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Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice

Frédéric Langevin[1], Gerry P. Crossan[1], Ivan V. Rosado[1], Mark J. Arends[2] & Ketan J. Patel[1,3]

Reactive aldehydes are common carcinogens. They are also by-products of several metabolic pathways and, without enzymatic catabolism, may accumulate and cause DNA damage. Ethanol, which is metabolised to acetaldehyde, is both carcinogenic and teratogenic in humans. Here we find that the Fanconi anaemia DNA repair pathway counteracts acetaldehyde-induced genotoxicity in mice. Our results show that the acetaldehyde-catabolising enzyme Aldh2 is essential for the development of *Fancd2*$^{-/-}$ embryos. Nevertheless, acetaldehyde-catabolism-competent mothers (*Aldh2*$^{+/-}$) can support the development of double-mutant (*Aldh2*$^{-/-}$*Fancd2*$^{-/-}$) mice. However, these embryos are unusually sensitive to ethanol exposure *in utero*, and ethanol consumption by postnatal double-deficient mice rapidly precipitates bone marrow failure. Lastly, *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice spontaneously develop acute leukaemia. Acetaldehyde-mediated DNA damage may critically contribute to the genesis of fetal alcohol syndrome in fetuses, as well as to abnormal development, haematopoietic failure and cancer predisposition in Fanconi anaemia patients.

Multicellular organisms are constantly exposed to common environmental toxins that cause genome damage. In addition, both aerobic respiration and metabolism generate many reactive chemical species that are capable of attacking DNA[1]. To counteract such endogenous threats to genome stability, cells catabolise these reactive molecules while simultaneously mobilizing DNA repair. The failure to respond to this threat leads to the catastrophic phenotypes caused by the genetic inactivation of certain DNA repair pathways. In Fanconi anaemia, biallelic mutations in any one of fourteen genes cause developmental defects, as well as progressive bone marrow failure and widespread cancer predisposition[2]. In this specific clinical instance we have little or no insight into the source of endogenous DNA damage, and we do not yet understand how this eventually contributes to the development of this phenotype.

In searching for a source of endogenous DNA damage, we focused on simple aldehydes. These molecules are ubiquitous organic compounds—common constituents of food sources—and are also produced within organisms as by-products of cellular metabolism[3]. Aldehydes are very reactive molecules and are therefore likely to constitute a serious threat to cellular integrity. In fact, these molecules can react avidly with DNA *in vitro*, causing a range of DNA modifications[4–6]. Whereas much is known about how aldehydes are detoxified in cells by a class of enzymes known as the aldehyde dehydrogenases[7,8], little is known about whether or not aldehydes damage DNA *in vivo* and, if so, how such DNA damage might be repaired. Here we present evidence indicating that the Fanconi anaemia DNA repair pathway has a crucial role in counteracting acetaldehyde-induced genotoxicity in mice.

Fanconi B cells are sensitive to acetaldehyde

A recent study tested chicken DT40 DNA repair mutant cell lines for hypersensitivity to formaldehyde[9]. The authors concluded that homologous recombination, translesion synthesis, and the Fanconi anaemia gene *FANCD2* protected against formaldehyde-induced clonogenic cell killing. Numerous studies have reported that cells exposed to acetaldehyde accumulate DNA damage[10,11]. Moreover, ethanol exposure (a dietary source of acetaldehyde) in mice and humans results in DNA base damage[10–13]. We compared the sensitivity of a chicken B-cell line, DT40, carrying a disruption of the Fanconi anaemia gene *FANCL* (Δ*FANCL*) to acetaldehyde, and found that this mutant strain is markedly sensitive to exogenous acetaldehyde (Fig. 1a). Four additional Fanconi anaemia mutants that operate both upstream (Δ*FANCB* and Δ*FANCC*; Fig. 1b) and downstream in the pathway (Δ*FANCJ* and Δ*RAD51C*; Fig. 1c)[9,14,15] were also sensitive to this aldehyde. To determine further the genetic requirements for protection against acetaldehyde genotoxicity, we exposed various DNA repair mutants representing distinct DNA repair pathways to acetaldehyde. As shown in Fig. 1d and e, mutants in homologous recombination (Δ*XRCC2*), translesion synthesis (Δ*REV1*), nucleotide excision repair (Δ*XPA*), non-homologous end joining (Δ*KU70*; also known as *XRCC6*) and mismatch repair (Δ*EXO1*) are not particularly sensitive to acetaldehyde.

