Rsu1 regulates ethanol consumption in *Drosophila* and humans

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Alcohol abuse is highly prevalent, but little is understood about the molecular causes. Here, we report that Ras suppressor 1 (Rsu1) affects ethanol consumption in flies and humans. *Drosophila* lacking Rsu1 show reduced sensitivity to ethanol-induced sedation. We show that Rsu1 is required in the adult nervous system for normal sensitivity and that it acts downstream of the integrin cell adhesion molecule and upstream of the Ras-related C3 botulinum toxin substrate 1 (Rac1) GTPase to regulate the actin cytoskeleton. In an ethanol preference assay, global loss of Rsu1 causes high naïve preference. In contrast, flies lacking Rsu1 only in the mushroom bodies of the brain show normal naïve preference but then fail to acquire ethanol preference like normal flies. Rsu1 is, thus, required in distinct neurons to modulate naïve and acquired ethanol preference. In humans, we find that polymorphisms in *RSU1* are associated with brain activation in the ventral striatum during reward anticipation in adolescents and alcohol consumption in both adolescents and adults. Together, these data suggest a conserved role for integrin/Rsu1/Rac1/actin signaling in modulating reward-related phenotypes, including ethanol consumption, across phyla.

addiction | alcohol | genetics | actin

Alcohol consumption has a worldwide prevalence of 42% (1), and alcohol is the third most serious risk factor for health loss worldwide (2). The genetic contribution to the development of alcohol use disorders (AUDs) has been estimated at 40–60% based on family, adoption, and twin studies (3, 4). Although several studies in humans and model organisms have described genes and molecular pathways involved in alcohol responses (5, 6), our molecular understanding of how AUDs develop is still incomplete.

The vinegar fly, *Drosophila melanogaster*, is a genetically tractable organism used to model addiction-relevant, ethanol-induced behaviors (7, 8). When exposed to ethanol vapor, flies display biphasic behaviors similar to those elicited in humans. Low ethanol doses induce a state of disinhibition and increased locomotor activity, whereas higher doses lead to loss of postural control and sedation (9, 10). Flies also display addiction-like behaviors similar to mammals. In an ethanol consumption and preference assay (11), for example, flies gradually acquire alcohol preference and will overcome an aversive stimulus to consume alcohol (12).

In addition to the similarities that mammals and flies display in their behavioral responses to ethanol, numerous genes and signaling pathways affect alcohol-induced behaviors across organisms. In vitro and in vivo studies in *Drosophila* and mammals

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A complete list of The IMAGEN Consortium can be found in the *SI Appendix*.

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Significance

Genetic factors play a major role in the development of human addiction. Identifying these genes and understanding their molecular mechanisms are necessary first steps in the development of targeted therapeutic intervention. Here, we have isolated the gene encoding Ras suppressor 1 (Rsu1) in an unbiased genetic screen for altered ethanol responses in the vinegar fly, *Drosophila melanogaster*. Our behavioral, genetic, and biochemical experiments show that Rsu1 links signaling from the integrin cell adhesion molecule to the small GTPase Rac1 in adult neurons to regulate actin dynamics and alcohol consumption preference. We also show that variants in human *RSU1* associate with altered drinking and brain activation during a reward prediction task, thereby validating the predictive power of our approach.

have revealed a link between alcohol and the actin cytoskeleton (13). When cultured primary mouse neurons are exposed to ethanol, there is a gradual decay in filamentous actin that correlates with decreased NMDA receptor current (14). Mice with a genetic KO of the actin-capping protein epidermal growth factor receptor kinase substrate 8 (EPS8), which displays reduced decay of both filamentous actin and NMDA receptor current in the presence of acute ethanol, show increased alcohol preference (14). Flies with mutations in the *arouser* gene, encoding an EPS8 homolog, also show an ethanol sensitivity phenotype (15).

A major regulator of actin cytoskeleton dynamics is the Rho family of small GTPases, including Rho, Rac, and Cdc42, and mutations in these genes affect alcohol-induced behaviors (13). Adult loss of Ras-related C3 botulinum toxin substrate 1 (Rac1) activity, for example, leads to enhanced sensitivity to alcohol-induced sedation, whereas loss of the Rac1 down-regulator RhoGAP18B causes reduced sensitivity (16). Although these studies have shown that Rho family GTPases play a role in alcohol responses, the upstream signaling pathways modulating their effects on actin cytoskeletal dynamics are not understood.

Here, we describe the identification and characterization of mutations in the *icarus* (*ics*) gene encoding Ras suppressor 1 (Rsu1), which exhibits reduced sensitivity to ethanol-induced sedation. Our experiments reveal that *ics* mediates normal behavioral responses to ethanol in the adult nervous system by regulating actin dynamics downstream of integrin and upstream of the Rac1 GTPase. Although WT flies gradually acquire ethanol consumption preference over several days, flies completely lacking Rsu1 show heightened naïve preference that does not increase further over the time of the assay. Conversely, flies lacking Rsu1 only in the mushroom bodies (MBs) show no naïve preference and also, fail to acquire preference over time, suggesting that distinct neural circuits mediate naïve and acquired ethanol preference. In humans, *RSU1* was associated with frequency of lifetime drinking in an adolescent sample and alcohol dependence in an independent adult replication sample. In adolescents, *RSU1* was also associated with altered functional MRI activation in the ventral striatum (VS) during reward anticipation. Our findings, thus, highlight Rsu1 and the integrin/Rsu1/Rac1 signaling pathway as important modulators of reward-related phenotypes, including ethanol consumption across phyla.

