Alcohol Interacts with Genetic Alteration of the Hippo Tumor Suppressor Pathway to Modulate Tissue Growth in Drosophila

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Abstract

Alcohol-mediated cancers represent more than 3.5% of cancer-related deaths, yet how alcohol promotes cancer is a major open question. Using Drosophila, we identified novel interactions between dietary ethanol and loss of tumor suppressor components of the Hippo Pathway. The Hippo Pathway suppresses tumors in flies and mammals by inactivating transcriptional co-activator Yorkie, and the spectrum of cancers associated with impaired Hippo signaling overlaps strikingly with those associated with alcohol. Therefore, our findings may implicate loss of Hippo Pathway tumor suppression in alcohol-mediated cancers. Ethanol enhanced overgrowth from loss of the expanded, hippo, or warts tumor suppressors but, surprisingly, not from over-expressing the yorkie oncogene. We propose that in parallel to Yorkie-dependent overgrowth, impairing Hippo signaling in the presence of alcohol may promote overgrowth via additional alcohol-relevant targets. We also identified interactions between alcohol and Hippo Pathway over-activation. We propose that exceeding certain thresholds of alcohol exposure activates Hippo signaling to maintain proper growth control and prevent alcohol-mediated mis-patterning and tissue overgrowth.

Introduction

Alcohol consumption is a significant risk factor in cancers of tissues that contact or metabolize alcohol (for example, upper aerodigestive tract and liver cancers) and also in other tissues such as breast cancer [1-16]. Alcohol-associated cancers are responsible for more than 3.5% of all cancer deaths, yet how alcohol causes cancer remains a major open question. Drosophila are amenable to rigorous functional genetic analysis and descriptive phenotypic characterizations. Drosophila models have been established to explore the role of alcohol in health contexts including models of fetal alcohol syndrome and alcohol addiction [17-23] as well as cancer-relevant phenotypes such as tissue overgrowth [24-26] in a whole-animal model making it a particularly relevant system to investigate the relationship between alcohol and cancer where systemic responses could underlie the pathogenesis of disease.

We identified novel interactions between dietary ethanol and the Hippo Tumor Suppressor Pathway, a signaling network highly conserved from flies to mammals. The Hippo Pathway acts as a master regulatory pathway to restrict growth and proliferation and to promote apoptosis, and its disruption is implicated in a number of cancers [27-34]. Hippo (Hpo; Mst1 and Mst2 in mammals) [35-39] is the upstream kinase in a core cassette in which activated Hpo kinase associates with Salvador (Sav1 or hWW45 in mammals) [40,41] and phosphorylates and activates downstream effector kinase Warts (Wts; Lats1 and Lats2 in mammals) [40,42,43] and Wts co-activator Mats (Mob1 in mammals) [44]. Wts phosphorylates and inhibits transcriptional co-activator Yorkie (Yki; YAP and TAZ in mammals) [45], a potent oncogene. Components of this core cassette can be regulated by distinct upstream factors to define the eventual biological outputs. For example, GPCR signaling regulates Wts directly [46], Sik kinases regulate Sav [47], and a set of FERM-domain proteins including Merlin (Mer)
and Expanded (Ex) act upstream to activate Hpo by an as yet undefined mechanism [48].

We found that alcohol exposure enhanced overgrowth upon Hippo Pathway attenuation in multiple organs in Drosophila. Surprisingly, alcohol did not enhance overgrowth from over-expressing yki, suggesting the Hippo pathway may target yki-independent growth regulators that are alcohol-responsive. We also found that high doses of alcohol enhanced phenotypes of hpo over-expression. Our studies reveal multiple interactions between alcohol and the Hippo Pathway and suggest a previously undescribed role for Hippo signaling to prevent tissue overgrowth upon alcohol exposure.

Materials and Methods

Drosophila tools

RNAi was achieved using inverted repeat alleles from the Transgenic RNAi Project for hpo (P[TRIP.JF02740]attP2, referred to here as hpoIRN), for Mer (P[TRIP.JF02841]attP2, MerIRN), and for ex (P[TRIP.JF03120]attP2, exIRN), and from the National Institute of Genetics for yki (12072R-2, wtsIRN) and for yki (4005R-2, ykiIRN). Materials and Methods

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Quantification

Mosaic eyes were visually scored as: "+" for mild over-representation of mutant tissue (white) compared to wild-type tissue (red), "++" for moderate over-representation, and "+++" for strongest over-representation. To rule out unintentional observer bias, trials were conducted blind with lab members scoring eyes without knowledge of genotypes. Adult wings were mounted, photographed, and traced to give pixel area. For engal4 wings, we measured area posterior to vein L4. For c765gal4, total wing area is shown. Area comparisons utilized T-tests; mosaic eye and wing abnormality comparisons utilized chi-squared tests. The data shown in the figures are representative experiments which have been performed independently a minimum of three times.

