc genes downregulated by p53 on Nutlin treatment were also downregulated in a p53-dependent manner on treatment with doxorubicin (Supplementary Fig. 10b). We then searched for confirmation of our results by analysing the data recently reported by Younger et al., who performed a genomic analysis that integrated transcriptome-wide expression levels, genome-wide p53-binding profiles and chromatin state maps to characterize the regulatory role of p53 in response to DNA damage<sup>37</sup>. Although this approach was designed to identify direct p53 targets, we reasoned that genes regulated by p53 indirectly, via p21/E2F4, might also be detected in their transcriptome-wide expression data. These experiments were performed on p53<sup>−/−</sup> and WT MEFs, treated or not with doxorubicin for 6 h (ref. 37), and our previous time-course experiments with Nutlin suggested that 6 h might be sufficient to observe a partial p53-mediated transcriptional downregulation<sup>2</sup>. Thus, we extracted the data of Younger et al. (Gene Expression Omnibus # GSE55727) to analyse the expression of the 12 Fanc genes that we had found downregulated by p53. In agreement with our results, this analysis showed that doxorubicin led to an overall decrease in the expression of Fanc genes in WT, but not p53<sup>−/−</sup> MEFs (Supplementary Fig. 11).

Transcriptome data mining was also used to find whether the downregulation of Fanc genes could correlate with p53 activation in haematopoietic cells. The Homeobox (Hox) transcription factors are important regulators of normal and malignant haematopoiesis, because they control proliferation, differentiation and self-renewal of haematopoietic cells. We analysed the data of Muntean et al. (Gene Expression Omnibus # GSE21299), who immortalized murine BMCs by transduction with Hoxa9-ER cells in the presence of tamoxifen (4-OHT), and observed that they undergo myeloid differentiation 5 days after 4-OHT withdrawal<sup>38</sup>. We found this differentiation to correlate with an induction of genes known to be transactivated by p53 (Cdkn1A/p21, Mdm2 and Fas), and with the downregulation of Fanc genes (Supplementary Fig. 12).

In sum, we found that 12 genes of the FA DNA repair pathway are downregulated by p53 via a p21/E2F4 pathway, and identified CDE/CHR motifs that are crucial for this regulation for three of these genes. Importantly, the genes are downregulated by p53 in response to Mdm2 inhibition or DNA damage, or on haematopoietic cell differentiation, and encode proteins involved in all parts of the FA DNA repair pathway, that is, proteins that