Results

***ics* Mutants Display Reduced Sensitivity to Ethanol-Induced Sedation.** To identify genes involved in ethanol-induced behaviors in *Drosophila*, we screened a collection of strains carrying random insertions of a transposable P element. We isolated one mutant that displayed reduced sensitivity to ethanol-induced sedation compared with controls (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). DNA sequencing analysis revealed that the Gal4-containing P element in this line is inserted in the *ics* gene, and we, thus, labeled it ics^{G4}. The *ics* gene had been previously identified because of its wing blister phenotype (17), and ics^{G4} mutant flies also exhibited wing blisters. The original mutant, ics^{BG}, carrying a P element insertion at the 3′ end of *ics* exon 3 (*SI Appendix*, Fig. S1*D*) showed reduced sensitivity to ethanol-induced sedation similar to that of ics^{G4} (*SI Appendix*, Fig. S1*A*). Heterozygous *ics* flies showed no phenotype and were used as controls in some of the experiments below. To confirm that the transposon inserted in ics^{G4} was, indeed, responsible for the ics^{G4} ethanol phenotype, we mobilized the ics^{G4} P element by supplying the transposase enzyme. Precise excision (ics^{x23}) of the P element reverted the mutant phenotype to the WT, whereas imprecise excision of the P element (ics^{x5}; resulting in a deletion of 1,353 bp) (*SI Appendix*, Fig. S1*D*) showed the *ics* mutant phenotype (*SI Appendix*, Fig. S1*B*). Expression of the Rsu1 protein was absent in *ics* mutants (ics^{G4} and ics^{x5}) and normal in the precise excision ics^{x23} (*SI Appendix*, Fig. S1*C*). The reduced ethanol sensitivity in *ics* mutants was not caused by altered pharmacokinetics, because ethanol absorption and metabolism were normal in ics^{G4}, ics^{BG}, and ics^{x5} flies compared with controls (*SI Appendix*, Fig. S1*E*). Flies carrying mutations in *ics* also showed normal locomotion (assessed by startle-induced phototaxis and negative geotaxis as well as spontaneous daily locomotion). These results suggest that *ics* mutations affect ethanol-induced behavior without generally disabling the flies.

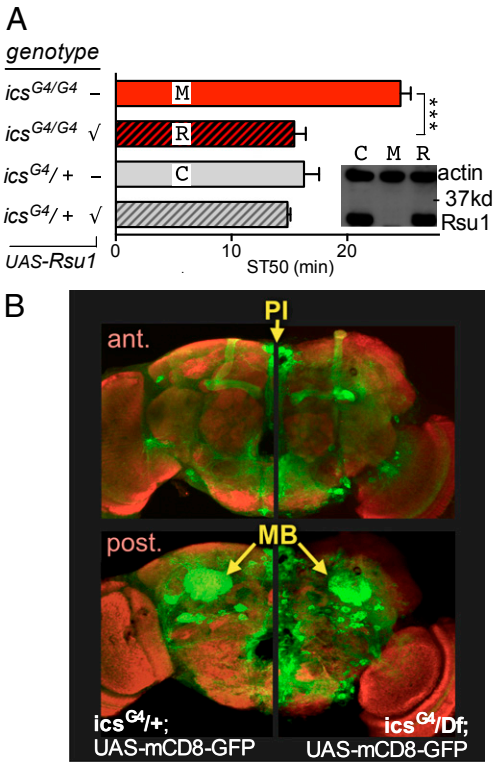


Fig. 1. *ics*, Encoding Rsu1, is required for normal ethanol responses. Here, flies were exposed to a 130:20 ethanol:airflow rate, and bars represent means ± SEM. ST50 stands for the median sedation time; increased ST50 indicates reduced ethanol sensitivity. (*A*) Mutant ics^{G4} flies show reduced sensitivity to ethanol-induced sedation. This phenotype and (*Inset*) the loss of Rsu1 protein are rescued with expression of Rsu1 cDNA (*UAS-Rsu1*; transgene presence indicated by √; $n = 8$). C, control; M, mutant; R, rescue. ***$P < 0.001$. (*B*) Brain expression pattern of ics^{G4} revealed by a membrane-bound GFP reporter (*UAS-mCD8-GFP*; green). *B* shows (*Upper*) anterior (ant.) and (*Lower*) posterior (post.) confocal stacks of ics^{G4} (*Left*) heterozygous WT and (*Right*) homozygous mutant flies. Expression includes neurosecretory cells in the pars intercerebralis (PI) as well as the MBs. Neuropil is counterstained with anti-Brp nc82 antibody (red).