Results and Discussion

Ethanol enhances tissue overgrowth associated with loss of the Hippo tumor suppressor

In an ongoing screen to elucidate the link between alcohol and cancer, we used the Gal4/UAS system [51] and the FLP/FRT system of mosaic analysis [49] to modulate levels of conserved growth regulators, tumor suppressors, and oncogenes in various tissues (eye, wing, etc.) in Drosophila exposed to a range of dietary ethanol. Wings from control flies (engal4) reared on food containing 1-10% ethanol generally showed no size increase, although consistent with previous reports [21] there were small but statistically significant size decreases in some cases (Figure 1A-B). Early in the wing screen, we identified the hpo tumor suppressor as a strong hit (Figure 1C-G). RNAI to hpo in the posterior wing increased size of this posterior compartment by more than 30% (Figure 1C-D,G). Posterior wing overgrowth was statistically significantly further increased by about 10% in flies reared on food containing 2-5% ethanol (Figure 1D-G) compared to hpo RNAI alone.

To establish if ethanol-mediated enhancement of tissue overgrowth was a wing-specific phenomenon or a more general tissue response, we examined the effects of dietary ethanol on hpo mutant eye tissue. Mosaic eyes containing control homozygous white wild-type tissue and homozygous
Figure 1. Dietary ethanol enhances overgrowth associated with loss of hpo. (A-B) Quantification of the effects of 1-10% ethanol on the posterior wing area (A) and whole wing area (B) of control flies (normalized to area of control wings on 0% ethanol). * indicates p=0.023995 for females at 5% and p=0.000633 for females at 10% in A and p=0.016029 for females at 5% and p=0.001122 for females at 10% in B. (C) Control wing. (D) hpo RNAi in the posterior wing. (E) Ethanol-mediated enhancement of wing overgrowth upon hpo knockdown. (F) Wing tracings from C-E highlight size changes. C-E, representative female wings. (G) Graph showing percent change in posterior wing area compared to controls at 0% ethanol for RNAi to hpo (black) and controls (blue) for food containing 0%, 1%, 2%, 5%, 7.5%, and 10% ethanol (normalized to areas of control flies treated with 0% ethanol). * indicates p=0.00224 (1%, females), p=0.00979 (2%, females), p=0.00589 (5%, females), p=0.00104 (7.5%, females), p=0.00730 (2%, males), p=0.02938 (5%, males), and p=0.01457 (7.5%, males). (H-J) Mutation in hpo (hpo<sup>MGH1</sup>) results in over-representation of mutant tissue (white) compared to wild-type tissue (red) [50]. Mosaic eyes fall into a range of over-representation from the mildest ratio scored as "*" (H) to moderate "**" (I) and severe "***" (J). (K) Ethanol enhanced over-representation of mutant tissue in a mosaic eye, noted by the increase in "**" and "***" eyes. This phenomenon was observed most strongly at 5% and 7.5% ethanol. Wild-type tissue in a mosaic eye serves as an internal control. We saw no gender differences; graph represents combined data for males and females. * indicates p=1.33E-10 (5%), p=3.97E-11 (7.5 %), and p=8.1E-6 (10%) compared to the same genotypes on 0%. (L-N) Images of wing discs expressing a GFP transgene under the control of engal4 in flies reared with 0%, 2% and 5% ethanol. Scale bar on wing disc images reflects 100 micrometers. (O) Graph reflecting quantification of GFP based on pixel intensity shows no change in GFP in flies reared on ethanol (p>0.05). (P) Control wing. (Q) Myc over-expression across the wing from a fly grown on control food. (R) Myc over-expression across the wing from a fly grown on food containing 5% ethanol. (S) Graph showing wing area for flies reared on 5% ethanol compared to controls at 0% ethanol for myc over-expression. Wing area is normalized to 100% for myc-expressing flies reared on 0% ethanol. Genotypes for this and subsequent figures are detailed in Materials and Methods. The number of flies analyzed in each experiment in this figure and subsequent figures is shown in parentheses on the base of each column. The bars in each graph in this figure and subsequent figures indicate Standard Deviation.

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Mosaic eyes containing tissue homozygous for hpo\textsuperscript{MGH1}, a strong hypomorphic allele, show over-representation of mutant tissue (white) compared to wild-type tissue (red) in a range of over-representation from mild (“+” = more white than red, about 65% of the eyes, Figure 1H) to moderate (“++” = strongly more white than red, about 20% of eyes, Figure 1I) to severe (“+++” = almost all white, about 10-15% of the eyes, Figure 1J).

