Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway

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Metabolism is predicted to generate formaldehyde, a toxic, simple, reactive aldehyde that can damage DNA. Here we report a synthetic lethal interaction in avian cells between *ADH5*, encoding the main formaldehyde-detoxifying enzyme, and the Fanconi anemia (FA) DNA-repair pathway. These results define a fundamental role for the combined action of formaldehyde catabolism and DNA cross-link repair in vertebrate cell survival.

Individuals afflicted with FA are prone to abnormal development and stem cell attrition and have a significant predisposition to cancer[1]. FA arises through germline inactivation of any one of 15 genes[2–4]. Most FA gene products operate together in an evolutionarily conserved pathway that eventually repairs DNA damage caused by certain chemotherapeutic agents that cross-link DNA[5–8]. However, such molecules cannot be the physiological source of DNA damage that precipitates the FA phenotype. Furthermore, chemotherapeutic cross-linkers cannot be the reason why the FA genes are conserved in all complex eukaryotes.

Recently we showed that acetaldehyde is a potential source of endogenous DNA damage necessitating repair by the FA-associated DNA-repair pathway[9]. However, acetaldehyde is not abundantly generated within cells, in contrast to formaldehyde (HCHO), the simplest reactive aldehyde[10]. Given that endogenous formaldehyde both is abundant and can attack DNA, we hypothesized that cells protect against this threat through the combined action of enzymatic detoxification and DNA repair. Here we examine the consequences of endogenous formaldehyde accumulation in DNA repair–deficient cell lines, revealing a fundamental role for the FA pathway in protecting cells against this reactive aldehyde.

Previous work has demonstrated that exogenous formaldehyde can be genotoxic. More specifically, chicken DNA repair–deficient cell lines deficient in the FA pathway downstream gene *FANCD2*, translesion synthesis (TLS) or homologous recombination (HR) show selective sensitivity to this aldehyde[11]. Formaldehyde-induced DNA damage has also been shown to be repaired by HR independently of the FA core complex[12]. We chose to extend these findings by testing formaldehyde genotoxicity in a human FA gene–deficient cell line (see **Supplementary Methods**). We used the human B cell line NALM-6 and its isogenic counterpart carrying an inactivation of the *FANCB* gene[13]. *FANCB*-deficient cells are very sensitive to formaldehyde (**Fig. 1a**),