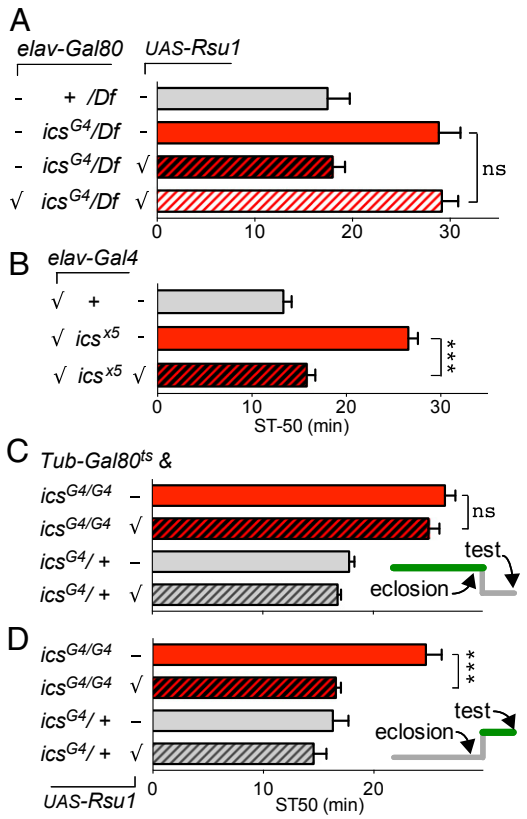


Fig. 2. Rsu1 is required in the adult nervous system for normal ethanol responses. (*A*) Suppression of Gal4 and *UAS-Rsu1* expression in the nervous system with *elav-Gal80* abrogates the behavioral rescue ($n = 6$–7). *Df*, genetic deficiency *Df(2L)BSC147* completely removing the *ics* gene locus; ns, not significant ($P > 0.91$). (*B*) Rsu1 expression exclusively in the nervous system through *elav^{c155}-Gal4* completely rescues the reduced ethanol sensitivity phenotype of *ics^{x5}* mutant flies. ***$P < 0.001$ ($n = 7$–9). (*C* and *D*) Adult expression (*D*) posteclosion but (*C*) not throughout development rescues the reduced ethanol sensitivity phenotype of *ics^{G4}* mutant flies. *UAS-Rsu1* expression was suppressed using ubiquitously expressed Gal80^{ts}, which inhibits Gal4 (and therefore, Rsu1 expression) at (*Inset*) 18 °C (gray) but not 29 °C (green). Flies were kept for 3 d at the test temperature before ethanol exposure. ns, Not significant ($P > 0.29$); ST50, median sedation time. ***$P < 0.001$ ($n = 6$–9).

Rsu1 Is Required in the Adult Nervous System for Normal Ethanol Sensitivity. To confirm that the reduced ethanol sensitivity of *ics* mutants was caused by loss of Rsu1 protein expression, we restored expression of Rsu1 by using the Gal4/UAS system (18) and introducing a *UAS-Rsu1* cDNA transgene. We drove expression of Rsu1 in *ics^{G4}* mutant flies by taking advantage of the transcriptional activator Gal4 contained within the inserted P element, which disrupts Rsu1 expression while also expressing Gal4 under the control of the endogenous *ics* promoter and enhancers. Homozygous *ics^{G4}* flies carrying the Gal4-transactivated *UAS-Rsu1* transgene showed WT ethanol sensitivity and restoration of WT Rsu1 protein expression levels (Fig. 1*A*). *ics^{G4}* drove expression of a *UAS-GFP* reporter in the brain, including in the MBs and neurosecretory cells of the pars intercerebralis; there were no obvious differences between *ics^{G4}* mutant and WT flies (Fig. 1*B*).

To investigate if *ics^{G4}*-driven expression in the nervous system was necessary for normal ethanol responses, we suppressed the expression of the *UAS-Rsu1* cDNA in neurons using a pan-neuronal inhibitor of Gal4, *elav-Gal80* (19). Neuronal suppression of Rsu1 expression prevented rescue of the *ics^{G4}* phenotype by the *UAS-Rsu1* transgene (Fig. 2*A*). To ask whether exclusive expression of Rsu1 in the nervous system was sufficient to rescue the *ics* mutant phenotype, we used the neuron-specific driver *elav-Gal4* to drive expression of *UAS-Rsu1* in the *ics* mutant background. As shown in Fig. 2*B*, reduced ethanol sensitivity of *ics^{x5}* was restored to WT levels when we expressed Rsu1 exclusively in neurons. Taken together, these data indicate that Rsu1 functions in the nervous system to regulate ethanol-induced behavior.