---

**Figure 2 | p53 activation promotes the binding of transcriptional repressor E2F4 at the Fancd2 gene.** (a) p21 is required for the downregulation of Fancd2 mRNAs from untreated or Nutlin-treated p21<sup>−/−</sup> MEFs were quantified. Results from three independent experiments. (b) Increased E2F4 binding at the Fancd2 promoter upon p53 activation. A map surrounding the Fancd2 transcription start site (TSS) is shown on the left (white box: UTR; Ex: exon 1); lollipops: putative E2F4-binding sites according to ref. 70 (Supplementary Fig. 3); arrows: ChIP PCR primers, and ChIP data on the right. ChIP assay for E2F4 binding was performed in Nutlin-treated p53<sup>−/−</sup> MEFs, and untreated or Nutlin-treated WT MEFs, with an antibody against E2F4 or rabbit IgG as a negative control. Immunoprecipitates were quantified using real-time PCR, fold enrichment was normalized to data over an irrelevant region, then E2F4 binding at Fancd2 in untreated WT cells was given a value of 1. Data from two independent ChIP experiments, each quantified in triplicates. (c) The p53-dependent regulation of Fancd2 occurs via a CDE/CHR motif. CDE/CHR motifs are required for gene repression by an E2F4-containing DREAM complex<sup>22</sup>. These motifs consist of a 6-bp long GC-rich CDE site (bound by E2F4) located 4-bp upstream of a 6-bp long AT-rich CHR site. On top, CDE/CHR motifs regulating the expression of five mouse genes are presented, as well as a putative CDE/CHR motif 23-38-bp downstream of the mouse Fancd2 TSS, and its mutated counterpart (with mutations in the CDE). Below, a 2-kb fragment centred around the Fancd2 TSS, containing a WT or mutant CDE/CHR, was cloned upstream a Luciferase gene and transfected into NIH-3T3 cells, treated or not with Nutlin, then Luciferase activity was measured after 24 h. Although the cell cycle kinetics of cells transfected with either plasmid were identical (Supplementary Fig. 4), Nutlin led to decreased luciferase activity only with the construct containing a WT CDE/CHR motif. Mutation of the putative CDE site increased Luciferase basal expression, and abrogated the effect of Nutlin. Results from three independent experiments. In all figures, means ± s.e.m. are shown; ***P ≤ 0.001, *P ≤ 0.05, NS, not significant by Student’s t-test.
Figure 3 | Several genes of the FA DNA repair pathway are downregulated upon p53 activation. (a) A comparison of p53−/−, wild-type and p53Δ31/Δ31 cells suggests a potential p53-dependent regulation for 11 additional genes of the Fanconi anaemia (FA) DNA repair pathway. mRNAs for the indicated Fanc genes were quantified as described in Fig. 1a, in four independent experiments. For 11 of the tested genes, mean mRNA levels were intermediate in WT cells compared with p53−/− and p53Δ31/Δ31 cells, with statistical significance by one-way analysis of variance (ANOVA). (b) The 11 genes are downregulated on murine p53 activation. mRNAs for the indicated Fanc genes were quantified in untreated or Nutlin-treated MEFs. Results from three independent experiments. (c) Increased E2F4 binding at several Fanc promoters upon p53 activation. ChIP assay for E2F4 binding was performed in untreated or Nutlin-treated WT MEFs, with an antibody against E2F4 or rabbit IgG as a negative control. Immunoprecipitates were quantified using real-time PCR, fold enrichment was normalized to data over an irrelevant region, and then E2F4 binding in untreated WT cells was given a value of 1. Data are from two to three independent ChIP experiments, each quantified in triplicates. Below the ChIP data are represented, as in Fig. 2b, sequences around the TSS for each gene, putative E2F4-binding sites (lollipops), and primers used for ChIP assays (arrows). In all figures, means ± s.e.m. are shown; ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05, NS, not significant by ANOVA or Student’s t-tests.
belong to the FA core complex (Fanca, Fancb and Fancm) and its accessory protein (FancI/UBE2T), the pivotal ID2 complex (Fancc2 and Fanci), or downstream effector proteins (Fanca1/Brca1, Fancc/Bach1/Rip1, Fancj/Palb2, Fancn/Brca2, Fancr/Rad51c, Fancs/Brca1)39-42. Together, these data suggested an important role for p53 in regulating the FA pathway.

p53 activation attenuates the repair of specific DNA lesions. A typical feature of FA cells is their inability to repair DNA interstrand crosslinks, as evidenced by an increased frequency of chromosomal aberrations, and more specifically tri- and quadri-radial chromosomes, after exposure to mitomycin C (MC)39. We compared the effects, on WT and p5331/31 cells, of a 48-h treatment with 50 nM MC. Such a treatment procedure was previously reported to differentially affect WT MEFs and MEFs with an impaired FA pathway43. Interestingly, we found that this procedure led to a rather subtle induction of p53 (suggested by a limited increase in p21 transactivation), which correlated with a twofold decrease in Fancd2 mRNA expression in p5331/31 MEFs, but no significant alteration of Fancd2 mRNA levels in WT cells (Supplementary Fig. 13). We next determined the frequencies of all types of chromosomal aberrations, or of radial chromosomes, in WT and p5331/31 cells before or after treatment with MC. In untreated cells, no significant difference was found between the two genotypes. Strikingly, however, chromosomal aberrations, and particularly radial chromosomes, were more frequent in p5331/31 cells after treatment with MC, consistent with a decreased capacity to repair interstrand crosslinks in the mutant cells (Fig. 5a). Accordingly, chromosomes with sister chromatid exchanges were also more frequent in MC-treated p5331/31 cells than in WT cells (Fig. 5b). These results suggested that the FA DNA repair pathway is attenuated in p5331/31 cells, presumably because these cells exhibit an increased p53 activity. Consistent with this, p5331/31 cells exhibited a decreased capacity to form Rad51 foci and an increased sensitivity to MC, and the pretreatment of cells with Nutlin appeared to further impact on these cellular phenotypes (Fig. 5c,d). Further evidence that the decreased DNA repair in p5331/31 cells resulted from increased p53 activity (rather than a loss of the p53 CTD per se) came from analysing Mdm2+/−/ Mdm4+/−/E6 MEFs. These MEFs express a WT p53 protein, but exhibit an increased p53 activity due to lower levels of p53 inhibitors44,45. Like p5331/31 MEFs, Mdm2−/−/ Mdm4−/−/E6 cells were more sensitive than WT cells to MC (Supplementary Fig. 14). In sum, a defective FA DNA repair pathway is known to activate p53 (ref. 46), but these results indicate that an increased p53 activity might reduce the expression of several FA genes and attenuate the FA DNA repair pathway. Taken together, these data indicate the existence of a positive regulatory feedback loop (Fig. 6).