Developmental role for Aldh2 in *Fancd2*$^{-/-}$ mice

Although Fanconi anaemia pathway-deficient cell lines are hypersensitive to exogenous acetaldehyde, this does not address whether aldehyde toxicity occurs within a physiological context. This is significant as acetaldehyde is produced as a by-product of natural metabolism (Fig. 1f). Such intracellular pools of aldehydes are eliminated through the action of many enzymes, including numerous aldehyde dehydrogenases[16,17], aldehyde oxidase[18] and cytochrome p450 (Fig. 1f)[19]. Although organisms use various means to catabolise aldehydes, we focused on the mitochondrial enzyme aldehyde dehydrogenase 2 because of its well-defined role in acetaldehyde catabolism in humans[7]. Surprisingly, disrupting *ALDH2* in Δ*FANCC* DT40 cells (Supplementary Fig. 1a) did not potentiate sensitivity to exogenous acetaldehyde (Supplementary Fig. 1b). We surmised that additional enzymes may compensate for *ALDH2* inactivation in these cells. However, the situation might be different if the same approach were tested in the context of a whole organism. Therefore, we set out to generate mice that harbour disruptions of the key Fanconi anaemia gene *Fancd2* in combination with *Aldh2*. *Fancd2* mice were chosen because they have the strongest phenotype among mice carrying

[1]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK. [2]University of Cambridge, Department of Pathology, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK. [3]University of Cambridge, Department of Medicine, Level 5, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK.

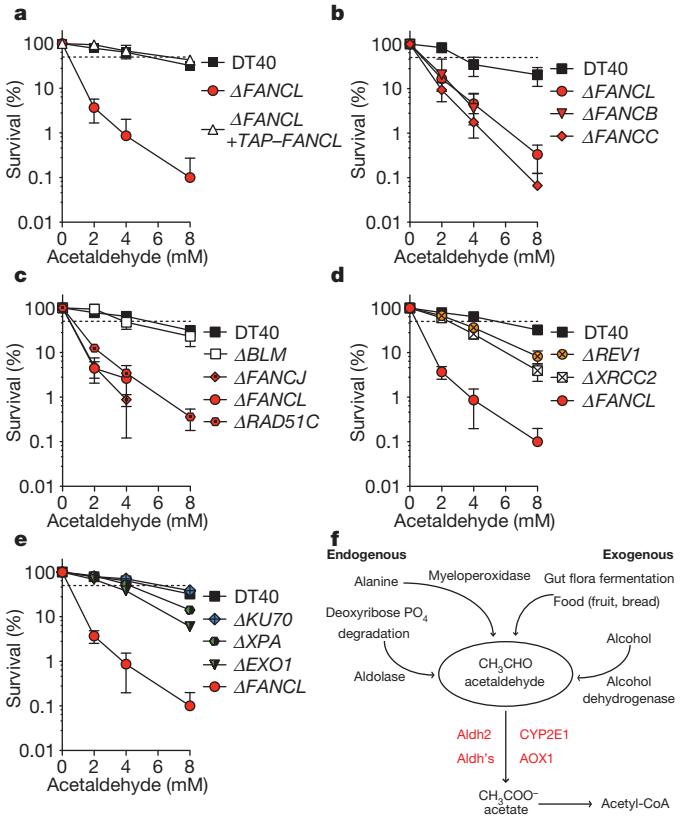

Figure 1 | Chicken Fanconi anaemia pathway knockout B cells are hypersensitive to acetaldehyde. **a**–**f**, Clonogenic survival of DT40 DNA repair mutants exposed to acetaldehyde. **a**, Fanconi anaemia: Δ*FANCL* and Δ*FANCL* complemented back with *TAP–FANCL* complementary DNA. **b**, Fanconi anaemia core complex components: Δ*FANCB* and Δ*FANCC*. **c**, Downstream Fanconi anaemia genes: Δ*FANCJ* and Δ*RAD51C*. **d**, Homologous recombination repair: Δ*XRCC2*; and translesion synthesis: Δ*REV1*. **e**, Non-homologous end joining: Δ*KU70*; nucleotide excision repair: Δ*XPA*; and mismatch repair: Δ*EXO1*. In **a**–**e**, each point represents the mean of three independent experiments, each carried out in duplicate; error bars represent s.e.m. **f**, Scheme outlining the potential sources contributing to the endogenous pool of acetaldehyde[42,43] and its catabolism.