Neurons expressing Gal4 in *ics^{G4}* mutant brains seemed no different from behaviorally normal *ics^{G4}*/+ heterozygotes (Fig. 1*B*), suggesting that Rsu1 is not needed to properly set up ethanol response neuronal circuits. Given that *ics* mutant flies show a developmental wing blister phenotype (17), it was possible that we could have missed subtle developmental defects. We, therefore, wished to directly test the requirement for Rsu1 in adult flies using Gal80^{ts}, which allows for temperature-dependent suppression of Gal4 driver activity (20). Using this system, the expression of Gal4 is suppressed at 18 °C but not at 29 °C. We first asked whether expression of *UAS-Rsu1* cDNA during development only was able to restore normal ethanol-induced sedation to *ics* mutant adults. We reared flies (*ics^{G4} UAS-Rsu1;Tub-Gal80^{ts}*) at 29 °C, allowed developmental expression of Rsu1, and then, suppressed expression during adulthood by shifting the flies to 18 °C for 3 d after eclosion. Expression of Rsu1 in this manner was unable to rescue the reduced ethanol sensitivity of *ics^{G4}* mutants (Fig. 2*C*). Conversely, when we raised flies at 18 °C, blocking Rsu1 expression during development but allowed Rsu1 expression in adulthood by shifting the flies to 29 °C for 3 d after eclosion, the phenotype of *ics^{G4}* mutants was completely rescued to WT levels (Fig. 2*D*). These data suggest that Rsu1 functions in the adult fly to regulate normal ethanol-induced behaviors and that Rsu1 is not required for the developmental wiring of neural circuits involved in regulating ethanol responses.

Rsu1 Functions Downstream of Integrin Signaling. Developmental experiments show that Rsu1 acts in concert with the scaffolding protein PINCH (particularly interesting new cysteine-histidine-rich protein) to inhibit the JNK signaling pathway downstream of the integrin signaling receptor (17). We, therefore, investigated whether perturbation of the integrin signaling pathway in *Drosophila* would alter ethanol sensitivity. As previously reported (21), flies heterozygous for mutations in the β-integrin–encoding gene

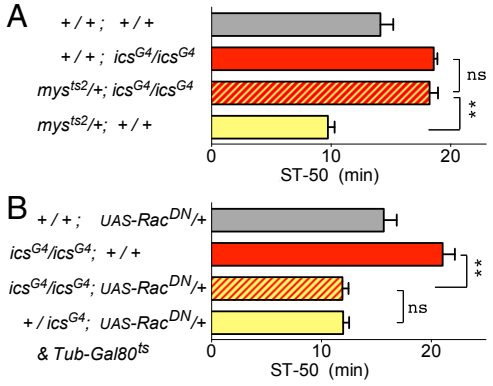


Fig. 3. Rsu1 links β-integrin to Rac1 signaling. (*A*) *ics^{G4}* homozygous mutants combined with heterozygous β-integrin loss-of-function mutants (*mys^{ts2}*) are as resistant to ethanol-induced sedation as *ics^{G4}* mutants alone, indicating that Rsu1 functions downstream of β-integrin. Females were grown at 29 °C for maximum *mys^{ts2}* effect. ns, Not significant ($P > 0.69$); ST-50, median sedation time. **$P < 0.01$ ($n = 7$–9). (*B*) *ics^{G4}* homozygous mutants combined with dominant negative Rac1 (*UAS-Rac1^{DN}*) are as sensitive as *Rac1^{DN}* mutants alone, suggesting that Rac1 functions downstream of Rsu1. Unexpressed *UAS-Rac^{DN}*/+ lacking a Gal4 driver served as a control. ns, Not significant ($P > 0.92$). **$P < 0.001$ ($n = 8$–10).

