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Citation for published version:
https://doi.org/10.1002/path.4869

Digital Object Identifier (DOI):
10.1002/path.4869

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
The Journal of Pathology

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Effects of long-term ethanol consumption and Aldh1b1 depletion on intestinal tumourigenesis in mice

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Word count of abstract: 281

Word count of main text (introduction – discussion): 3999

Keywords: colorectal cancer, intestine, alcohol, Aldh, acetaldehyde, proliferation, DNA damage, EUCOMM
Abstract

Ethanol and its metabolite acetaldehyde have been classified as carcinogens for the upper aerodigestive tract, liver, breast and colorectum. Whereas mechanisms related to oxidative stress and Cyp2e1 induction seem to prevail in the liver and acetaldehyde has been proposed to play a crucial role in the upper aerodigestive tract, pathological mechanisms in the colorectum have not been clarified yet. Moreover, all evidence for a pro-carcinogenic role of ethanol in colorectal cancer is derived from correlations observed in epidemiological studies or from rodent studies with additional carcinogen application or tumour suppressor gene inactivation. In the current study, wildtype mice and mice with depletion of Aldehyde dehydrogenase 1b1 (Aldh1b1), an enzyme which has been proposed to play an important role in acetaldehyde detoxification in the intestines, received ethanol in drinking water for one year. Long-term ethanol consumption led to intestinal tumour development in wildtype and Aldh1b1-depleted mice, but no intestinal tumours were observed in water-treated controls. Moreover, a significant increase in DNA damage was detected in the large intestinal epithelium of ethanol-treated mice of both genotypes compared with the respective water-treated groups, along with increased proliferation of the small and large intestinal epithelium. Aldh1b1 depletion led to increased plasma acetaldehyde levels in ethanol-treated mice, to a significant aggravation of ethanol-induced intestinal hyperproliferation, and to more advanced features of intestinal tumours, but it did not affect intestinal tumour incidence. These data indicate that ethanol consumption can initiate intestinal tumourigenesis without any additional carcinogen treatment or tumour suppressor gene inactivation, and we provide evidence for a role of Aldh1b1 in protection of the intestines from ethanol-induced damage, as well as for both carcinogenic and tumour-promoting functions of acetaldehyde, including increased progression of ethanol-induced tumours.
Introduction

Alcohol is one of the most commonly used recreational drugs, which in 2002 was consumed by more than 1.9 billion adults worldwide, with 22% of men and 3% of women drinking 40 g ethanol or more per day [1]. Ethanol and its metabolite acetaldehyde have been classified as a group one carcinogens by the International Agency for Research on Cancer for the locations upper aerodigestive tract, liver, breast and colorectum [1].

We aim to further elucidate the role of ethanol in colorectal carcinogenesis. Evidence for a pro-carcinogenic function of ethanol in colorectal cancer (CRC) is mainly derived from epidemiological studies. A pooled meta-analysis of eight prospective cohort studies reported a multivariate relative risk of 1.41 for persons who consumed 45 g ethanol or more per day [2] (5.6 UK alcohol units), which is supported by two other meta-analyses of prospective cohort and case-control studies with similar results [3, 4]. Furthermore, CRC mutation analysis in the EPIC-Norfolk study revealed a significant association of high alcohol intake and p53 mutations in patients with advanced Dukes’ stage colorectal cancer [5] as well as a significant association of high alcohol intake and truncating mutations of adenomatosis polyposis coli (APC) [6].

Direct evidence for the genotoxicity of ethanol in the intestines along with mechanistic data from rodent models is scarce. Most data are derived from rodent studies where ethanol led to increased tumour formation when co-administered with a chemical carcinogen (reviewed in [1, 7]). Furthermore, two studies reported an increased polyp number and size after ethanol treatment of ApcMin mice, which are pre-disposed to intestinal tumour development due to a germline mutation in the Apc gene [8, 9]. However, there are presently no long-term ethanol consumption studies demonstrating intestinal tumour induction in rodents by ethanol treatment alone.
There is a growing body of mechanistic hypotheses for genotoxic as well as co-carcinogenic effects of ethanol in the intestines derived from human, rodent and tissue culture studies, which are systematically reviewed elsewhere [7]. Hypotheses include adverse effects of acetaldehyde, generation of free radicals, cytochrome P450 induction, increased efficiency of carcinogens and hyperproliferation. However, the exact mechanisms remain unclear and findings have been inconsistent.

This study focussed on the direct genotoxic effects of ethanol in vivo. DNA damage after ethanol consumption has been suggested to arise from reactive (oxygen and nitrogen) species, whose generation is mainly driven by cytochrome P450 2e1 (CYP2E1), as well as from DNA adducts of acetaldehyde [10, 11]. Ethanol is oxidised to highly reactive acetaldehyde by alcohol dehydrogenases (ADH) and also by CYP2E1 in the liver. Aldehyde dehydrogenases (ALDH) catalyse further oxidation of acetaldehyde to acetate. Systemic ethanol clearance occurs in the liver. The intestinal epithelium is exposed to acetaldehyde generated by mucosal ADHs and from the bloodstream as well as from the intestinal lumen, where acetaldehyde generation is linked to the intestinal microbiota [10-12]. Whereas oxidative stress is believed to be most important in the liver, there is also evidence for a role of CYP2E1 in intestinal oxidative stress after ethanol consumption [11, 13].