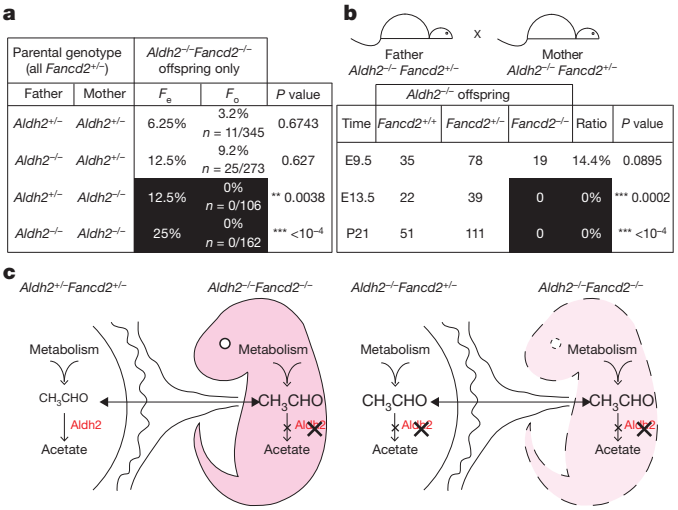

Figure 2 | A single maternal allele of *Aldh2* is essential for the development of *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice. **a**, Crosses consisting of various allelic combinations at the *Aldh2* locus (always *Fancd2*$^{+/-}$) were set up to obtain *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ pups. F_e and F_o are expected and observed Mendelian frequencies, respectively, of *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ pups. Shaded regions highlight the cross for which the frequency is 0% when it is expected to be 12.5% or 25%. χ^2 test, confidence interval 5%. **b**, Observed frequencies of *Fancd2* genotypes in *Aldh2*$^{-/-}$ embryos at E9.5, E13.5 and 21 days postpartum (P21) for the intercross outlined above the table. Each column represents the number of embryos obtained for each genotype. The last column defines the percentage of *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice. Shaded region highlights cross for which the frequency is 0% when it is expected to be 25%. χ^2 test, confidence interval 5%. **c**, Schematic interpretation of how maternal or fetal Aldh2 may contribute to the development of *Fancd2*$^{-/-}$ mice. Light pink shading and dashed line indicate that the embryo dies.

mutations in Fanconi anaemia genes[20], with the exception of *Slx4* mice[21]. Furthermore, acetaldehyde exposure in cell lines induces the monoubiquitination of this key Fanconi anaemia protein[22] (Supplementary Fig. 1c) and mouse *Fancd2*$^{-/-}$ cells are sensitive to exogenous acetaldehyde (Supplementary Fig. 2).

First, we created *Aldh2*$^{+/-}$ mice using embryonic stem cells harbouring a targeted mutation in the *Aldh2* locus obtained from the EUCOMM consortium (*Aldh2*$^{tm1a(EUCOMM)Wtsi}$; Supplementary Fig. 3a, b). Germline transmission of the knockout allele and subsequent crosses generated *Aldh2*$^{-/-}$ mice in a C57BL/6J background. Homozygous mice were born at the expected Mendelian ratios and show no overt phenotype (Supplementary Fig. 3c), consistent with previously published *Aldh2* knockout mice[23]. Second, we crossed *Aldh2*$^{-/-}$ with *Fancd2*$^{+/-}$ mice made in the 129S4 background[24]. To obtain *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice, we set up four types of crosses where two allelic configurations of the disrupted *Aldh2* locus (*Aldh2*$^{+/-}$ or *Aldh2*$^{-/-}$) were combined with *Fancd2* heterozygosity (*Fancd2*$^{+/-}$) in both male and female mice (Fig. 2a). This was done because *Fancd2*$^{-/-}$ mice are sterile. The observed genotypic frequencies presented in Fig. 2a show that when the maternal genotype was *Aldh2*$^{-/-}$, double-mutant offspring were not produced (associated with a reduction in mean litter size; Supplementary Fig. 4a). This is in contrast with the expected Mendelian segregation of 12.5% and 25% when the paternal genotypes are *Aldh2*$^{+/-}$ and *Aldh2*$^{-/-}$, respectively (Fig. 2a and Supplementary Fig. 4b). However, when the females were *Aldh2*$^{+/-}$, then *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ pups were born. Timed *Aldh2*$^{-/-}$*Fancd2*$^{+/-}$ male mice × *Aldh2*$^{-/-}$*Fancd2*$^{+/-}$ female mice crosses were set up to determine when *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ embryos died. Dams from such crosses were killed on embryonic days (E)9.5 and E13.5 and embryos were genotyped. At E9.5, *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ embryos were observed at a frequency of 14.4% (Fig. 2b), but at E13.5 they had all died. This suggests that fetal attrition occurs between E9.5 and E13.5. These results indicate that the maternal *Aldh2* genotype profoundly influences the development of double-mutant embryos *in utero*. Figure 2c and Supplementary Fig. 4c illustrate the various situations whereby survival of *Fancd2*$^{-/-}$ embryos is and is not supported; and the extent to which these results are dependent on maternal and fetal *Aldh2* genotypes. We conclude that, in the absence of the Fanconi anaemia pathway, either the mother or the fetus requires Aldh2 to catabolise acetaldehyde to enable fetal development. Acetaldehyde is a volatile small molecule, thereby allowing it to passively diffuse across the placental membranes. This could then enable the mother to break down acetaldehyde, hence compensating for *Aldh2* deficiency in the fetus. Thus, a single *Aldh2* allele is sufficient to allow the development of *Fancd2*$^{-/-}$ embryos.