Ethanol doses of 1-10% decreased the percentage of eyes in the mild “+” category and increased the percentage of eyes that were scored as moderate and severe (“++” or “+++”) (Figure 1K). At 5% ethanol, approximately 50% of eyes were severe (compared to less than 15% reared on 0% ethanol); at 7.5% ethanol, this increased to over 60% of eyes.

We find it unlikely that RNAi off-target effects or alcohol regulation of the GAL4/UAS system cause this phenomenon.
Figure 3. Ethanol promotes Hippo signaling in Drosophila. (A) Control wing. Representative wings over-expressing hpo reared on (B) 0% ethanol and (C) 10% ethanol. (D) Tracings of wings in A-C. (E) Quantification of the effects of ethanol treatment on wing area (normalized to control wing treated with 0% ethanol). Significant area effects were seen at 5% in some but not all experiments. * indicates p=0.0004738091994 (males, 5%), p=0.000003379435 (females, 10%), and p=5.50153E-11 (males, 10%). (F-G) Wings from flies reared on (F) 5% and (G) 10% ethanol showed increased patterning abnormalities. (H) Quantification of the effects of ethanol on proximal-distal positioning of the intersection of L2 and L3. Comparing control and Hpo over-expressing wings at 0% ethanol, * indicates p=0.0132429375674621 (females) and p=0.00003467107 (males). Comparing Hpo over-expressing wings at 0% to 5%, * indicates p=0.000391195433611 (females), p=0.03843393 (males). Comparing Hpo over-expressing wings at 0% to 10%, * indicates p=0.0000000059140932 (females) and p=0.000442676 (males). (I) Quantification of weakening and loss of segments within L2, L3, and L4 longitudinal veins. Comparing control and Hpo over-expressing wings at 0% ethanol, * indicates p=2.45092E-22 (females) and p=0.00000659349 (males). Comparing Hpo over-expressing wings at 0% to 5%, * indicates p=0.02424 (males), Comparing Hpo over-expressing wings at 5% to 10%, * indicates p=0.0550172 (females). Comparing Hpo over-expressing wings at 0% to 10%, * indicates p=0.0001108737 (females) and p=0.04258 (males). (J) Control wing. (K-L) Representative wings undergoing RNAi to yki. Obvious wing size reduction is observed at 21°C (K) which increases at the higher temperature of 25°C. (M) Overlay of tracings of wings in J-L. (N) Representative wing undergoing RNAi to yki reared on 10% ethanol. (O) Overlay of tracings of wings in (K) and (N). (P) Quantification of the effects of ethanol treatment on wing area at 21°C (normalized to control wing treated with 0% ethanol). * indicates p=0.00433037 (females), p=0.030839921 (males). Wings shown are from females.

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the phenomenon is not specific to wing tissue but may represent a folding prevented accurate quantification. We therefore used enhanced over-representation of yki and hpo knockdown in the posterior wing (Figure 2A). Additionally, significantly enhanced overgrowth upon ex RNAi (Figure 2B) indicates that alcohol could reflect interaction with alcohol-exposed signaling through the pathway or could result from the complicated way in which upstream inputs direct pathway outputs. Signaling through the pathway is complex and not strictly linear; for example, Ex promotes signaling through Hpo [48] and also binds and inhibits Yki directly [52]. Loss of Mer or ex singly has distinct phenotypes from loss of core components hpo or wts [53]. However, simultaneous loss of Mer and ex phenocopies loss of hpo or wts in other contexts [48]. The interaction between alcohol and ex knockdown but not Mer knockdown can be further resolved as future work in the field elucidates how upstream factors activate the pathway to define distinct biological outputs.

To address downstream components, we examined knockdown of wts and over-expression of yki. RNAi to wts in the posterior wing with enga4 led to such overgrowth that wing folding prevented accurate quantification. We therefore used weaker pan-wing driver c765gal4. Ethanol enhanced wing overgrowth from RNAi to wts in females (Figure 2C).