Strong evidence for a role for acetaldehyde in ethanol-related cancers comes from individuals with polymorphisms in ADH and ALDH enzymes that lead to increased acetaldehyde levels causing an increased cancer risk [12]. ALDH isoforms with highest affinity for acetaldehyde are ALDH2 (K_M<1µM) > ALDH1B1 (K_M 30µM) > ALDH1A1 (K_M 50-180 µM) [14]. ALDH2 is the predominant isoform involved in acetaldehyde detoxification in the liver. A common polymorphism of ALDH2 in Asians is linked to an increased risk of cancers of the upper aerodigestive tract and colorectum [12].
ALDH1B1 is highly expressed in the gastrointestinal epithelium and has been proposed to play an important role in acetaldehyde detoxification in the intestine [15, 16]. A polymorphism of ALDH1B1 with reduced activity [17] has been reported to be associated with drinking habits and alcohol sensitivity in Caucasians [18, 19]. Recently, Aldh1b1 knockout mice were found to have increased plasma acetaldehyde levels after a single dose of ethanol [16].

In order to find direct evidence for genotoxic effects of ethanol in the intestine, along with mechanistic insights into the roles of acetaldehyde and Aldh1b1, we examined tumourigenesis in wildtype mice as well as in mice with Aldh1b1 depletion after long-term ethanol treatment.
Materials and Methods

Long-term ethanol treatment of mice

All experiments described in this study were performed under a project licence issued under the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Aldh1b1<sup>tm2a(EUCOMM)Wtsi</sup> mice were sourced from the EUCOMM project [20] and maintained on a C57BL/6 background. The “knockout-first” allele tm2a contains a lacZ promoterless trapping cassette inserted into the intron of the Aldh1b1 gene and a LoxP site in exon 2, which disrupt gene function.

Homozygous Aldh1b1<sup>tm2a(EUCOMM)Wtsi</sup> mice (abbreviated to Aldh1b1<sup>tm2a</sup> hereafter) and wildtype littermates received either normal drinking water or ethanol in drinking water ad libitum for one year, starting at an age of 6-7 weeks (n=15 males and females in equal numbers). Ethanol treatment started with one month of 10% (v/v) ethanol in drinking water, followed by one month of 15% (v/v) ethanol in drinking water and 20% (v/v) ethanol in drinking water for the remaining ten months (Figure S1).

Western Blot analysis

Protein expression in intestinal epithelial cell lysates [21] was analysed by Western Blot using anti-Gapdh (#2118, Cell Signalling) and anti-Aldh1b1 (15560-1-AP, Proteintech) antibodies as detailed in Supplementary material and methods.

Acetaldehyde assay

Plasma was prepared from heparinised blood by 15 min centrifugation with 3,000g at 4°C immediately after withdrawal, snap-frozen on dry ice and stored at -80°C until analysis. Plasma acetaldehyde concentrations were determined using an enzymatic acetaldehyde assay kit (K-ACHYD, Megazyme) according to the manufacturer’s instructions.
Histology and immunohistochemistry

Immunohistochemistry using rabbit anti-Aldh1b1 (15560-1-AP, Proteintech), rabbit anti-Aldh2 (AB PB9472, Picoband), rabbit anti-Aldh1a1 (ab52492, abcam), rabbit anti-Ki67 (ab16667, abcam), rabbit anti-P53 (CM1, gift from Phil Coates, [22]), rabbit anti-phospho-histone H2A.X (Ser139) (γH2AX) (#2577, Cell Signaling) and rabbit anti-Cyp2e1 (ADI-100-MFO, Enzo Life Sciences) primary antibodies is described in Supplementary material and methods along with details on antibody validation and quantitative analysis of immunostaining signals.

Statistical analysis

Data were analysed using GraphPad Prism® 6 software (version 6.04) and are shown as either mean or dot plot with SD error bars. Groups were compared using unpaired, two-tailed Student’s t-test or Mann-Whitney test as appropriate, or two-way ANOVA with Bonferroni’s multiple comparisons correction test, as detailed in the figure legends. Tumour incidence is depicted as bar charts and was analysed using Fisher’s exact test. Differences between groups were considered significant if p ≤ 0.05.
Results

Ethanol treatment of mice

Ethanol administration in drinking water mimics human patterns of ethanol intake, but ethanol clearance in mice is five times faster than in humans, so a high ethanol consumption is required in order to observe the same toxicity and pathological effects as in humans [23-26]. C57BL6 mice have been shown to consume concentrations of up to 18% v/v ethanol voluntarily [27, 28], so the strain is well suited for ethanol administration in drinking water. In the present study, acceptance of ethanol in drinking water was good and mice consumed between 3.5-4.5 ml of liquid containing 10-20% v/v ethanol per day (Figure S2A). Whereas body weights of females did not differ, ethanol-fed wildtype and Aldh1b1tm2a males had significantly lower body weights than respective water-treated males starting from Week 27 in Aldh1b1tm2a and Week 35 in wildtype mice (Figure S2B, C).