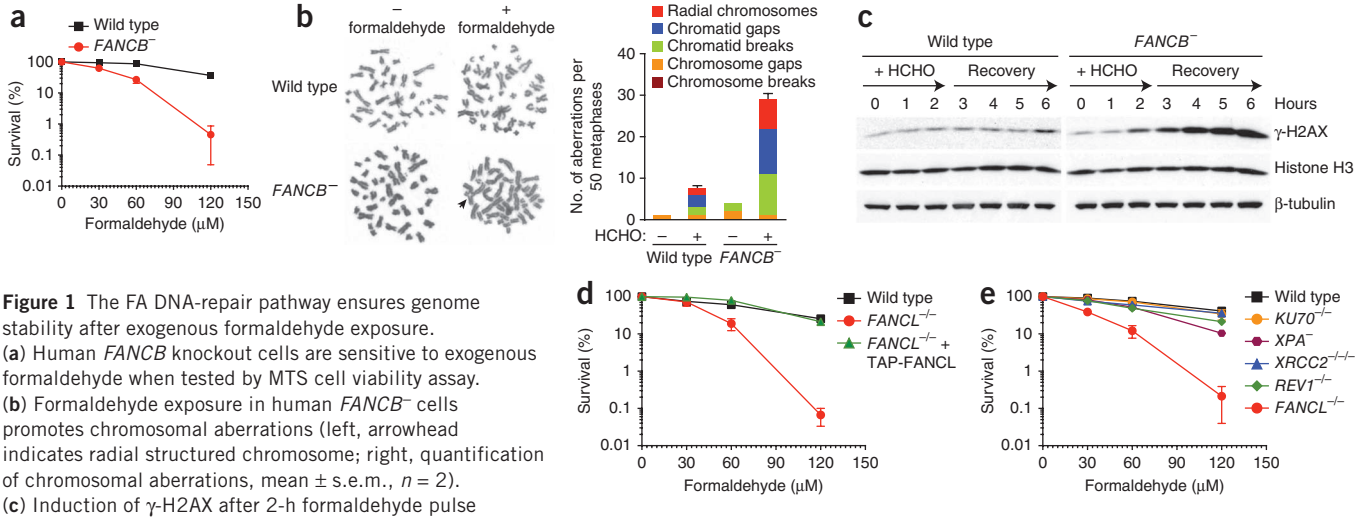

Figure 1 The FA DNA-repair pathway ensures genome stability after exogenous formaldehyde exposure. (**a**) Human *FANCB* knockout cells are sensitive to exogenous formaldehyde when tested by MTS cell viability assay. (**b**) Formaldehyde exposure in human *FANCB*$^{-}$ cells promotes chromosomal aberrations (left, arrowhead indicates radial structured chromosome; right, quantification of chromosomal aberrations, mean ± s.e.m., $n = 2$). (**c**) Induction of γ-H2AX after 2-h formaldehyde pulse treatment and release. (**d**) *FANCL* DT40 knockout cells are hypersensitive to formaldehyde; *FANCL*-complemented cell line is resistant to formaldehyde. (**e**) DT40 knockouts *XRCC2*, *KU70* and *REV1* are not hypersensitive to formaldehyde; *XPA*-deficient cells are mildly sensitive to formaldehyde.

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Received 29 June; accepted 16 September; published online 13 November 2011; doi:10.1038/nsmb.2173

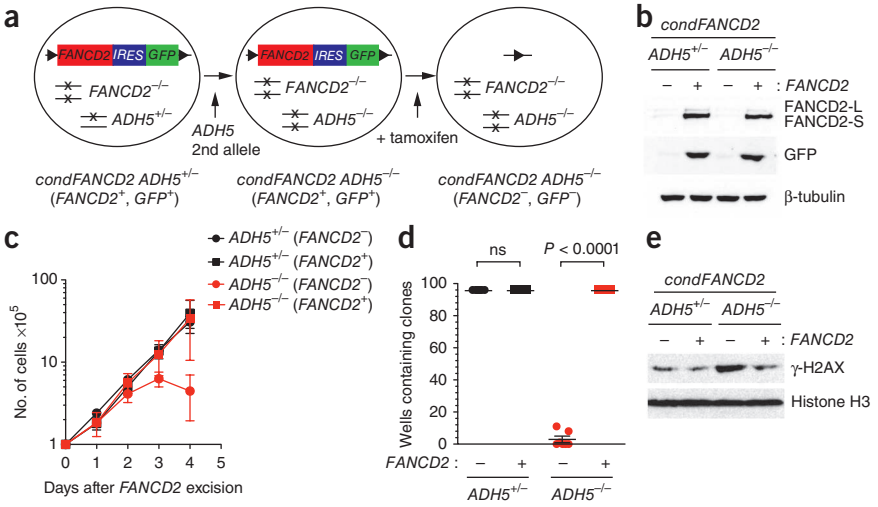

Figure 2 *FANCD2* and *ADH5* are synthetically lethal in DT40 cells. (**a**) Strategy outlining the conditional deletion of *FANCD2* in *ADH5*-null DT40 cells by addition of 4-OH tamoxifen. (**b**) Western blot of FACS GFP-positive (+) or -negative (–) sorted cells after 4-OH tamoxifen treatment for FANCD2 (top), GFP (middle) and β-tubulin (bottom). (**c**) Growth curve of FACS GFP$^+$ and GFP$^-$ sorted cells representing mean values of two independent experiments. Error bars define s.e.m. ($n = 2$). (**d**) Clonogenic survival assay of FACS GFP$^+$ and GFP$^-$ sorted cells plated at 50 cells per well into 96-well plates; mean values obtained from seven plates from two independent experiments. ($P < 0.0001$, Fisher's exact test. ns, nonsignificant.) (**e**) Induction of γ-H2AX in FACS GFP$^-$ and GFP$^+$ sorted cells lacking both *FANCD2* and *ADH5*.

and this sensitivity correlated with the accumulation of chromatid-type chromosome breakage and radial structure formation (**Fig. 1b**). These cells also showed enhanced induction of Ser139 phosphorylation of histone H2AX (γ-H2AX) (**Fig. 1c**), a marker of double strand breaks (DSBs).