Figure 4 | CDE/CHR motifs are important for the p53-dependent repression of Fanci and Fancr. (a) Identification of candidate CDE/CHR motifs in Fanci and Fancr with a positional frequency matrix. The CDE/CHR motifs in six mouse genes were used to define the positional frequency matrix shown on top, which was then used to identify candidate CDE/CHR motifs in Fanci and Fancr (for details, see Supplementary Fig. 7). The candidate CDE/CHRs map 38 (Fanci)- and 15 (Fancr)-bp downstream of the transcription start site (TSS) of each gene. Also shown here are the mutated CDE/CHRs that were tested in luciferase assays in c. (b) p53 activation leads to decreased Fanci and Fancr protein levels. Protein extracts, prepared from untreated or Nutlin-treated MEFs, were immunoblotted with antibodies against Fanci, Fancr and actin, then bands were quantified and the amounts of Fanci or Fancr proteins in unstressed WT cells were assigned a value of 1. (c) The p53-dependent regulation of Fanci and Fancr occurs via a CDE/CHR motif. For each gene, a 1-kb fragment centred around the TSS, containing a WT or mutant CDE/CHR, was cloned upstream of a luciferase gene and transfected into NIH-3T3 cells, treated or not with Nutlin, then luciferase activity was measured after 24 h. Mutation of the putative CDE site increased luciferase basal expression and abrogated the effect of Nutlin. Results from two independent experiments; means ± s.e.m. are shown; **P ≤ 0.001, ***P ≤ 0.01, NS, not significant by Student’s t-test.

Human p53 also regulates FA genes. We next tested whether the FA genes that were found regulated by murine p53 were similarly regulated in human cells. We compared human primary WT cells

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms11091 | www.nature.com/naturecommunications
Figure 5 | A decreased capacity to repair mitomycin C-induced DNA lesions in cells with increased p53 activity. (a) p53<sup>Δ31/Δ31</sup> MEFs exhibit increased frequencies of mitomycin C-induced chromosomal aberrations. Frequencies of total chromosomal aberrations, or tri- and quadri-radial chromosomes, were determined in wild-type (WT) and p53<sup>Δ31/Δ31</sup> (Δ31) MEFs at passage 3, untreated or after treatment with mitomycin C (MC). On top, typical examples of MC-treated WT and Δ31 metaphases presenting chromosomal aberrations (arrowheads: chromosome breaks; arrow: radial chromosome; scale bars, 2 μm). Below, results were plotted from 107 (WT untreated), 99 (WT MC-treated), 112 (Δ31 untreated) and 98 (Δ31 MC-treated) metaphases. To prevent any potential bias, cell preparations were dropped onto code-labelled slides (to mask the genotypes of cells to be analysed) and the same metaphases were independently observed by two experimenters. (b) p53<sup>Δ31/Δ31</sup> MEFs exhibit increased frequencies of MC-induced sister chromatid exchanges. As in a, an unbiased procedure was used to determine the percentage of chromosomes presenting one or several sister chromatid exchanges (SCEs). On top, representative examples of chromosomes from MC-treated WT (left) or p53<sup>Δ31/Δ31</sup> (right) metaphases displaying SCEs (scale bars, 2 μm). Below, results plotted from an analysis of 3,013 (WT untreated), 1,287 (WT MC treated), 1,905 (Δ31 untreated) and 340 (Δ31 MC treated) chromosomes. *P = 0.059. (c) p53 activation correlates with a decreased capacity to form Rad51 foci in response to mitomycin C. Rad51 foci were counted in cells treated with MC or MC + Nutlin. On top, typical nuclei are shown (scale bars, 2 μm); below, results from >300 nuclei per genotype. The reduced capacity to form Rad51 foci might result from p53-dependent decreases in the expression of Fancr/Rad51 as well as other Fanc genes. (d) Effects of p53 activation on the cellular sensitivity to MC. Cells were treated or not with Nutlin 2.5 μM for 24 h, then with MC at 0, 0.01, 0.1 and 1 μg ml<sup>−1</sup> for 48 h, then counted. For each genotype, the final number of untreated cells was given a value of 1 and used as reference. Results from three experiments. Means ± s.e.m. are shown; ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05 by Student’s t-test.
with p53-deficient cells, and observed that out of the 12 p53-regulated FA genes identified in mouse cells, 9 are also downregulated upon p53 activation in human MRC5 cells: FANCA, FANCB, FANCD1, FANCD2, FANCI, FANCIJ, FANCJ, FANCN, and FANCQ (Fig. 7a). Interestingly, one of these genes, FANCJ, was recently identified as one of 210 genes most likely to be downregulated by p53 in an E2F4-dependent manner. Furthermore, candidate CDE/CHR motifs could be found for each of these genes (Supplementary Fig. 15a), and the CDE/CHRs in Fancd2, Fancn, and Fancr were highly conserved in the human FANC homologous genes (Supplementary Fig. 15b). Consistent with this, we next found that human p53 activation leads to increased E2F4 binding at the FANCd2, Fanci, and FANCr promoters (Supplementary Fig. 16a), and that mutation of the CDE/CHRs in these promoters abolished their p53-dependent regulation (Supplementary Fig. 16b). The p53-dependent downregulation of FANC genes could also be observed in response to DNA damage in MRC5 cells (Supplementary Fig. 17), and we verified that the CDE/CHR motif in FANCd2 is important for its DNA damage-induced downregulation (Supplementary Fig. 18). In addition, the data mining of a transcriptome-wide analysis were again consistent with our results (Supplementary Fig. 19). BLM, DEK, FEN1, TIMELESS, and RECQL4 were also downregulated in human cells upon p53 activation, further indicating an overall conservation of the regulatory pathways identified in murine cells (Supplementary Fig. 20).