Expression of ethanol-metabolising enzymes

Protein expression of the Aldh isoforms with highest affinity for acetaldehyde, Aldh2, Aldh1b1 and Aldh1a1 [14, 29], was analysed in the mouse intestines by immunohistochemistry. Aldh2 had a cytosolic expression pattern and was expressed ubiquitously in the small and large intestines (Figure 1A-D). Aldh1b1 expression in wildtype mice was mostly confined to intestinal epithelial cells. The granular, cytosolic immunostaining was strongest in crypt epithelial cells and diminished towards the villus tip in the small intestines (Figure 1E). A similar gradient was observed in the distal and mid large intestines, with the highest staining intensity in crypt bases, decreasing towards the surface (Figure 1G), whereas immunostaining intensity of intestinal epithelial cells was uniform along the crypt axis of the proximal colon (not shown). An opposite gradient was observed for Aldh1a1 expression. Aldh1a1 immunostaining in the small intestines was cytosolic, and was confined exclusively to villus epithelial cells with no expression in the
crypt epithelium (Figure 1I, J). Likewise, in the large intestines, Aldh1a1 expression was confined to epithelial cells of the upper half of the crypt of the proximal and mid colon, whereas crypt bases were devoid of immunostaining (Figure 1K, L). Staining intensity decreased from proximal to distal large intestine.

*Aldh1b1* murine intestines were mostly devoid of Aldh1b1 immunostaining. However, occasional single crypts and clusters of crypts in the small and large intestines were found to have a faint residual granular cytosolic immunostaining for Aldh1b1, which was strongest in crypts / crypt bases of the small / large intestines, thus exactly mimicking the expression pattern in wildtype murine intestines (Figure 1F, H). No compensatory up-regulation of Aldh2 or Aldh1a1 expression was observed by immunohistochemistry in *Aldh1b1* mice treated with water (Fig. 1B, D, J, L) or ethanol (not shown) and ethanol did not influence Aldh2, Aldh1b1 or Aldh1a1 immunostaining intensity in the small or large intestines of wildtype or *Aldh1b1* mice (not shown).

As the Aldh1b1 immunostaining pattern in *Aldh1b1* intestines suggested patchy residual expression, we analysed Aldh1b1 expression in isolated intestinal epithelium by Western Blot, loading four times as much protein of *Aldh1b1* than of wildtype samples in order to detect even low levels of residual protein expression. A weak band was observed, confirming very low level residual Aldh1b1 protein expression in *Aldh1b1* mouse intestinal epithelial cells (Figure 2), indicating that this mouse is a hypomorph rather than complete knockout of Aldh1b1 expression.

Cyp2e1 is involved in ethanol metabolism in the liver, where it is thought to play a central role in adverse ethanol effects, and a similar role in the intestines has been suggested [11, 13]. Immunohistochemical Cyp2e1 analysis verified enhanced Cyp2e1 protein expression in the ethanol-treated liver, as described in the literature [30]. We detected increased
Cyp2e1 immunostaining in livers of ethanol-treated mice, but only very faint immunostaining in the intestines with no apparent effect of ethanol or Aldh1b1 depletion (Figure S3).

**Effects of ethanol treatment on plasma acetaldehyde levels, tissue morphology, proliferation and DNA damage**

Adverse effects of acetaldehyde produced during ethanol metabolism are one of the proposed carcinogenic mechanisms of ethanol consumption [11]. ALDH1B1 is highly expressed in the human and murine intestines [15, 31] and has been suggested to play a role in intestinal ethanol metabolism [15, 16]. Aldh1b1 knockout mice have previously been shown to have higher plasma acetaldehyde levels than wildtypes after a single dose of ethanol [16], but no long-term ethanol treatments have been performed yet. By analysing Aldh1b1tm2a mice, we aimed to clarify the importance of Aldh1b1 in ethanol metabolism and to elucidate the role of acetaldehyde in intestinal damage related to ethanol consumption. Plasma acetaldehyde levels did not differ in water- and ethanol-treated wildtype mice, but there was a significant increase in plasma acetaldehyde levels in ethanol-treated Aldh1b1tm2a mice, compared with water-treated Aldh1b1tm2a mice and with ethanol-treated wildtype mice (Figure 3).