Ethanol teratogenicity in *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice

Next, we wanted to establish whether or not acetaldehyde toxicity was sufficient to cause fetal attrition. Previous studies have shown that C57BL/6J mice were susceptible to the teratogenic effects of ethanol, providing a mouse model for human fetal alcohol syndrome[25]. This teratogenicity is largely due to acetaldehyde, a by-product of ethanol catabolism[26,27]. Because we knew that an *Aldh2*$^{-/-}$*Fancd2*$^{+/-}$ male × *Aldh2*$^{+/-}$*Fancd2*$^{+/-}$ female cross generated the highest frequency of viable double-mutant pups (~9%; Fig. 2a, c), this cross was used to assess the impact of *in utero* exposure to acetaldehyde derived from

ethanol catabolism. It is noteworthy that the double-mutant pups born from this cross often carry subtle defects such as kinked tails and eye defects (Supplementary Fig. 5a–c). Figure 3a outlines our experimental protocol, which is adapted from previous work[25]. Briefly, pregnant dams were administered a total dose of 5 g kg^{-1} of 28% ethanol by intraperitoneal injection on E7.5. Control mice consisted of females from the same type of cross, subjected to injections of 0.9% saline solution. On E16.5, embryos were dissected and genotyped. The data in Fig. 3b and Supplementary Fig. 5d show that ethanol exposure reduces the frequency of double-mutant mice to 2.9% (7/240) compared to 14.5% (20/138) in the saline-treated group. A significant proportion of double-mutant embryos exposed *in utero* to ethanol suffered developmental defects, most drastically manifested as exencephaly (Fig. 3c–e, Supplementary Fig. 5f). The teratogenic events observed in embryos characterized in this experiment are compiled in Supplementary Fig. 5e. It is also noteworthy that a proportion of *Aldh2*$^{+/-}$*Fancd2*$^{-/-}$ embryos showed developmental defects in response to ethanol exposure (8/25; Fig. 3c, d and Supplementary Fig. 5e). In conclusion, *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ embryos are very sensitive to exposure to an unambiguous exogenous precursor of acetaldehyde.

Ethanol impairs haematopoiesis in *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice

Bone marrow failure is a hallmark of patients with Fanconi anaemia. It is also known that chronic ethanol abuse is associated with bone marrow dysfunction[28,29] and that exposing bone marrow cells to ethanol or acetaldehyde compromises blood forming units[30]. We therefore investigated the susceptibility of *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice to ethanol-induced myelotoxicity, the expectation being that ethanol exposure would lead to acetaldehyde accumulation and that this would then cause bone marrow dysfunction. We exposed 6–8-week-old mice corresponding to four possible genotypes (wild type, *Aldh2*$^{-/-}$, *Fancd2*$^{-/-}$ and *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$) to continuous oral ethanol in their drinking water supply. The protocol is outlined in Fig. 4a: it consists of 5 days of 15% ethanol/water followed by 5 days with 20% ethanol/water. The data in Supplementary Fig. 6 show that ethanol treatment causes a decrease in all three blood constituents. This is most notable in double-mutant mice where there is a profound drop in red blood cells and haemoglobin (Supplementary Fig. 6a, d). To establish the basis for this anaemia, we quantified the bone marrow cellularity in treated mice compared to untreated control animals. Figure 4b shows a significant decrease in the number of nucleated cells per femur in the ethanol-exposed double-mutant mice. To establish if DNA damage contributed to this ethanol sensitivity, bone marrow aspirates from ethanol-exposed mice were tested for the induction of phosphorylated histone γH2AX—an established marker of DNA damage. The western blot in Fig. 4c shows a clear induction of γH2AX in bone marrow cells obtained from double-mutant compared to control genotypes. We then set out to determine if progenitor colony-forming units (pre-B cells, granulocytes and erythrocytes) are affected by ethanol. Equal cell numbers of bone marrow cells were plated onto clonogenic plates, and it is clear that colony-forming units corresponding to all three cell types are reduced in double-mutant mice after ethanol treatment (Fig. 4d). Finally, we analysed the bone marrow histology, comparing untreated control mice with corresponding treated genotypes. The microscopy shown in Fig. 4e shows that ethanol consumption causes an almost complete obliteration of bone marrow haematopoiesis in *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice.