We comprehensively tested formaldehyde sensitivity in both upstream and downstream components of the FA DNA-repair pathway using mutant chicken DT40 cell lines. Mutations in all the components of the FA pathway so far tested sensitized cells to formaldehyde (**Fig. 1d** and **Supplementary Fig. 1**). Cell lines deficient in the other major pathways of DNA repair were largely resistant to formaldehyde, with the exception of those deficient in nucleotide excision repair (specifically, mutant for the gene *XPA*), which showed mild sensitivity (**Fig. 1e**). In contrast to earlier results by others[11], we did not observe enhanced formaldehyde sensitivity in cells lines mutant for translesion synthesis (*REV1* and *REV3*) or homologous recombination (*XRCC2*, *XRCC3*, *RAD51C*, *RAD52* and *RAD54*) (**Supplementary Fig. 2**). This suggests that formaldehyde-induced DNA damage may not include interstrand cross-links.

Formaldehyde is generated endogenously within the nucleus as a byproduct of histone demethylation by the Jumonji demethylases and dealkylation of methylated DNA bases by AlkB orthologs (**Supplementary Fig. 3**)[10,14,15]. The cell prevents accumulation of endogenously generated formaldehyde through the action of alcohol dehydrogenase 5 (encoded by *ADH5*)[16]. To test whether the endogenous formaldehyde pool can be genotoxic, we generated an *ADH5*-knockout DT40 strain through gene disruption (**Supplementary Fig. 4a**,**b**). The *ADH5*$^{-/-}$ strain was readily obtained; this mutant's growth was not compromised, nor did it show increased sensitivity to exogenous formaldehyde under these conditions (**Supplementary Fig. 4c**). Next we attempted to inactivate the FA pathway in this strain by disruption of either the *FANCC* (one allele) or *FANCL* genes (two alleles). This proved unsuccessful (0/138 for *FANCC* and 0/781 for *FANCL*), suggesting that the combined inactivation of the FA pathway and *ADH5* could be synthetically lethal. We therefore constructed a DT40 *FANCD2*$^{-/-}$ strain that carries a conditional *FANCD2-IRES-GFP* expression cassette flanked by *lox*P sites (hitherto referred to as *condFANCD2*) (**Fig. 2a**). Owing to the presence of a 4-OH-tamoxifen–inducible Cre recombinase, 4-OH-tamoxifen treatment leads to excision of the *FANCD2-IRES-GFP* cassette, rendering the cells *FANCD2* deficient and *GFP* negative (*FANCD2*$^-$*GFP*$^-$). We then disrupted the *ADH5* gene in this strain to create the *condFANCD2 ADH5*$^{-/-}$ cell line, which lost FANCD2 and GFP expression upon treatment with 4-OH-tamoxifen, thereby generating *FANCD2*$^-$ *GFP*$^-$ cells. We purified this population by FACS (**Fig. 2b**) and followed the cells' fate in culture. The *FANCD2*$^-$ *GFP*$^-$ cells ceased growing after 3 d in culture (**Fig. 2c**), indicating that *FANCD2* has an essential role in cells lacking *ADH5*. Additionally, when *FANCD2*$^-$ *GFP*$^-$ cells were plated to isolate double-mutant clones, no viable *FANCD2*$^-$ *GFP*$^-$ cells were obtained in the *ADH5*$^{-/-}$ background (**Fig. 2d**). Finally, we observed a clear induction of γ-H2AX in the *FANCD2*$^-$ *GFP*$^-$ *ADH5*$^{-/-}$ cells but only a marginal increase in *FANCD2*$^-$ *GFP*$^-$ *ADH5*$^{+/-}$ cells (**Fig. 2e**).