During histopathological analysis of H&E-stained sections of liver, pancreas, oesophagus, stomach and caecum of water- and ethanol-treated wildtype and Aldh1b1tm2a mice, no abnormalities were found to be associated with either genotype or treatment. Intestinal morphology was analysed using H&E-stained swiss rolls of the small and large intestines. In accordance with a previous analysis of untreated Aldh1b1 knockout mice [16], we found no abnormalities in water-treated wildtype or Aldh1b1tm2a mice. However, there was an increased number of branching or budding crypts in the large intestines of some ethanol-treated animals of both genotypes (Figure S4). There were no apparent differences in intestinal epithelial cells with apoptotic morphology [32] between the groups, but ethanol-
treated wildtype and Aldh1b1tm2a mice showed more mitotic cells in higher regions of the crypt-villus axis of the small intestines or crypt axis of the large intestines (representative images in Figure S4). To quantify effects on proliferation, the length of the zone of epithelial cells with nuclear immunostaining for the proliferation marker Ki67 in relation to the total crypt length was determined (Figure 4). There were no significant differences in intestinal Ki67-positive zones between any of the groups in the distal colon and rectum (not shown), however the proliferative, Ki67-positive zones in the proximal small and large intestines of ethanol-treated wildtype mice were significantly longer than in water-treated wildtype mice. This effect was more pronounced in ethanol-treated Aldh1b1tm2a mice, where the Ki67-positive zones were significantly longer compared with water-treated Aldh1b1tm2a in the proximal small and large intestines and also in the distal small intestines and mid colon. Moreover, the Ki67-positive zones in the proximal and distal small intestines and in the mid colon were significantly longer in ethanol-treated Aldh1b1tm2a mice compared with ethanol-treated wildtype mice, indicating a higher susceptibility of Aldh1b1tm2a mice to ethanol-induced intestinal hyperproliferation.

We further assessed C-terminally phosphorylated histone H2AX (termed γH2AX) immunostaining. This marker for DNA double-strand breaks arises quickly after DNA damage and can be detected for several months thereafter, making it a suitable long-term marker [33]. There were no differences in the small intestines, but a highly significant increase in γH2AX-positive nuclei was found throughout the epithelium of the large intestines of ethanol-treated wildtype and Aldh1b1tm2a mice compared with the respective water-treated groups (Figure 4).

**Tumour development**

Irrespective of genotype or treatment, 60-85% of females and 25-50% of males developed lymphoma (Figure S5), a common background lesion in the C57BL6 strain [34]. 4/14
wildtype mice and 4/15 Aldh1b1tm2a mice developed intestinal epithelial tumours after one year of ethanol treatment. One tumour was located in the proximal small intestine, whereas all other intestinal tumours in both groups were either found at the transition of the proximal colon to the mid colon or at the proximal end of the colon just after the caecum (Figure 5). One ethanol-treated Aldh1b1tm2a mouse developed hepatocellular carcinoma and one water-treated wildtype mouse was diagnosed with a benign squamous papilloma of the oesophagus during histopathological analysis. No tumours at other sites were found during necropsy or histopathological analysis. The effect of ethanol on intestinal tumour development was significant (p<0.05) in the wildtype and there was a trend (p=0.0996) in Aldh1b1tm2a mice. Ethanol effects on intestinal tumourigenesis were highly significant when combining both wildtype and Aldh1b1tm2a mice in order to analyse the ethanol effect irrespective of genotype (Figure 5, Table 1). There was no difference between ethanol-treated wildtype and Aldh1b1tm2a mice regarding tumour number or size (Figure S5).

Most intestinal tumours were adenomas with low-grade (mild to moderate) dysplasia. Some tumours of the ethanol-fed Aldh1b1tm2a mice displayed signs of focal high-grade dysplasia and showed foci of invasive adenocarcinoma (Figure S5). Adenomatous crypts were characterised by extensive nuclear Ki67 immunostaining. Many tumours had areas with widespread nuclear p53-immunostaining that showed an increased intensity compared with normal intestinal epithelium. These areas often contained more nuclei with γH2AX positive nuclei. (Figure 6, Figure S6)
Discussion

Rodent studies (overview in [1]) prompted the classification of ethanol as a carcinogen in experimental animals in different locations, including the colorectum. However, evidence for CRC was derived exclusively from rodent models with additional carcinogen treatment or genetic inactivation of tumour suppressor genes (e.g. Apc), whereas intestinal tumourigenesis after ethanol treatment alone has not been reported previously [7]. In the current study, we found for the first time that long-term ethanol treatment of mice leads to intestinal tumour development. Possible reasons why this has not been reported in previous studies include: i) ethanol treatment duration usually being much shorter than one year; and ii) less ethanol consumption in the few existing long-term studies despite the rapid ethanol elimination in rodents that necessitates higher ethanol consumption to observe the same physiological and pathological effects as in humans [26]. In addition, none of the publications mentioned that intestines were longitudinally opened, stained and analysed as whole mount specimens, so it is possible that intestinal tumours were missed.