Further evidence of this conservation was obtained using the Oncomine software (www.oncomine.org). Tumour samples from the Australian Ovarian Cancer Study revealed that the p53 pathway is functional in low-grade ovarian serous tumours, but frequently lost in high-grade ovarian carcinomas. Evidence for this first came from using a transcriptomic signature of p53 target genes. Formal demonstration was later obtained by TP53 sequencing, which identified p53 mutations in 0% of low-grade serous tumours and 96.7% of high-grade carcinomas. We analysed the transcriptome data of Anglesio et al., who characterized 90 ovarian samples from the Australian Ovarian Cancer Study, including 60 high-grade adenocarcinomas. As expected, the expression of genes activated by p53 (CDKN1A/p21, MDM2, DDB2 and SESN1) was decreased in high-grade tumours. On the opposite, FANCd2, and other genes known to be repressed by E2F4 in a p53-dependent manner (BIRC5, CDC6, and CDC23C), were more expressed in high-grade tumours (Fig. 7b). Increased FANCd2 expression also correlated with increases in the expression of other FA genes (FANCA, FANCJ, FANCQ, FANCN, and FANCQ), as well as additional genes regulated by p53 in our experiments (BLM, FEN1 and TIMELESS; Fig. 7b). Similar results were obtained when we analysed data from liver cancers (Supplementary Fig. 21) and adenocortical tumours (Supplementary Fig. 22), providing evidence that human p53 downregulates several genes of the FA pathway in many tissues, and that loss of p53 function leads to an increased expression of FANC genes in advanced human cancers.

We next found that Nutlin sensitized human primary WT cells, but not their p53-deficient counterparts, to MC (Fig. 7c). Likewise, the sensitivity to MC of human cancer cells expressing a WT p53 was markedly increased by Nutlin (Fig. 7d), suggesting a potential therapeutic relevance of our findings.

**Discussion**

In this report, we further analysed the consequences of a deletion of the p53 carboxy-terminal domain. Our previous analysis indicated that most p53<sup>331/331</sup> mice exhibit a full set of features characteristic of DC. At the molecular level, the increased p53 activity in p53<sup>331/331</sup> MEFs correlated with the downregulation of four genes implicated in telomere syndromes: Dkc1, Ret11, Ter1, and Tin2 (ref. 2). Here we show that several other genes involved in telomere metabolism are downregulated in p53<sup>331/331</sup> cells: Blm, Dek, Fanca2, Feni, Gar1, Recqa4 and Timeless, strengthening the notion that p53 plays a major role in the regulation of telomere metabolism.