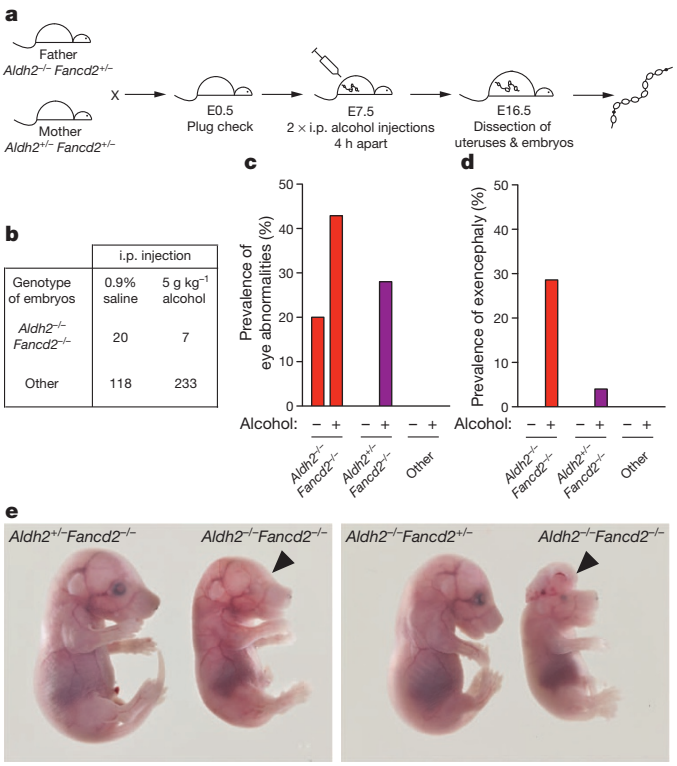

Figure 3 | Maternal ethanol exposure aborts the development of *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ embryos. **a**, Schematic representation of the experiment to expose pregnant *Aldh2*$^{+/-}$*Fancd2*$^{+/-}$ mice to ethanol. **b**, Numbers of *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ embryos after exposure to ethanol or saline solution. ***$P < 10^{-4}$, Fisher's exact test, confidence interval 1%. **c**, **d**, Prevalence of eye abnormalities (**c**) and exencephaly (**d**) for the various *Aldh2* and *Fancd2* genotypes in the saline (−) and ethanol (+) groups. **e**, Developmental abnormalities (left, anophthalmia; right, exencephaly).

Leukaemogenesis in weaned double-mutant mice

Lastly, we wanted to determine the fate of the unexposed weaned *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice. Despite subtle developmental defects, double-mutant mice were nevertheless healthy. However, within 3–6 months a significant proportion of animals succumbed to an acute illness presenting itself as rapid weight loss and lethargy (Fig. 5a). These mice were killed and the majority of necropsies revealed a large mediastinal mass, splenomegaly and blast-like lymphoid cells in the peripheral blood film and bone marrow aspirate (Fig. 5b and Supplementary Fig. 7). In three instances, these cells represented a clonal expansion of early T cells as they were double positive for CD8 and CD4 markers and in one instance, CD8 alone (Supplementary Fig. 8a). Furthermore, immunohistochemistry revealed that the neoplastic cells strongly stained for the pan-T-cell-marker CD3 (Supplementary Fig. 8b). Because bone marrow aspirates indicated that >30% of the cellular population were blasts, we conclude that this illness is akin to acute lymphoblastic leukaemia.

Discussion

Children with Fanconi anaemia suffer developmental defects, bone marrow failure and cancer predisposition because of endogenous DNA damage[2]. Aldehydes are likely to be a significant source of such damage, contributing to the genesis of the Fanconi anaemia clinical phenotype. Aldehyde accumulation is particularly toxic to Fanconi anaemia pathway-deficient bone marrow cells. Although the most obvious target is the haematopoietic stem cell pool, it is also possible that cells contributing to the marrow niche might also be susceptible. Our work also raises new therapeutic approaches to treat Fanconi anaemia in humans. For instance, it might be possible to induce the catabolism of aldehydes by induction of the relevant enzymes. This could be done simply—and somewhat paradoxically—by regular low-dose ethanol exposure or barbiturates[31,32]. Alternatively, small-molecule agonists of Aldh2, like the Alda1 molecule, might raise acetaldehyde catabolic activity[33,34]. Lastly, it will be important to determine the natural sources of aldehydes. These metabolic pathways could then be modulated so as to subvert toxic accumulation.