Besides other mechanisms, the two predominant routes previously suggested to explain pro-carcinogenic properties of ethanol are related to acetaldehyde and to Cyp2e1 induction, which can both lead to genotoxic damage [1, 7, 35, 36]. Pro-carcinogenic functions of Cyp2e1 include generation of reactive oxygen species which can result in oxidative DNA damage, stimulation of proliferation, procarcinogen activation and perturbation of retinoic acid metabolism [11, 35]. Acetaldehyde leads to genotoxic damage via DNA adducts and induces hyperproliferation [37, 38]. N²-ethylidenedeoxyguanosine adducts (stabilised and detected by reduction to N2-ethyldeoxyguanosine) are abundant and weakly mutagenic and have been used as a biomarker for ethanol-induced DNA damage. N²-propanodeoxyguanosine adducts are less abundant and could not be detected in all biomarker studies of ethanol exposure, however they are highly mutagenic, causing DNA
inter- and intra-strand crosslinks as well as DNA-protein crosslinks. Mutations can also be caused by ethenobase adducts, related to lipid peroxidation stimulated by acetaldehyde or also by reactive oxygen species generated by Cyp2e1 [1, 37, 38]. Mechanisms related to Cyp2e1 and oxidative stress are mostly described in relation to ethanol-associated liver damage [35], whereas most evidence and mechanistic details relating to acetaldehyde are discussed for cancers of the upper aerodigestive tract [38]. Both mechanisms have been suggested to play a role in intestinal carcinogenesis [7, 11, 39].

Cyp2e1 is highly expressed in the liver, but protein expression in the intestine is controversial [40, 41]. Basal expression of Cyp2e1 protein has been reported in some studies for human [42] and rodent [13, 43, 44] colon and rat small intestines [45], whereas other studies found no evidence of Cyp2e1 protein expression in human or rodent colon [46, 47] or human [48, 49] or rodent [50] small intestines. Cyp2e1 protein induction by ethanol has been detected in rodent colon [13, 43, 45] and small intestines [45, 51] suggesting a role for Cyp2e1 in intestinal ethanol effects. We found increased Cyp2e1 immunostaining in ethanol-treated wildtype and Aldh1b1tm2al livers. However, we could not detect any notable differences in the intestines, so an involvement of Cyp2e1 in intestinal ethanol effects is unlikely in our model.

ALDH2 is crucial for acetaldehyde metabolism in human liver, and ethanol-treated Aldh2 knockout mice were found to have increased plasma acetaldehyde levels compared with wildtypes [52]. They were also shown to have higher N²-ethyldenedeoxyguanosine levels than wildtypes in the liver [53], stomach [54], oesophagus [55, 56], tongue and submandibular gland [55] after ethanol treatment. We have previously shown that mice with synchronous disruption of the Fanconi anaemia pathway and Aldh2 were most susceptible to ethanol-induced haematopoietic toxicity, with marrow aplasia and leukaemia resulting
from DNA damage in haematopoietic cells, and foetuses were more susceptible to ethanol teratogenicity [36, 57].

Aldh1b1 has been proposed to play an important role in acetaldehyde detoxification in the intestine [15, 16]. We observed the highest Aldh1b1 expression in the proliferative compartments of the murine small and large intestinal epithelium. Previously published figures [15] showed a similar pattern in large intestinal crypts and were interpreted as indicating stem cell positivity. It is tempting to speculate that Aldh1b1 might be particularly important for protection of the crypt basal stem cell and proliferating compartments of the intestinal epithelium (which is most prone to tumour formation) from genotoxic DNA damage by acetaldehyde after ethanol consumption. We have found increased plasma acetaldehyde levels in ethanol-treated Aldh1b1tm2a mice compared with ethanol-treated wildtypes in accordance with previous observations in Aldh1b1 knockout mice [16] and we hypothesise that Aldh1b1 deletion might also lead to locally increased acetaldehyde levels in the proliferative intestinal epithelium after ethanol consumption.

The gene trap approach of the knockout first strategy that was applied to generate Aldh1b1tm2a mice [20] can generate hypomorphs as well as complete gene knockouts, for example when alternative splicing occurs over the gene trap cassette [58-60]. In the case of the Aldh1b1tm2a allele, the entire protein coding region is contained in Aldh1b1 exon 2 and is not disrupted by either the transgene cassette or distal LoxP site, which explains the low level residual protein expression that we observed, indicating that this is a hypomorph with very low intestinal Aldh1b1 expression. Accordingly, Aldh1b1tm2a mice might not display the maximum effect of a complete Aldh1b1 deletion. However, residual expression in the intestines was at a very low level and only present in occasional single crypts or small patches of crypts and most of the intestinal epithelium was devoid of staining. Moreover, the increased plasma acetaldehyde levels indicated functional effects of Aldh1b1 marked
reduction, making Aldh1b1\textsuperscript{tm2a} mice a useful model for studying the role of acetaldehyde and Aldh1b1 depletion.