In order to address whether the synthetically lethal interaction observed between *FANCD2* and *ADH5* was specific to the downstream component *FANCD2* or generalizable to core complex components, we generated a transcriptionally repressible *FANCL* knockout strain (**Fig. 3a**). The *ADH5*$^{+/-}$ cell was modified to express a *TAP-FANCL* transgene driven by a tetracycline-repressible promoter (*TetO*::*TAP-FANCL*). Both alleles of *FANCL* were knocked out in this strain, giving a *FANCL*$^{-/-}$*ADH5*$^{+/-}$ *TetO*::*TAP-FANCL* strain. We then proceeded to disrupt the second allele of *ADH5*, yielding the strain *FANCL*$^{-/-}$ *ADH5*$^{-/-}$ *TetO*::*TAP-FANCL*. Extinction of *TAP-FANCL* expression, and thus inactivation of the FA pathway, was observed upon addition of doxycycline to the culture medium. Within 2 d of doxycycline addition, TAP-FANCL expression was greatly diminished (**Fig. 3b**). This coincided with a cessation of proliferation and a marked decrease in viability only among cells deficient for *ADH5* (**Fig. 3c**). To quench endogenously produced formaldehyde, we exploited the chemistry between β-mercaptoethanol (β-ME) and aldehydes. Formaldehyde reacts readily with the thiol group of β-ME, giving a less reactive and more stable product, 2-((hydroxymethyl)thio) ethanol (**Supplementary Fig. 5**). Addition of 100 μM β-ME to the growth medium rescued the defect in cell proliferation and improved viability after repression of *TAP-FANCL* in *ADH5*$^{-/-}$ cells (**Fig. 3d**). Finally, using γ-H2AX as a marker of DSBs, we noted that cells lacking *ADH5* greatly induced γ-H2AX upon *FANCL* repression (**Fig. 3e**), in comparison to the *ADH5*-proficient cells. As predicted, addition of 100 μM β-ME suppressed this accumulation.

To summarize, we have shown by two independent approaches that inactivation of formaldehyde catabolism by disruption of *ADH5* results in synthetic lethality with upstream (*FANCL*) or downstream (*FANCD2*) components of the FA pathway. Our observation that FA-deficient chicken DT40 cells have an essential requirement for *ADH5* (and hence formaldehyde catabolism) contrasts with the requirement for *ALDH2* (encoding the enzyme that catabolizes acetaldehyde),