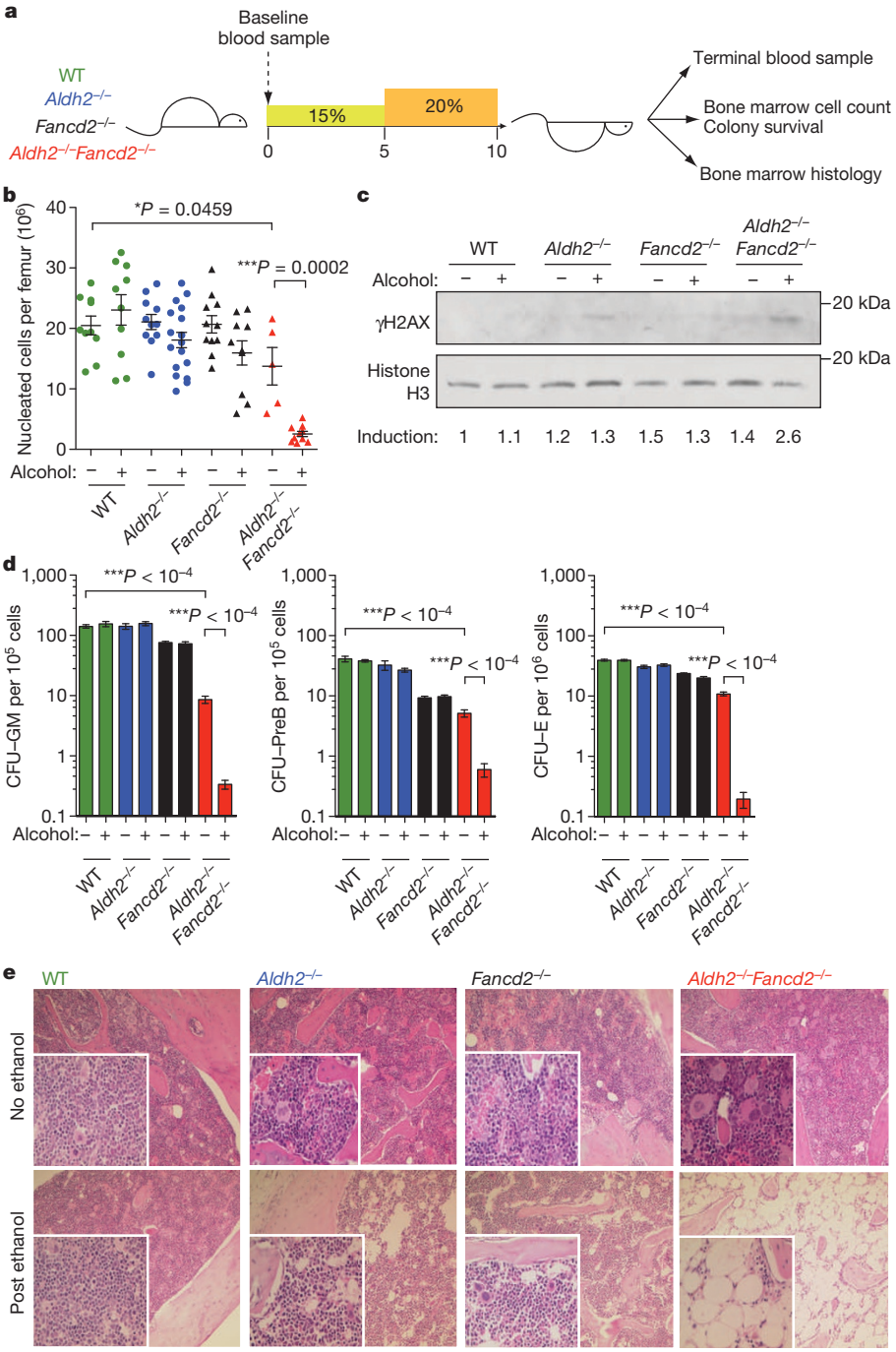

Figure 4 | Ethanol-induced bone marrow failure in *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice. **a**, Schematic outline of the oral alcohol experiment. **b**, Bone marrow cellularity in untreated (−) and treated (+) mice. Unpaired *t*-test, central bar represents mean; error bars define s.e.m. **c**, Anti-γH2AX western blot in untreated (−) and ethanol-exposed (+) bone marrow. **d**, Clonogenicity of untreated (−) and ethanol-exposed (+) bone marrow progenitors. Left: colony-forming-unit–granulocyte-monocyte cells (CFU–GM); middle: CFU–pre-B cells; right: CFU–erythrocytes (CFU–E). Each bar represents the mean of three independent experiments, with two mice per experiment, each plated in duplicate; error bars define s.e.m. ***$P < 10^{-4}$, unpaired *t*-test. **e**, Haematoxylin and eosin staining of bone marrow sections from mice not exposed (top) or exposed to ethanol (bottom). For each genotype/treatment unit, the lower magnification is ×100, inset is ×400.

Although it is currently unclear how aldehydes damage DNA *in vivo*, they directly modify bases *in vitro*, which can lead to DNA–protein and DNA–DNA crosslinks (Fig. 5c). Aldh2 efficiently oxidizes acetaldehyde, but this enzyme also detoxifies other reactive aldehydes, such as 4-hydroxynonenal, acrolein, propionaldehyde and butyraldehyde[34]. All these reactive molecules are generated through metabolism and may also contribute to DNA damage[5,8]. It is also possible that aldehyde-mediated genotoxicity may be indirect; for example, it may deplete cellular NAD^+ pools or stimulate the formation of other free radical species. Nevertheless, our work indicates that the Fanconi anaemia pathway genes seem to be specifically required for cellular resistance to acetaldehyde. However, other genes working in homologous recombination (except *Rad51C*), and in translesion synthesis do not appear to confer cellular resistance to acetaldehyde. This is a surprise because genetic and biochemical work have identified a critical role for homologous recombination and translesion synthesis genes in DNA interstrand crosslink repair[14,35]. It is therefore possible that aldehyde-mediated DNA damage does not result in DNA interstrand crosslinks.