Increased γH2AX levels have been detected in ethanol-treated mice with a combination of Aldh2 deletion and a defective Fanconi anaemia pathway [36] and in acetaldehyde treated cells [61]. DNA double-strand breaks might arise from N2-propano-2'-deoxyguanosine DNA-acetaldehyde adducts after ethanol treatment, producing DNA interstrand crosslinks and DNA-protein crosslinks [37]. We detected a highly significant increase in nuclear γH2AX immunostaining by ethanol treatment in the entire large intestinal epithelium, but not in the small intestinal epithelium of wildtype and Aldh1b1\textsuperscript{tm2a} mice, indicating genotoxic damage after ethanol consumption. Furthermore, a co-localisation of Ki67, γH2AX and p53 immunostaining in adenomatous crypts reflected the DNA damage and response in ethanol-induced tumours. There was no genotype difference in γH2AX immunostaining, tumour incidence, number or size between ethanol-treated wildtype and Aldh1b1\textsuperscript{tm2a} mice. However, some intestinal tumours of Aldh1b1\textsuperscript{tm2a} mice had evidence of high-grade dysplasia, with focal invasive adenocarcinoma, whereas wildtype tumours displayed only low-grade dysplasia. The difference in local cellular acetaldehyde levels might not have been sufficient to result in large detectable differences in DNA damage, while acetaldehyde still affected intestinal proliferation and tumour progression.

Hepatic ethanol-induced hyperproliferation has been linked with increased activator protein-1 levels and retinoic acid depletion related to the induction of Cyp2e1 [11, 35, 62-64]. Stimulation of proliferation by ethanol has also been reported in the oesophagus [65], small intestines [66] and large intestines [66-68] of ethanol-treated rats as well as in human alcoholics [69], but has not been detected in the distal small intestine of ethanol-treated Apc\textsuperscript{Min} mice [8]. Increased acetaldehyde levels after ethanol consumption have been suggested to play a role in ethanol-induced hyperproliferation [70], as acetaldehyde
treatment of rats induced proliferation of the upper gastrointestinal tract mucosa [71] and large intestinal proliferation in ethanol-treated rats was found to correlate with mucosal acetaldehyde levels [68].

In the current study, we found histological features of increased intestinal epithelial proliferation after ethanol consumption, such as increased and ectopic mitotic figures and crypt branching or budding, and this was confirmed by observing longer Ki67-positive zones in ethanol-treated murine intestines, which were significantly longer in ethanol-treated Aldh1b1tm2a mice compared with ethanol-treated wildtypes. This increase in ethanol-induced intestinal hyperproliferation after depletion of the acetaldehyde-metabolising enzyme Aldh1b1 provides the first direct causal evidence for a role of acetaldehyde in intestinal hyperproliferation after ethanol consumption. It further shows that Aldh1b1 contributes to the protection of the intestinal epithelium from damage related to ethanol consumption.

In conclusion, we show for the first time that long-term ethanol consumption without additional carcinogen treatment or prior tumour suppressor gene inactivation, leads to intestinal hyperproliferation, DNA damage and intestinal tumourigenesis in wildtype and Aldh1b1-depleted mice. Interestingly, apart from one small intestinal tumour, all intestinal tumours were found in the mid and proximal colon, which are the same sites where ethanol-induced hyperproliferation was observed. Aldh1b1 depletion and the related increase in blood acetaldehyde (together with a likely local increase in acetaldehyde levels within the intestinal epithelium) aggravated both intestinal hyperproliferation and histopathological features of tumour progression, but did not affect tumour incidence. Thus, ethanol and its metabolite acetaldehyde appear to act as both carcinogen and tumour promoter – acetaldehyde acts at lower levels in wildtype and higher levels in Aldh1b1 depleted mice to promote intestinal hyperproliferation at lower and higher levels respectively, indicating a tumour promoter role for acetaldehyde. Ethanol / acetaldehyde acting as DNA damaging
carcinogen appears to have similar effects on intestinal tumour initiation in both wildtype and Aldh1b1 depleted mice. Furthermore, we provide evidence that increased levels of acetaldehyde (in Aldh1b1 depleted mice) appear to be important in intestinal tumour progression.
Acknowledgements

We thank KJ Patel for providing mouse models, P Coates for providing the p53 CM1 antibody, A Cederbaum, AP Kipp and N Oberbeck for providing FFPE specimens for antibody validation and H Caldwell and E McLay for technical support and sample processing.
Conflict of interest declaration

The authors declare that there are no conflicts of interest.
Abbreviations

ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase; Aldh1b1tm2a:
Aldh1b1tm2a(EUCOMM)Wtsi; APC: adenomatous polyposis coli; CRC: colorectal cancer;
CYP2E1: cytochrome P450 2e1; FFPE: formalin-fixed, paraffin-embedded; LI: large
intestines, SI: small intestines, WT: wildtype
Statement of author contributions

MJA and MFM conceived the experiments. MFM and YZ carried out experiments. MJA and MFM carried out histopathological analysis and MFM analysed the data and produced the figures. MFM and MJA wrote the manuscript. DJA provided the mouse model, gave experimental advice and revised the manuscript.
Reference list


### Tables

Table 1.