Over-expressing yki in the posterior wing or across the whole wing promotes tissue overgrowth. We tested conditions that led to overgrowth similar to loss of hpo seen in Figure 1 for wild-type yki transgene UAS-ykiV5. Surprisingly, ethanol did not reproducibly enhance ykiV5-mediated overgrowth (Figure 2D). Therefore we tested a distinct wild-type yki transgene, UAS-FLAGyki. As with RNAi to wts, overgrowth produced by the FLAGyki transgene using enga4 was too extensive to quantify, so we assessed overgrowth with c765gal4. Consistent with our findings for ykiV5, dietary ethanol did not enhance FLAGyki-mediated overgrowth (Figure 2E). Because wild-type versions of yki would be subject to inhibition by endogenous Hippo signaling, we also tested the transgene UAS ykiSI168AGFP.HA. The S168A mutation cannot be phosphorylated by Wts at the 14-3-3 site, so is insensitive to Wts-induced inactivation via translocation out of the nucleus [54]. Dietary ethanol did not enhance the overgrowth due to ykiSI168AGFP.HA expression (Figure 2F). If alcohol-mediated enhancement of overgrowth upon loss of ex, hpo, or wts occurs via interaction with Yki protein or its targets, we would predict alcohol to enhance Yki over-expression-induced overgrowth. Therefore, our genetic interaction studies suggest that alcohol may interact with Hippo signaling at or downstream of hpo and wts, possibly parallel to yki. This is particularly unexpected because signaling through Yki is reported to be crucial to overgrowth from loss of Hippo Pathway tumor suppression. Our findings may reveal a role for Yki-independent Hippo Pathway targets in promoting growth upon alcohol exposure. Alternatively, alcohol may be acting in a less straightforward way to regulate Yki protein not revealed by our wild-type and mutant Yki over-expression studies.
wing patterning and wing size) did not enhance the wing size reduction due to RNAi of yki (Figure 3N-P). This may suggest that alcohol further enhances Hpo-mediated growth regulation through targets other than yki.

A role for Hippo Pathway Tumor Suppression in response to stress?

If Hippo signaling is activated by alcohol as part of a stress response, it seems reasonable to speculate that Hippo signaling is acting to prevent some of the deleterious effects of alcohol. If this is the case, then impairing Hippo signaling in the presence of alcohol would mean those deleterious effects of alcohol would take place. Taking our findings into account with this logic, we propose that in addition to tissue homeostasis regulated by Hippo signaling under normal conditions (Figure 4A), alcohol does not promote the overgrowth of Drosophila eye and wing tissues in part because alcohol promotes activation of Hippo signaling (Figure 4B). However, in contexts where Hippo signaling is impaired and tissue undergoes established Yki-dependent overgrowth (Figure 4C), we propose alcohol can then also act to promote additional tissue overgrowth, possibly by interacting with Yki-independent targets of Hippo signaling (Figure 4D) because yki over-expression is not sufficient to recapitulate this phenomenon.

The ability of alcohol to promote Hippo signaling in flies and for oxidative stress to promote Hippo signaling in mammalian systems [55-58] suggests there may be a highly conserved role for stress to activate the Hippo Pathway. This raises interesting questions regarding the origins of Hippo signaling to respond to alcohol. We speculate that activation of Hippo signaling by alcohol developed in a common ancestor as a mechanism to maintain proper patterning and growth regulation even upon exposure to environmental stressors; maintenance of this mechanism may have been selected for in species like Drosophila that develop in the presence of alcohol, such as on fermenting fruit or other species that encounter environmental exposure to related stressors, to prevent stress-mediated tissue overgrowth.

Our findings that alcohol enhances both Hippo Pathway loss of function and over-expression phenotypes are consistent with a role for dietary alcohol (i.e. whole animal exposure) to have specific effects on target organs undergoing Hippo Pathway modulation. This could be particularly relevant in prescribing lifestyle changes and for designing therapies for patients depending on whether or not their tumors maintain intact Hippo signaling. Moreover, our findings suggest that impaired Hippo Pathway tumor suppression may underlie the pathogenesis of specific alcohol-mediated cancers. Notably, there is striking overlap between Hippo-associated cancers [27-34] and alcohol-associated cancers, including colorectal, liver, and breast cancers [59-64].

Yki is the best-characterized target of the Hippo Pathway; its homologs YAP and TAZ are widely accepted to play an important role in cancer. Therefore, we were surprised that alcohol did not enhance tissue overgrowth upon yki over-expression. Alcohol may act in a novel way to promote Yki accumulation or activity. Indeed, alcohol promoted Hippo signaling which normally antagonizes yki; this could have
masked alcohol-mediated enhancement of wild-type yki phenotypes and resulted in the lack of observed interaction between dietary ethanol and wild-type yki over-expression (Figure 2D). However (1), we did not observe any significant phenotypic enhancement of over-expressing hpo at dietary alcohol concentrations of 2% (not shown), where we did not see interactions between yki over-expression and ethanol and (2) alcohol did not enhance the organ size reduction caused by knockdown of yki. Therefore, we believe the simplest model to explain our data is that in the range of alcohol concentrations tested in our study, alcohol interacts with Yki-independent targets of Hpo and/or Wts. Our report may represent another context of Hippo Pathway functions that are Yki-independent including reported roles in F-actin regulation [65] and polarity [66]. Moreover, controversial reports propose YAP acts as a breast cancer tumor suppressor [67-69]. Thus, Hippo Pathway targets parallel to YAP could be particularly relevant to alcohol-mediated breast cancers.

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