Importantly, some of these genes are involved in DNA repair, and we next found p53<sup>331/331</sup> cells to exhibit decreased mRNA levels for 11 additional genes mutated in FA, and a reduced capacity to repair DNA interstrand crosslinks. Because DC and FA are both inherited bone marrow failure syndromes in humans, these new findings raised the possibility that an attenuated FA pathway might contribute to the bone marrow failure that affects p53<sup>331/331</sup> mice. Importantly, however, mice carrying knocked out alleles of Fanca genes exhibit little or no haematological abnormalities in the absence of additional stress (for example, aldehyde-mediated DNA damage), whereas aplastic anaemia occurs spontaneously in mouse models of telomere dysfunction (for example, Pot1<sup>−/−</sup> mTR<sup>−/−</sup> mice) and in p53<sup>331/331</sup> mice. Furthermore, p53<sup>331/331</sup> mouse cohorts of mixed genetic backgrounds previously indicated that a gene linked to the Agouti locus, on chromosome 2, had an impact on their survival. None of the Fanca genes maps on chromosome 2, whereas mRNA levels for Ret1, located 26 cm away from Agouti, affected the survival of mutant mice. Ret1 encodes a Fanca-like helicase that might participate in DNA repair, but that mainly acts as a dominant regulator of telomere length. Accordingly, Ret1 is mutated in telomere syndromes, including severe DC and pulmonary fibrosis. Together, these data indicate that telomere dysfunction most likely plays a predominant role in the aplastic anaemia that affects p53<sup>331/331</sup> mice.

Interestingly, aplastic anaemia is not the only clinical trait shared by patients with FA and DC: abnormal skin pigmentation,
Figure 7 | Human p53 also regulates multiple genes of the Fanconi anaemia DNA repair pathway. (a) Human p53 activation leads to the downregulation of several FANC genes. mRNAs were prepared from human diploid lung fibroblasts (MRC5) and their SV40-transformed derivative cells (SVM), untreated or treated with Nutlin, and mRNAs were quantified using real-time PCR, normalized to control mRNAs, then the amount in untreated MRC5 cells was assigned a value of 1. For each gene, results are from three independent experiments; means ± s.e.m. are shown; ***P \leq 0.001, **P \leq 0.01, *P \leq 0.05, NS, not significant by Student’s t-test. (b) In human ovarian cancers, loss of p53 function correlates with an increase in the expression of FANC genes. Analysis of transcriptome data from ref. 47 with the Oncomine software indicates that ovarian serous cancer progression correlates with a decreased expression of p53-transactivated genes (for example, CDKN1A and MDM2), and an increased expression of several FANC genes (FANCA, FANCD2, FANCI, FANCJ, FANCR and FANCT). (c) p53 activation sensitizes cells to mitomycin C. MRC5 and SVM cells were treated and analysed as in Fig. 5d. Results from three independent experiments. (d) Human cancer cells expressing a WT p53 can be sensitized to mitomycin C by a treatment with Nutlin. Colon carcinoma cells HCT116 (HCT) and their p53−/−-derivative cells (HCT p53 KO) were treated and analysed as in Fig. 5d. Results from three independent experiments.
short stature and testicular hypoplasia may affect patients with either syndrome. Furthermore, telomere dysfunction was reported for at least some patients with FA, and cells from patients with DC appeared hypersensitive to MC in a few studies. In fact, although DC and FA are distinct clinical disorders caused by mutations in different genes, their clinical similarities initially led to some confusion, and recent evidence of misdiagnosis can still be found occasionally. As mentioned above, because a defective FA pathway may activate p53, our data also provide a rationale for the combination of Nutlin with therapeutic agents between these syndromes. Independently, our data also suggest a positive-feedback loop between p53 and telomere metabolism. Together, our analyses of p53 and FANC D2 (FANC D2) mutant cells raise the intriguing possibility that a sustained p53 activation might contribute to the clinical overlap between DC and FA, notably by leading to a concomitant downregulation of genes important for telomere metabolism and genes of the FA DNA repair pathway (Supplementary Fig. 23). Because the p53 pathway is affected by single-nucleotide polymorphisms in many genes including TP53, MDM2, MDM4 and CDKN1A, we further presume that the strength of the regulatory loops that affect p53, telomere-related and FA genes should vary among humans, and that this might contribute, in patients with identical disease-causing mutations, to the variability in clinical overlap between these syndromes. Independently, our data also provide a rationale for the combination of Nutlin with therapeutic agents inducing DNA interstrand crosslinks, to efficiently kill cancer cells that retain a functional p53 pathway.