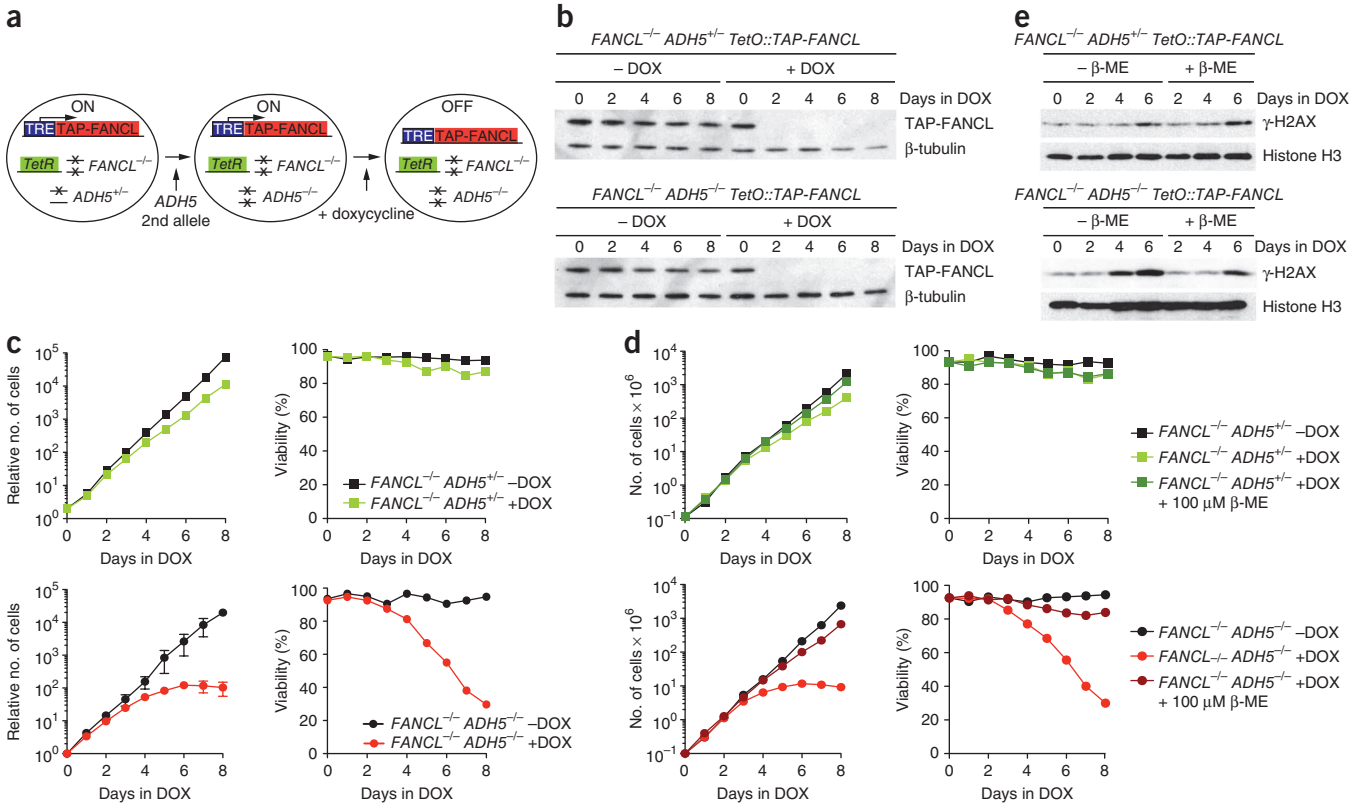

Figure 3 The FA core complex gene *FANCL* and *ADH5* are synthetically lethal in DT40 cells. (**a**) Strategy for conditional *FANCL* repression in *ADH5*-null DT40 cells by addition of doxycycline. (**b**) Conditional depletion of TAP-FANCL protein in doxycycline (DOX)-treated *FANCL*$^{-/-}$ *ADH5*$^{+/-}$ *TetO::TAP-FANCL* or *FANCL*$^{-/-}$ *ADH5*$^{-/-}$ *TetO::TAP-FANCL* cells. Whole cell extracts were western blotted with anti-tubulin IgG antibody (Sigma-Aldrich), which cross-reacts with TAP-FANCL. (**c**) Growth curves and viability plots of *FANCL* conditionally repressed cells in *ADH5*$^{+/-}$ and *ADH5*$^{-/-}$; each point represents mean values of two independent experiments. Error bars, s.e.m. (**d**) Growth curves of viable cells and viability plots of conditionally repressed *FANCL*$^{-/-}$ *ADH5*$^{+/-}$ or *ADH5*$^{-/-}$ *TetO::TAP-FANCL* cells in the presence of 2-mercaptoethanol (β-ME); each point represents mean values of two independent experiments. Error bars, s.e.m. (**e**) Induction of γ-H2AX following *FANCL* repression in *ADH5*$^{-/-}$ DT40 cells and its suppression following addition of β-ME.

whose absence is not synthetically lethal[9]. Thus, endogenous formaldehyde appears to be either more abundant and/or more potently genotoxic than acetaldehyde. It is striking that the full complement of FA genes is only found in higher eukaryotes; perhaps the expansion of FA genes coevolved with multicellularity and the emergence of stem cell pools[17,18]. Because we know that formaldehyde is produced as a byproduct of histone demethylation, our results lead us to predict that certain cell lineages with a propensity for extensive histone modifications might be particularly reliant on FA pathway repair. Endogenous formaldehyde genotoxicity might therefore explain the progressive attrition of germ and blood stem cells in FA.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

We thank N. Adachi and H. Koyama (Kihara Institute for Biological Research, Japan) for kindly providing NALM-6 human cell lines. We are grateful to J. Sutherland and K. Lang (MRC Laboratory of Molecular Biology) for chemical insight and to M. Daly and F. Zhang (MRC Laboratory of Molecular Biology) for invaluable help with flow cytometry. I.V.R. and F.L. are funded by the Fanconi Anaemia Research Fund and Children's Leukaemia Trust, respectively.

AUTHOR CONTRIBUTIONS

I.V.R. and K.J.P. designed the study and the experiments, and wrote the paper. I.V.R. performed the majority of the experiments presented. F.L. contributed to DT40 clonogenic assays and assisted in the generation of *ADH5*-deficient cell lines. G.P.C. helped with analysis of chromosome breaks. M.T. generated and provided the FANCD2 inducible cell line.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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