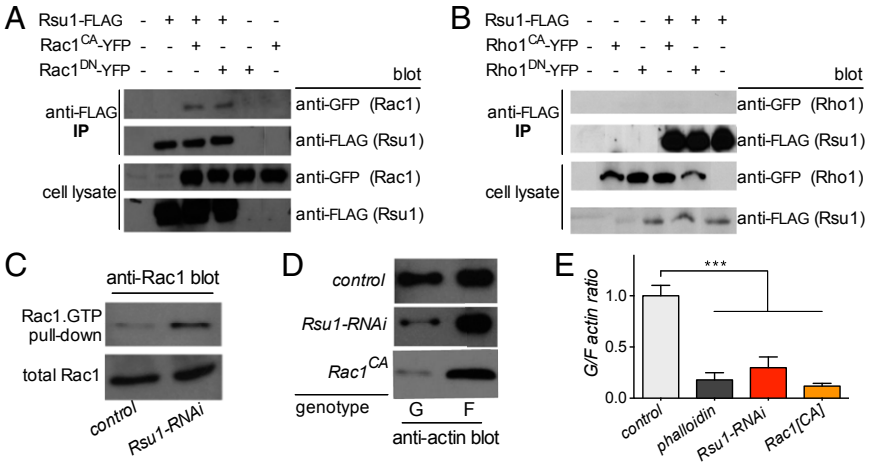


Fig. 4. Rsu1 binds to Rac1 and affects actin dynamics in *Drosophila* S2 cells. (*A* and *B*) Rsu1 binds to (*A*) both the GTP-locked forms of Rac1 ($Rac1^{CA}$) and GDP-locked forms of Rac1 ($Rac1^{DN}$) but (*B*) not to Rho1 GTP- ($Rho1^{CA}$) or GDP-locked ($Rho1^{DN}$) forms. (*C*) Rac1.GTP pull-down experiments shows that RNAi-mediated knockdown of Rsu1 leads to increased Rac1.GTP loading. (*D* and *E*) Globular (G) to filamentous (F) actin assay measuring the ratio of actin in free globular to assembled filamentous form showing that RNAi-mediated knockdown of Rsu1 causes an approximately threefold decrease in G/F actin ratio, whereas overexpression of constitutive active $Rac1^{CA}$ causes an approximately ninefold decrease in G/F actin ratios compared with controls. The actin stabilizer phalloidin also decreases the G/F ratio and served as a positive control. IP, immunoprecipitation. ***$P < 0.001$ ($n = 4$–9).

myospheroid (mys^{ts2}) showed increased sensitivity to the sedating effects of ethanol compared with the WT (Fig. 3*A*) (mys^{ts2} homozygotes are not viable). When we introduced the ics^{G4} mutation into this genetic background, the mys^{ts2} ics^{G4} double-mutant flies showed the same reduced ethanol sensitivity as ics^{G4} mutant flies (Fig. 3*B*), suggesting that Rsu1 controls ethanol-induced behavior downstream of the integrin receptor. We also observed genetic interactions between *ics* alleles and mutants in the genes encoding PINCH and integrin-linked kinase (ILK) (*SI Appendix*, Fig. S2), further supporting our hypothesis that Rsu1 affects ethanol-induced behaviors by regulating the integrin signaling pathway.

Rsu1 Acts Upstream of Rac1 and Affects Actin Dynamics. Because Rsu1 acts in concert with PINCH to inhibit JNK activity during development (17), we tested for potential genetic interactions between mutations in *ics* and *basket* (encoding JNK). We were unable to find any such interaction or a sedation phenotype in *basket* mutants, which is consistent with two previous studies reporting the absence of an ethanol sedation phenotype in *basket* mutants (22, 23). Other than JNK, other downstream targets of integrin signaling include Rho family GTPases. Depletion of human Rsu1 in a human breast cancer cell line elevates the levels of activated Rac (Rac.GTP) (24), suggesting that Rsu1 reduces Rac1 activation. We, therefore, investigated whether Rsu1 functions through Rac1 to affect ethanol-induced responses. Expressing dominant negative (DN) Rac1 in *ics-Gal4*–expressing cells (ics^{G4}/+;$UAS\text{-}Rac1^{DN}$/+) resulted in increased sensitivity to ethanol-induced sedation (Fig. 3*B*). This increased sensitivity remained the same in the ics^{G4} homozygous mutant background, suggesting that Rac1 regulates ethanol responses downstream of Rsu1.

We next determined whether Rsu1 physically interacts with Rac1 by cotransfecting *Drosophila* Schneider (S2) cells with FLAG-tagged Rsu1 and various Rho GTPases tagged with YFP. Immunoprecipitation with an anti-FLAG antibody pulled down both GTP-locked $Rac1^{G12V}$ [constitutively active (CA)] and GDP-bound $Rac1^{T17N}$ (DN) (Fig. 4*A*). It did not, however, coimmunoprecipitate Rho1 (Fig. 4*B*) or Cdc42, suggesting that Rsu1 is a specific binding partner for Rac1 in the Rho family of small GTPases.

Our genetic data indicated that Rsu1 acts upstream of Rac1 to oppose the latter's activity. We, therefore, hypothesized that, in the absence of Rsu1, there would be increased Rac1 activation. We found that knockdown of Rsu1 with RNAi in S2 cells (*SI Appendix*, Fig. S3) increased levels of Rac.GTP loading (Fig. 4*C*). In addition, both overexpression of $Rac1^{CA}$ and Rsu1 knockdown caused a decrease in the globular to filamentous actin ratio (Fig. 4 *D* and *E*). Taken together, these data indicate that Rsu1 binds to Rac1 and destabilizes actin filaments through Rac1 inhibition.

***ics* Mutants Show Increased Alcohol Preference in *Drosophila*.** We next asked whether *ics* mutant flies exhibit an alcohol-drinking phenotype. Flies were tested in an ethanol consumption preference assay [capillary feeder (CAFÉ)] (11, 12). WT flies gradually acquire preference for ethanol over 3 d, showing that alcohol is reinforcing consummatory behavior (*SI Appendix*, Fig. S4), which likely involves reward pathways. Conversely, ics^{G4} mutant flies showed significant naïve preference for ethanol on day 1, which remained unchanged over 4 d of the assay (Fig. 5*A*). This enhanced preference in *ics* mutants was caused by an increase in ethanol consumption, whereas the total food consumption volume was no different from the WT (*SI Appendix*, Fig. S4). Introducing *UAS-Rsu1* driven by ics^{G4} into the mutant flies restored this phenotype to WT (i.e., gradual acquisition of preference over the first few days of the assay) (Fig. 5*A* and *SI Appendix*, Fig. S4).