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<td>4/15 (27%)</td>
<td>11</td>
<td>8 large intestinal adenomas and 3 early adenocarcinomas of the large intestine</td>
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Table and figure legends

Table 1.

Tumours in wildtype and $Aldh1b1^{tm2a}$ mice after 1 year of ethanol- or water-treatment.

Figure 1.

Representative images of Aldh immunostaining of the small intestines (A, B, E, F, I, J) and large intestines (C, D, G, H, K, L) of water-fed wildtype (A, C, E, G, I, K) and $Aldh1b1^{tm2a}$ (B, D, F, H, J, L) mice: Aldh2 (A-D), Aldh1b1 (E-H), Aldh1a1 (I-L) (scale bar 50 µm). Images F and H were chosen to represent focal areas with residual staining in $Aldh1b1^{tm2a}$ intestines. They are not representative for the overall immunostaining of the intestinal epithelium, which was mostly blank. Dashed lines indicate outlines of crypts and villi. Arrows show residual immunostaining in F and H and lack of immunostaining in K and L.

Figure 2.

Representative Western Blot analysis images of Aldh1b1 and Gapdh protein expression in isolated intestinal epithelial cells of the large intestine (A) and small intestine (B) of mice treated with 20% (v/v) ethanol in drinking water or regular drinking water for three weeks. 5 µg of total protein were loaded for wildtype (WT) samples, whereas 20 µg protein were loaded for $Aldh1b1^{tm2a}$ (tm2a) samples in order to detect very low levels of residual Aldh1b1 protein expression in $Aldh1b1^{tm2a}$ mice. C) Illustration of the $Aldh1b1^{tm2a}(EUCOMM)Wtsi$ targeted $Aldh1b1$ gene with “knockout first cassette” in $Aldh1b1^{tm2a}$ mice. Modified image from [72, 73]. The protein coding region (light grey bar) is not interrupted by the loxP site within exon 2.
Figure 3.
Plasma acetaldehyde concentrations of water- and ethanol-treated wildtype (WT) and Aldh1b1tm2a mice. Data are shown as mean and SD error bars, n=7. Significant ethanol effects are denoted as ** p ≤ 0.01, and genotype effects are denoted as # p ≤ 0.05 as determined with unpaired, two-tailed Student’s t-tests.

Figure 4.
Representative images of Ki67 immunostaining of the small intestine (A-D) and colon (E-H) and of γH2AX immunostaining of the colon (I-L) of wildtype (A, B, E, F, I, J) and Aldh1b1tm2a (C, D, G, H, K, L) mice treated with water (A, C, E, G, I, K) or ethanol (B, D, F, H, J, L). Scale bar for A-D: 100µm. Scale bar for E-H: 50µm. Scale bar for I-L: 50µm. (M-P) Quantification of length of zone with Ki67-positive intestinal epithelial cell nuclei as percentage of total crypt length in wildtype and Aldh1b1tm2a mice treated with water or ethanol, in the proximal small intestine (M), distal small intestine (N), proximal colon (O) and mid colon (P). (Q) Quantification of nuclear γH2AX for the large intestine using a score from 0-3. Data are shown as dot plots with mean and SD error bars, n=14-15. Significant ethanol effects are denoted as * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and genotype effects are denoted as # p ≤ 0.05 as determined with unpaired, two-tailed Student’s t-tests.

Figure 5.
Analysis of intestinal tumours. Representative images of tumour analysis of the large intestine with (A) whole mount image of methylene blue-stained large intestine. Sequential images were combined to produce a whole-length image. White arrowheads indicate tumours (scale bar: 5mm). Magnification (B) of tumours indicated in “(A)” (scale bar: 1mm). (C) H&E sections of intestinal tumours (scale bar: 500µm). (D) Bar chart of number of
wildtype and \textit{Aldh1b1}^{tm2a} mice that developed intestinal tumours after receiving ethanol in drinking water or regular drinking water for one year. Fisher’s exact test was calculated to compare ethanol effects separately in wildtype mice (\( * p \leq 0.05 \)) and in \textit{Aldh1b1}^{tm2a} mice (\( p=0.0996 \)).

E) Bar chart of ethanol effects irrespective of genotype. Ethanol-treated wildtype and \textit{Aldh1b1}^{tm2a} mice were combined into one group and compared with water-treated wildtype and \textit{Aldh1b1}^{tm2a} mice using Fisher’s exact test (\( ** p \leq 0.01 \)).