Methods

Cells and cell culture reagents. MEFs, isolated from 13.5-day embryos, were cultured for ≤6 passages in a 5% CO2 and 3% O2 incubator, for 24 h, then pulse-labeled for 1 h with bromo-deoxy uridine (BrdU) (10 μM), fixed with 70% ethanol, double stained with fluorescein isothiocyanate anti-BrdU and propidium iodide, and sorted using a LSRII cytometer. Data were analysed using FlowJo.

 Luciferase expression assays. To construct the Luciferase reporter plasmids, we cloned a 2-kb fragment (for Fancd2) or 1-kb fragment (for Fanc, Fancd2, and Fanci) around the transcription start site upstream of the Firefly luciferase gene in a pGL3-basic vector (Promega), or a variant fragment generated by PCR mutagenesis of the putative CDE/CHR motif (details on request). Next, NIH-3T3 cells were transfected using lipofectamine 2000 by 3 μg of a Fanc-luciferase reporter plasmid and 30 ng of renilla luciferase expression plasmid (pGL4.73, Promega) for normalization, and treated or not with 10 μM Nutlin 3a or 0.5 μg ml⁻¹ doxorubicin. Transfected cells were incubated for 24 h, then trypsinized, resuspended in 75 μl culture medium with 7.5% FBS and transferred into a well of an optical 96-well plate (Nunc). The dual-glo luciferase assay system (Promega) was used according to the manufacturer’s protocol to lyse the cells and read firefly and renilla luciferase signals. Results were normalized, then the average luciferase activity in cells transfected with a WT Promoter and not treated with Nutlin were assigned a value of 1.

 Cell cycle assays. Log phase cells, treated or not with Nutlin, were incubated for 24 h, then pulse-labeled for 1 h with bromo-deoxy uridine (BrdU) (10 μM), fixed with 70% ethanol, double stained with fluorescein isothiocyanate anti-BrdU and propidium iodide, and sorted using a LSRII cytometer. Data were analysed using FlowJo.

 Immunofluorescence. Cells were spread onto coverslips, treated or not with Nutlin 10 μM, then MC 0.1 μg ml⁻¹ for 1 h, and left to recover for 12 h. Twenty-four hours after Nutlin treatment, cells were fixed and permeabilized. Coverslips were incubated with a Rad51 antibody (Ab-1 Calbiochem) for 1 h at 37 °C in a humid chamber, then with secondary Alexa Fluor anti-rabbit antibody (Invitrogen). Slides were mounted in Vectashield with 0.2 μg ml⁻¹ 4,6-diamidino-2-phenylindole. Images were captured on a Zeiss Axiopt2 microscope using equal exposure times for all images.

 Cellular sensitivity to mitomycin C. Cells were seeded into wells of a 96-well plate (500 cells per well, in triplicates). After adhesion, cells were treated or not with Nutlin 2.5 μM for 24 h, then with MC for 48 h at 0, 0.01, 0.1 and 1 μg ml⁻¹. Cells were then counted using the CyQUANT kit (Life Technologies) and a microplate reader according to the supplier’s recommendations.

 Statistical analyses. Differences between two groups were analysed by Student’s t-test. difference between three groups were analysed by one-way analysis of variance, and values of P ≤ 0.05 were considered significant.

References

1. Khincha, P. P. & Savage, S. A. Genomic characteristic of the inherited bone marrow failure syndromes. Semin. Hematol. 50, 333–347 (2013).
2. Simeonova, I. et al. Mutant mice lacking the p53 C-terminal domain model telomere syndromes. Cell Rep. 3., 2046–2058 (2015).
3. Hamard, J. P. et al. The C terminus of p53 regulates gene expression by multiple mechanisms in a target- and tissue-specific manner in vivo. Genes Dev. 27, 1868–1885 (2013).
4. Armanios, M. & Blackburn, E. H. The telomere syndromes. Nat. Rev. Genet. 13, 693–704 (2012).
5. Tummal, H. et al. Poly(A)-specific ribonuclease deficiency impacts telomere biology and causes dyskeratosis congenita. J. Clin. Invest. 125, 2151–2160 (2015).
6. Holohan, B., Wright, W. E. & Shay, J. W. Telomereopathies: an emerging spectrum disorder. J. Cell Biol. 205, 289–299 (2014).

ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms11091 | www.nature.com/naturecommunications
8. Kocak, H. NATURE COMMUNICATIONS | 7:11091 | DOI: 10.1038/ncomms11091 • www.nature.com/naturecommunications
9. Codd, V.
10. Jain, D., Malik, A. A., Kumar, A., Malik, B. K. & Raina, V. Variations in exon-2
12. Rooney, S.
14. Ting, A. P., Low, G. K., Gopalakrishnan, K. & Hande, M. P. Telomere attrition
11. Venteicher, A. S., Meng, Z., Mason, P. J., Veenstra, T. D. & Artandi, S. E.
16. Lin, W.
25. Chawla, R.
26. Crabbe, L., Jauch, A., Naeger, C. M., Holtgreve-Grez, H. & Karlseder, J.
27. Sengupta, S.
28. Joksic, I.
30. Benson, E. K.
34. Mjelle, R.
33. Fischer, M., Quaas, M., Steiner, L. & Engeland, K. The p53-p21-DREAM-CDE/
18. Batenburg, N. L., Mitchell, T. R., Leach, D. M., Rainbow, A. J. & Zhu, X. D.
32. Fischer, M., Quaas, M., Wintsche, A., Muller, G. A. & Engeland, K. Polo-like kinase 4 transcription is activated via CRE and NRF1 elements, repressed by
17. Craddock, L., Blau, C., Naeger, C. M., Holgrev-Greitz, H. & Katslser, J. Telomere dysfunction as a cause of genomic instability in Werner syndrome. Proc. Natl Acad. Sci. USA 104, 2205–2210 (2007).
36. Qian, L., Yuan, F., Rodriguez-Tello, P., Padaonkar, S. & Zhang, Y. Human Fanconi anemia complementation group A protein stimulates the 5’ fl annexidase activity of FEN1. PloS ONE 6, e26666 (2011).
37. Younger, S. T., Kenzlemann-Bro, D., Jung, H., Attardi, L. D. & Rinn, J. L. Integrative genomic analysis reveals widespread enhancer regulation by p53 in response to DNA damage. Nucleic Acids Res. 43, 4447–4462 (2015).
38. Muntean, A. G. et al. The PAF complex synergizes with MLL fusion proteins at HOX loci to promote leukemogenesis. Cancer Cell 17, 609–621 (2010).
39. Longerich, S., Li, J., Xiong, Y., Sung, P. & Kupfer, G. M. Stress and DNA repair biology of the Fanconi anemia pathway. Blood 124, 2812–2819 (2014).
40. Sawyer, S. L. et al. Biallelic mutations in BRCAl cause a new Fanconi anemia subtype. Cancer Discov. 5, 135–142 (2015).
41. Hira, A. et al. Mutations in the gene encoding the E2 conjugating enzyme UBE2T cause Fanconi anemia. Am. J. Hum. Genet. 96, 107–1107 (2015).
42. Wang, A. T. et al. A dominant mutation in human RAD51 reveals its function in DNA interstrand crosslink repair independent of homologous recombination. Mol. Cell 59, 478–490 (2015).
43. Crossan, G. P. et al. Disruption of mouse Slx4, a regulator of structure-specific nuclease, phenopolis Fanconi anemia. Nat. Genet. 43, 147–152 (2011).
44. Montes de Oca Luna, R., Wagner, D. S. & Lozano, G. Rescue of early embryonic lethality in mdk2-deficient mice by deletion of p33. Nature 378, 203–206 (1995).
45. Bardot, B. et al. Mice engineered for an obligatory Mdm4 exon skipping express higher levels of the Mdm4-S isoform but exhibit increased p53 activity. Oncogene 34, 2943–2948 (2015).
46. Contreras, D. et al. Bone marrow failure in Fanconi anemia is triggered by an exacerbated p53/p21 DNA damage response that impairs hematopoietic stem and progenitor cells. Cell Stem Cell 11, 36–49 (2012).
47. Anglesio, M. S. et al. Mutation of ERBB2 provides a novel alternative mechanism for the ubiquitous activation of RAS-MAK in ovarian serous low malignant potential tumours. Mol. Cancer Res. 6, 1678–1690 (2008).
48. Hunter, S. M. et al. Molecular profiling of low grade serous ovarian tumours identifies novel candidate driver genes. Oncotarget 6, 37663–37677 (2015).
49. Ahmed, A. A. et al. Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. J. Pathol. 221, 49–56 (2010).
50. Bakker, S. T., de Winter, J. P. & te Riele, H. Learning from a paradox: recent insight into Fanconi anemia through studying mouse models. Dis. Model Mech. 6, 40–47 (2013).