The MBs are a brain center in *Drosophila* involved in higher-order processing, such as associative olfactory learning (25) and ethanol-reinforced odor preference (26). We next asked whether Rsu1 was required in the MB for normal ethanol preference in the CAFÉ assay. Using an *MB-Gal80* transgene, we inhibited MB expression of Rsu1 in ics^{G4};*UAS-Rsu1* flies (*SI Appendix*, Fig. S5) (27). Like the WT, these flies showed no naïve ethanol preference, but unlike the WT, they did not acquire ethanol preference over the 4-d span of the experiment (Fig. 5*B*). To confirm that loss of Rsu1 from the MB caused this lack of acquired ethanol preference, we knocked down Rsu1 expression specifically in adult MB. Using a mifepristone-inducible *MB-GeneSwitch* driver (28), we found that adult expression of both *UAS-Rsu1-RNAi* and $UAS\text{-}Rac1^{CA}$ overexpression led to a complete loss of ethanol preference (Fig. 5*C*). Together, our data show that flies globally lacking Rsu1 display high naïve preference that does not change over time. Conversely, flies lacking Rsu1 only in the MB show neither naïve nor acquired preference. Both are in contrast to WT flies, which show no naïve preference but gradually

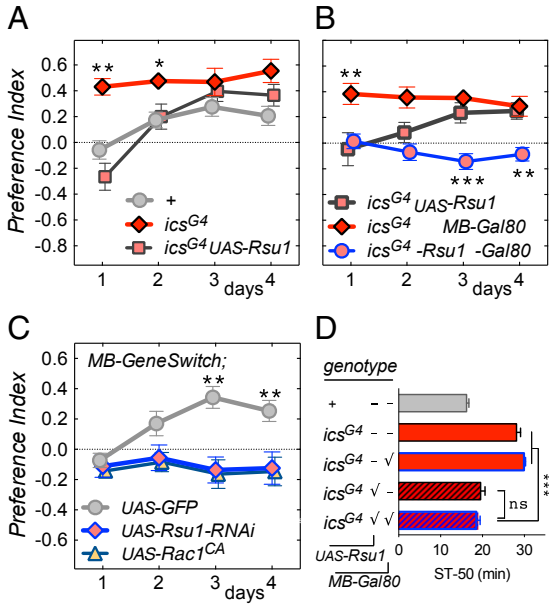


Fig. 5. Alcohol consumption preference phenotypes in flies lacking Rsu1. (*A*) *ics* Mutant flies show increased naïve ethanol preference compared with control in the two-bottle choice CAFÉ assay. This phenotype is rescued by expression of *UAS-Rsu1* in all icsG4-expressing cells. (*B* and *D*) *ics* Rescue flies lacking Rsu1 expression in the MBs only (*ics^{G4} UAS-Rsu1 MB-Gal80*) do not develop acquired ethanol preference but (*B*) have normal naïve preference on day 1 and (*D*) ethanol-induced sedation. (*C*) Adult MB-specific knockdown of Rsu1 or overexpression of $Rac1^{CA}$ causes loss of acquired ethanol preference. The transgenes were expressed in adults using a mifepristone-inducible *MB-GeneSwitch* driver. ns, Not significant; ST-50, median sedation time. *$P < 0.05$; **$P < 0.01$ (ics^{G4} vs. control); ***$P < 0.001$.

acquire preference in the CAFÉ assay over a few days. Flies lacking Rsu1 in the MB only showed normal ethanol-induced sedation (Fig. 5*D*). These findings indicate that naïve responses to ethanol, such as naïve preference and sensitivity to sedation, are mediated by Rsu1 in neurons outside the MB, whereas within the MB, Rsu1 is essential for gradual acquisition of preference.

***RSU1* Genotypes Are Associated with Reward Anticipation and Alcohol Consumption in Human Adolescents.** We next sought to translate our *Drosophila* findings to humans. Alcohol drinking activates the reward system, and alcohol preference and drinking behavior are associated with reward anticipation (29–35). Reward anticipation can be reliably measured during the monetary incentive delay (MID) task (36), where subjects must press a button on seeing an object on the screen. The form of the object determines whether subjects can accrue a large, small, or no monetary win if pressing the button in time. To test a possible association of SNPs in human *RSU1* with reward anticipation, we measured brain activation with functional MRI blood oxygenation level dependent (BOLD) responses during the MID task. We first conducted neuroimaging analyses in 1,303 adolescents of the IMAGEN cohort who were assessed at age 14 y old. We observed extensive activation in the brain comparing the anticipation of a large win with no win, including in the VS, a region crucial for reward processing (37). In this region of interest, we detected an association of the minor T allele of rs7078011 in *RSU1* with increased VS activation, which remained significant after controlling for the 70 SNPs present at the *RSU1* locus in the IMAGEN dataset ($P_{10,000\text{permutation}} = 0.046$) (Fig. 6*A*). However, we did not detect association of rs7078011 with frequency of lifetime drinking at 14 y old in the IMAGEN sample. Because rs7078011 is localized in the seventh intron of human *RSU1* (Fig. 6*B*), we hypothesized that it may be a marker for an unidentified linked causal variant in the vicinity. Of 70 SNPs identified in *RSU1*, 22 SNPs were in linkage disequilibrium (LD) with rs7078011. These SNPs covered the eighth exon of the gene (Fig. 6*B* and *SI Appendix*, Fig. S6). To investigate if rare variants are present in the gene locus covered by these SNPs, we analyzed whole-genome sequencing data of the eighth exon in reference datasets (National Heart, Lung, and Blood Institute and 1000 Genomes Phase III). Here, we detected several rare variants (minor allele frequency < 1%) with a predicted disruption of protein function. These variants are either missense [i.e., rs144428707 (SNP), rs375646999 (SNP), rs375416941 (SNP), rs372364335 (SNP), and rs199904406 (SNP)] (*SI Appendix*, Table S1) or splicing related [i.e., rs373104238 (indel)]. However, our datasets did not have sufficient power to allow stable association analyses of these potentially causative polymorphisms.