**Figure 6.**

Representative immunostaining images of tumours of the large intestine of ethanol-treated \textit{Aldh1b1}^{tm2a} mice (A-F) and small intestine of an ethanol-treated wildtype mouse (G-I). Immunostaining for Ki67 (A, D, G), p53 (B, E, H) and \( \gamma \text{H2AX} \) (C, F, I) (scale bar: 100\( \mu \text{m} \)).

**Figure S1.**

Experimental setup for long-term ethanol treatment of mice. Each group consisted of 15 male and female \textit{Aldh1b1}^{tm2a} mice or 15 wildtype littermates that received either normal drinking water or ethanol in drinking water for one year starting at an age of 6-7 weeks. Ethanol treatment started with one month of 10\% (v/v) ethanol in drinking water, followed by one month of 15\% (v/v) ethanol in drinking water, and 20\% (v/v) ethanol in drinking water for the remaining ten months. *One ethanol-treated wildtype mouse was found dead at an early time point and was excluded from the analysis.

**Figure S2.**

A) Mean liquid consumption per day of mice receiving regular drinking water or ethanol in drinking water. Liquid consumption per mouse was calculated from drinking water use per
cage over the entire duration of the experiment (n=4-6 cages with 2-5 mice each per treatment and sex, wildtype and \textit{Aldh1b1}\textsuperscript{tm2a} littermates were housed together). Data are shown for males and females as mean and SD error bars. Differences were evaluated using two-way ANOVA with Bonferroni’s multiple comparisons test. (B, C) Body weights of water- and ethanol-treated wildtype (WT) and \textit{Aldh1b1}\textsuperscript{tm2a} (tm2a) mice over the course of the experiment. Group means and SD error bars are shown for females (B) and males (C), n=7-8. Differences were evaluated using two-way ANOVA with Bonferroni’s multiple comparisons test. Body weights of males were significantly different between water and ethanol-treated mice of the same genotype: 1) Body weights of ethanol-treated wildtype males were significantly lower than body weights of water-treated wildtype males in weeks: 35 (*), 41(*), 42 (*), 43 (*), 44(*), 45 (*), 46 (*), 48 (*), 49(**), 50 (**), 51 (**), 52 (**). 2) Body weights of ethanol-treated \textit{Aldh1b1}\textsuperscript{tm2a} males were significantly lower than body weights of water-treated \textit{Aldh1b1}\textsuperscript{tm2a} males in weeks: 27(*), 30(*), 31 (*), 32 (*), 33(*), 34 (*), 35 (*), 39 (*), 40 (**), 41(***), 42 (**), 43 (**), 44(***), 45 (*), 46 (**), 47 (*), 48 (*), 49(**), 50 (*), 51 (*), 52 (*). Significant ethanol effects are denoted as * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

**Figure S3.**

Representative images of Cyp2e1 immunostaining of the small intestine (A, B), large intestine (C, D) and liver (E, F) of wildtype mice treated with water (A, C, E) or ethanol (B, D, F). Scale bar for A, B, E, F: 100µm. Scale bar for C, D: 50µm.

**Figure S4.**

Representative H&E images of mitotic figures (arrows) in the upper part of large intestinal crypts (A) and small intestinal crypts (B); and (C) crypt branching or budding which was
observed in the large intestine of 4/14 wildtype and 2/15 Aldh1b1^tm2a_ mice treated with ethanol for one year (scale bar: 50µm).

**Figure S5.**

(A, B) Bar charts of lymphoma incidence in female (A) and male (B) mice. (C) Number of intestinal epithelial tumours per mouse after one year of water- or ethanol treatment of wildtype and Aldh1b1^tm2a_ mice. Data are shown as dot plots with mean, n=14-15. No significant differences were found between ethanol-treated wildtype and Aldh1b1^tm2a_ mice when analysing with Mann-Whitney test. (D) Diameter of intestinal tumours (mm) in ethanol-treated wildtype and Aldh1b1^tm2a_ mice. Data are shown as mean and SD error bars, n=7-8 (data on tumour size were not available for one ethanol-treated wildtype mouse with one intestinal tumour and one ethanol-treated Aldh1b1^tm2a_ mouse with 3 intestinal tumours). No significant differences were found between ethanol-treated wildtype and Aldh1b1^tm2a_ mice when analysing with unpaired, two-tailed Student’s t-test. (E, F) Representative H&E images of an ethanol-treated Aldh1b1^tm2a_ mouse intestinal tumour showing early invasion. (E) Image (scale bar 500µm) with area shown in magnification indicated by a box. (F) Magnification of the area highlighted in E (scale bar 50µm), with an arrow indicating early invasion through the muscularis mucosae (asterisks).

**Figure S6.**

Nuclear p53 immunostaining of intestinal tumours of ethanol-treated wildtype and Aldh1b1^tm2a_ mice. Tumours (n=17) were categorised by comparing p53 immunostaining in relation to adjacent normal intestinal epithelium as either normal level p53, widespread faint up-regulation of p53 in relation to normal tissue or widespread strong up-regulation of p53 in relation to normal tissue.