51. Langevie, F., Crossan, G. P., Rosado, I. V., Arends, M. J. & Patel, K. J. FanCD2 counteracts the toxic effects of naturally produced aldehydes in mice. Nature 475, 53–58 (2011).
52. Garaycochea, I. J. et al. Genotoxic consequences of endogenous aldehydes on mouse haematopoietic stem cell function. Nature 489, 571–575 (2012).
53. Hockemeyer, D., Palm, W., Wang, R. C., Couto, S. S. & de Lange, T. Engineered telomere degradation models dyskeratosis congenita. Genes Dev. 22, 1773–1788 (2008).
54. Uringa, E. J. et al. RTEL1 contributes to DNA replication and repair and telomere maintenance. Mol. Biol. Cell 23, 2782–2792 (2012).
55. Ding, H. et al. Regulation of murine telomere length by RTEL: an essential gene encoding a helicase-like protein. Cell 117, 873–886 (2004).
56. Ballew, B. J. et al. A recessive founder mutation in regulator of telomere elongation helicase 1, RTEL1, underlies severe immunodeficiency and features of Hoyeraal Hreidarsson syndrome. PLoS Genet. 9, e1003695 (2013).
57. Walne, A. J., Vulliamy, T., Kirwan, M., Plagnol, V. & Dokal, I. Constitutional mutations in RTEL1 cause severe Dyskeratosis congenita. Ann. J. Hum. Genet. 92, 448–453 (2013).
58. Le Guen, T. et al. Human RTEL1 deficiency causes Hoyeraal-Haridsson syndrome with short telomeres and genome instability. Hum. Mol. Genet. 22, 3239–3249 (2013).
59. Stuart, B. D. et al. Exome sequencing links mutations in PARN and RTEL1 with familial pulmonary fibrosis and telomere shortening. Nat. Genet. 47, 512–517 (2015).
60. Leutertre, F. et al. Accelerated telomere shortening and telomerase activation in Fanconi’s anaemia. Br. J. Haematol. 105, 883–893 (1999).
61. Nagasawa, H. & Little, J. B. Suppression of cytotoxic effect of mitomycin-C by superoxide dismutase in Fanconi’s anaemia and dyskeratosis congenita fibroblasts. Carcinogenesis 4, 795–799 (1983).
62. McDonald, R. & Goldschmidt, B. Pancytopenia with congenital defects (Fanconis Anaemia). Arch. Dis. Child. 35, 367–372 (1960).
63. Bodalski, J., Defenicinsa, E., Judkiewicz, L. & Pacanowska, M. Fanconis anaemia and dyskeratosis congenita fibroblasts. Carcinogenesis 12, 330–342 (1963).
64. Steier, W., Van Voolen, G. A. & Selmanowicz, V. I. Dyskeratosis congenita: relationships to Fanconi’s anaemia. Blood 39, 510–512 (1972).
65. Sirinavin, C. & Trowbridge, A. A. Dyskeratosis congenita: clinical features and genetic aspects. Report of a family and review of the literature. J. Med. Genet. 12, 339–354 (1975).
Acknowledgements

We thank J. Leemput for technical help in ChIP assays. The ‘Genetics of Tumour Suppression’ laboratory is an ‘Equipe Labellisée Ligue Nationale Contre le Cancer’, with support from the Ligue Headquarter and from the Comité du Cantal. The project was initiated by grants from the Fondation de France (Comité Tumeurs), the Ligue Nationale contre le Cancer (Comité Ile de France) and the Fondation ARC. PhD candidates were supported by fellowships from the Ministère de l’Enseignement Supérieur et de la Recherche (S.J and E.T.), and the Ligue Nationale contre le Cancer (S.J).

Author contributions
S.J. and E.T. performed quantitative PCRs, ChIP and cell cycle assays, western blots, immunofluorescence, and chromosome breakage and cellular sensitivity assays; V.L. performed molecular cloning and luciferase assays, and contributed to chromosome breakage assays and bioinformatics analyses; B.B. contributed to ChIP assays; F.T. designed the project, performed bioinformatics analyses and wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Jaber, S. et al. p53 downregulates the Fanconi anaemia DNA repair pathway. Nat. Commun. 7:11091 doi: 10.1038/ncomms11091 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of the license, visit http://creativecommons.org/licenses/by/4.0/