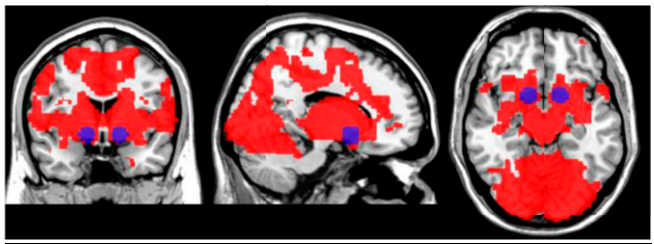
We, therefore, carried out a linear kernel-based association analysis (38) of the SNPs in strong linkage disequilibrium with rs7078011. Kernels combine the contribution of genetic variations, thus enabling detection of genetic effects that cannot be represented by a single SNP alone (39). Although kernel analyses do not indicate directionality of an association, they are particularly sensitive in reliably detecting associations with potentially causal rare variants. We found significant associations of the *RSU1* kernel with both VS activation [generalized mean correlation (mc) = 0.020, $P_{1,000\text{permutations}} = 0.0480$] (Fig. 6*A* and *SI Appendix*, Fig. S7*A*) and the frequency of lifetime drinking (mc = 0.020, $P_{1,000\text{permutations}} = 0.0140$) in the IMAGEN sample at 14 y old (Fig. 6*C* and *SI Appendix*, Fig. S7*B*).

To investigate if *RSU1* might be a risk factor for alcohol addiction, we analyzed 1,149 alcohol-dependent patients and 1,360 controls of Caucasian descent (*SI Appendix*, Table S2) from the Study of Addiction: Genetics and Environment (SAGE) (40). We found a significant association of the *RSU1* kernel with alcohol dependence (mc = 0.018, $P_{10,000\text{permutations}} = 5.40 \times 10^{-3}$) (Fig. 6*C* and *SI Appendix*, Fig. S7*C*). We also measured association of the *RSU1* kernel with alcohol drinking in 4,604 adults age 31 y old of the population-based North Finish Birth Cohort 1966 (NFBC1966) (41). However, we found no significant association with the quantity of alcohol consumption (Fig. 6*C* and *SI Appendix*, Fig. S7*D*).

In addition, we carried out an analysis of haplotype block 5 involving rs7078011. It is noteworthy that the allele frequencies of NFBC are very different from those of IMAGEN ($P = 2.03 \times 10^{-48}$, $\chi^2_{df=21} = 286.19$) and the SAGE ($P = 1.35 \times 10^{-52}$, $\chi^2_{df=20} = 303.80$), whereas the latter two are very similar ($P = 0.922$, $\chi^2_{df=21} = 12.59$) (*SI Appendix*, Table S3), indicating distinct genetic backgrounds of the samples. There was a nominally significant association of haplotype phase 4 (Hap4) of block 5 with increased frequency of drinking in the IMAGEN sample at age 14 y old ($P = 0.0343$) and a significant association of the *RSU1* haplotypes with alcohol dependence in the SAGE dataset (omnibus test; $P = 5.99 \times 10^{-3}$ from 10,000 permutations). Although the association of the *RSU1* haplotypes with alcohol dependence in the SAGE dataset was driven by Hap3 ($P = 2.71 \times 10^{-3}$), there was a trend for an association of Hap4 ($P = 0.0856$) (Fig. 6*C* and *SI Appendix*, Table S4). We also found a nominally significant association of Hap4 with quantity of alcohol consumption in the NFBC1966 dataset at age 31 y old ($P = 0.0360$) (Fig. 6*C* and *SI Appendix*, Table S4).