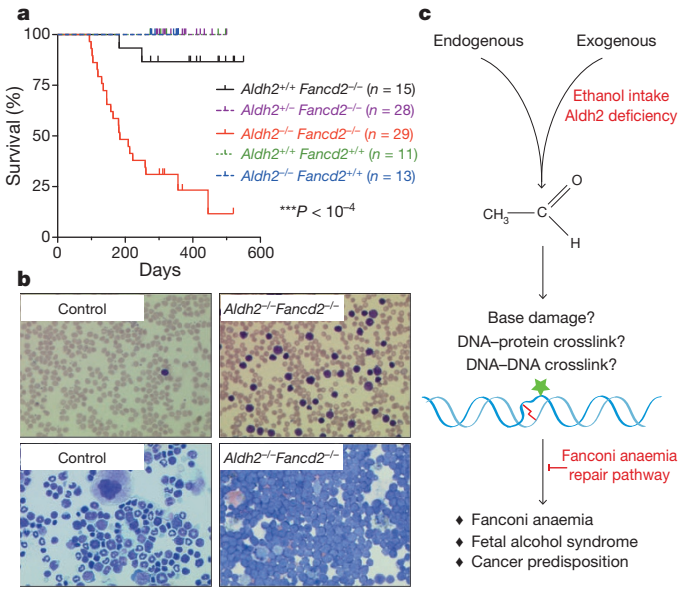

Figure 5 | Acute leukaemia in *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice. **a**, Kaplan–Meier survival curve for the cohort of weaned *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ mice and relevant genotype controls (black, purple, green and blue). ***$P < 10^{-4}$. **b**, Analysis of blood films (top; ×400) and bone marrow aspirates (bottom; ×400) obtained from a control (left) and a sick *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$ (right) mouse, revealing leukaemic blast cells. **c**, Cartoon summarizing the genetic interaction between acetaldehyde metabolism with DNA repair and the consequences of their dysfunction in humans.

In humans, excessive maternal alcohol consumption causes fetal alcohol syndrome, a prevalent worldwide cause of congenital learning disability[36]. Our study potentially links fetal alcohol syndrome to DNA damage and therefore has far-reaching implications for the understanding of the pathogenesis of this affliction in humans. Although at present it is not known whether fetal alcohol syndrome is associated with cancer predisposition, recent epidemiological evidence indicates that maternal ethanol exposure may correlate with an increased risk of acute childhood leukaemia[37,38]. The further relevance of this study to human health is also supported by the high frequency of *ALDH2* deficiency in South East Asians. In fact, up to 8% (540 million) of the world's population carries a dominant-negative mutation in *ALDH2* (ref. 39). Alcohol consumption in these individuals is strongly associated with a risk of aerodigestive tract cancer[39,40] and, in the light of this study, it is very likely that acetaldehyde-mediated DNA damage drives cancer development. This might also apply to Fanconi anaemia patients, who have a high incidence of such cancers[41]. In summary, the findings reported here not only provide fresh insights into the interplay between metabolism and cancer predisposition, but also may have profound implications for the clinical care of Fanconi anaemia patients as well as overall public health.

METHODS SUMMARY

DT40 and mouse cells. Cells were grown in RPMI medium complemented with 3% chicken serum, 7% fetal calf serum, 50 μM β-mercaptoethanol and penicillin/streptomycin mix, at 37 °C. Homology arms for the *GgAldh2* targeting vector were amplified from DT40 genomic DNA and cloned in pBluescript (Supplementary Fig. 1a). Sensitivity clonogenic assays were performed on methylcellulose after exposure for 2 h and 10–14 days incubation (see Methods). Lymphocytes purified from mouse spleens were stimulated with 40 μg ml^{-1} LPS and plated with acetaldehyde. Viability was measured by Trypan blue exclusion after 7 days using a ViCell XR cell counter.

Mice. *Fancd2* mice were a gift from M. Grompe. *Aldh2*$^{+/-}$ embryonic stem cells were injected in blastocysts from C57BL/6J mice. Chimaeric males were bred with C57BL/6J females to obtain germline transmission. Mice were maintained in specific pathogen-free conditions. All animal experiments undertaken in this study were done with the approval of the UK Home Office and the MRC Centre Ethical Review Committee.

Alcohol administration in mice. Pregnant females were injected intraperitoneally with 5 g kg^{-1} of a 28% ethanol solution at E7.5. For the 10-day oral exposure experiment, water supply was replaced by a 15% and 20% ethanol solution and was ingested orally *ad libitum* by the mice.

Haematological studies. Peripheral blood counts were collected in EDTA tubes and analysed on a VetABC analyser. Haematopoietic colony forming unit assays were performed as described previously[21] on bone marrow cells from mice exposed to alcohol for 10 days.

Histology. Tissue biopsies were fixed in 10% formalin, paraffin embedded and 4-μm sections were cut before haematoxylin and eosin staining. Blood smears and bone marrow cytospins were stained with May–Grünwald and Giemsa stains.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

DT40 cell culture and acetaldehyde sensitivity assays. DT40 cells were maintained in RPMI medium supplemented with 50 μM β-mercaptoethanol, 7% fetal calf serum, 3% chicken serum and penicillin/streptomycin mix (Gibco) in a 10% CO_2 incubator at 37 °C. Sensitivity to acetaldehyde (Fluka; catalogue no. 00070) was measured by colony survival assays. 2×10^5 cells were treated with acetaldehyde for 2 h at 37 °C before seeding of 3 dilutions on methylcellulose-DMEM medium in 6-well plates. After 10–14 days incubation, colonies were scored and survival was plotted relative to the untreated control. Each experiment represents the average of 3 independent experiments.

Generation of Δ*ALDH2* cells. Δ*ALDH2* construct: the 5′ homology arm was amplified from DT40 genomic DNA and cloned as a 2.7-kb XhoI-BamHI fragment in pBluescript (aldh2_5F1, 5′-TTGAAACTCTGGATAATGGCAAC; and aldh2_5R1, 5′-AGCTTTCTTGATCAGGTGCCCAAC). The 3′ homology arm was amplified from genomic DNA and cloned as a 2.4-kb BamHI-NotI fragment in pBluescript (aldh2_3F1, 5′-ATAGCCTATGTCTGCTTTGGATCCACA; and aldh2_3R1, 5′-TGACGTCTGAAGAGTGCAGCTCCT). Before transfection, the targeting construct was linearized by NotI restriction digest. Drug-resistant clones were screened by Southern blot using a 900-bp fragment as a probe amplified from genomic DNA (aldh2sbpbF1, 5′-ATACCGATTCCTCAAGGTTGGAT; and aldh2sbpbR1, 5′-CCACTATGTCATCTGACAGGTTGA).

Mouse strains. *Fancd2* mice (*Fancd2*^{tm1Hou}, MGI code: 2673422, 129S4/SvJae) were a gift from M. Grompe. *Aldh2* mice were generated from embryonic stem cells purchased from EUCOMM (*Aldh2*$^{tm1a(EUCOMM)Wtsi}$; MGI code: 4431566, C57BL/6N). Details of the targeting construct can be found in Supplementary Fig. 3 and http://www.knockoutmouse.org/about/targeting-strategies. Embryonic stem cells were injected in blastocyst to generate chimaeric animals. Germline transmission of the targeted allele was monitored by PCR using Aldh2 forward (5′-TGGACATGGTCCTGAAATGTCTCC-3′) and Aldh2 reverse (5′-GACTAGACTGCCAGAAACCATGAA-3′); 95 °C for 3 min, 95 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s (35 cycles), 72 °C for 3 min. In individual experiments, all mice were matched for age and gender. Mice were maintained in specific pathogen-free conditions. All animal experiments undertaken in this study were done with the approval of the UK Home Office and the MRC Centre Ethical Review Committee.

Pregnancy experiment. Timed matings of *Aldh2*$^{-/-}$*Fancd2*$^{+/-}$ males and *Aldh2*$^{+/-}$*Fancd2*$^{+/-}$ females were set up. Females were checked for the presence of a vaginal plug the following morning, considered as day E0.5. Plugged females were injected with either 5 g kg^{-1} of a 28% ethanol solution or 0.9% saline solution equivalent at E7.5, in two separate intraperitoneal injections of 2.5 g kg^{-1}, 4 h apart. At E16.5, pregnant females were killed and uteruses taken for dissection of embryos.

Oral alcohol experiment. Six-to-eight-week-old mice of four possible genotypes (wild type, *Aldh2*$^{-/-}$, *Fancd2*$^{-/-}$ and *Aldh2*$^{-/-}$*Fancd2*$^{-/-}$) were exposed to continuous oral alcohol exposure for 10 days. For the first 5 days, the drinking water supply was replaced by a 15% ethanol/water solution, followed by a 20% ethanol/water solution for the last 5 days. A baseline blood sample was taken from tails before alcohol exposure and by cardiac puncture at the end of the experiment, in order to measure full blood counts. Femurs were dissected for histological analysis and to determine bone marrow cellularity. Bone marrow of individual femurs was flushed out in 400 μl of PBS using a 26-gauge needle. Nucleated cells were quantified using 3% acetic acid and methylene blue and a ViCell XR counter (Beckman Coulter).

Peripheral blood counts. Blood was collected in EDTA microvette tubes (Sarstedt) and analysed on a VetABC analyser.

Haematopoietic CFU assays. These were performed using total bone marrow cells harvested from the femurs and tibias of untreated mice or after 10 days of oral alcohol exposure. Nucleated cells were enumerated using 3% acetic acid and methylene blue. 10^7, 10^6 and 10^5 nucleated cells were plated in Methocult M3334 (Stem Cell Technologies) and CFU–E was counted after 9 days. 10^6, 10^5, and 10^4 nucleated cells were plated in either Methocult GF M3534 (Stem Cell Technologies) for CFU–GM or Methocult M3630 (Stem Cell Technologies) for CFU–pre-B and counted after 7 days[21].

Sensitivity assays of primary mouse B cells. These were performed on lymphocytes purified from the spleen using Lympholyte M (Cederlane). Lymphocytes were stimulated with LPS (Sigma L4391) at a final concentration of 40 μg ml^{-1}. 4×10^5 cells were plated with acetaldehyde in one well of a 24-well plate. After 7 days the viable cells were enumerated using trypan blue exclusion counting 100 images using a ViCell XR (Beckman Coulter). Each data point represents the mean of two independent experiments each carried out in triplicate.

Immunoblot. γH2A.X antibody (Millipore, JBW301) was used at 1:3,000. Histone H3 polyclonal antibody (Abcam) was used at 1:2,000. FANCD2 antiserum was used at 1:3,000. Fold-induction of γH2A.X relative to H3 was calculated by densitometry.

Histology. Tissue biopsies were paraffin embedded and 4-μm sections were cut before haematoxylin and eosin staining. Blood smears and bone marrow cytospins were stained with May–Grünwald and Giemsa stains.