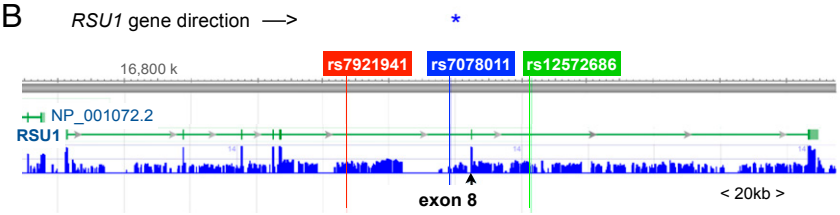
Last, we evaluated gray matter volume of the VS and white matter connectivity of brain structures related to the reward system and associative learning, which are both known to contribute to the development of addiction (42). There was no association with rs7078011 or the *RSU1* kernel with VS volume ($P_{1,000\text{permutations}} = 0.449$) or fractional anisotropy measures of diffusion tensor imaging in fiber tracts linking the hippocampus with the limbic system (fornix crescent: $P_{1,000\text{permutations}} = 0.554$; fornix body: $P_{1,000\text{permutations}} = 0.711$; VS: $P_{1,000\text{permutations}} = 0.176$) (*SI Appendix*, Fig. S8). These anatomical findings suggest

A



	rs7078011 of RSU1 (10000 Permutation)	Linear Kernel of RSU1 (1000 Permutation)
Ventral Striatum	P=0.046	P=0.048

B



C

	IMAGEN Life-time frequency of drinking (N=1908)	**NFBC** Quantity of alcohol consumption (g/d) (N=4604)	**SAGE** (Caucasian) Alcohol dependence vs. dontrol (N=2346)
Linear Kernel Analysis	*MC=0.020 P=0.0140	Not Significant	MC=0.018 $P=5.40\times10^{-3}$
Haplotype Analysis	Hap4 (P=0.0343)	Hap4 (P=0.0360)	Hap3 ($P=2.71\times10^{-3}$) Omnibus ($P=5.99\times10^{-3}$)

*MC stands for generalized mean correlation as a measure of effect size.

Fig. 6. Genetic studies in humans. (*A*) Whole-brain analysis of reward anticipation large win vs. no win during the MID task shows positive BOLD response (PBR) during reward anticipation (family-wise error $P < 0.05$). The location of the VS (±15, 9, −9; 9-mm radius) is depicted in blue. The results of association analyses between VS and RSU1 gene are summarized. ROI, region of interest. (*B*) Exon/intron schematic of the *RSU1* gene. The first SNP of haplotype block 6 (rs7921941; red), the last SNP of haplotype block 5 (rs12572686; green), and the main SNP (rs7078011; asterisk and blue) are highlighted. The eighth exon is indicated with an arrow. (*C*) Summary of genetic analyses of alcohol drinking in the human datasets IMAGEN, SAGE, and NFBC1966.

that the *RSU1* variants alter behavior without changing human neuroanatomy, consistent with our findings in *Drosophila*, where no obvious developmental abnormality was observed in fly brains lacking Rsu1, thus underscoring the concordance of our *Drosophila* and human findings.

Discussion

Role of Rsu1 and Integrin Signaling in Ethanol Sensitivity. The Rho family of small GTPases is known to regulate ethanol-induced behaviors (13), but the upstream pathways that signal to these GTPases in this context are largely unknown. In this report, we characterize the effects of *ics*/*RSU1* on ethanol-related behaviors. We isolated mutations in the *Drosophila ics* gene because of their reduced sensitivity to ethanol-induced sedation. *Drosophila* Rsu1, like its human homolog RSU1, is a 32-kDa protein with a C-terminal domain that contains seven leucine-rich repeats and binds to the integrin effector PINCH to inhibit JNK signaling. In flies, absence of Rsu1 leads to abnormalities during wing development and dorsal closure (17). Indeed, the *ics* mutants that we isolated exhibit wing blisters similar to the ones caused by the loss of integrin, PINCH, and ILK, suggesting that Rsu1 acts in concert with these proteins in integrin-dependent cell adhesion (17). Our data indicate that, in the regulation of adult ethanol behaviors, Rsu1 acts downstream of integrin to antagonize integrin signaling, which was suggested by the fact that loss of Rsu1 leads to reduced ethanol sensitivity, whereas loss-of-function mutations of integrin, PINCH, and ILK result in the opposite phenotype: enhanced ethanol sensitivity. Thus, Rsu1 has modulatory roles on integrin signaling that are context-dependent. During wing development, Rsu1 mediates integrin signaling to antagonize JNK (17), whereas in the adult nervous system, Rsu1 antagonizes integrin signaling to suppress Rac1 activity.

Rsu1 Regulates Actin Dynamics. We were unable to observe any genetic interaction between Rsu1 and JNK mutants. We, therefore, hypothesized that Rsu1 might act through the small Rho family GTPase Rac1 to regulate ethanol-induced behaviors, because depletion of Rsu1 enhanced Rac1 activation and cell migration (24). We found that Rsu1 acts upstream of Rac1 to antagonize Rac1 activity in both flies and cell culture. Rsu1 coimmunoprecipitated specifically with Rac1 (but not Rho1 or Cdc42) from *Drosophila* S2 cells but did not show a preference for either GTP- or GDP-bound Rac1. Because Rsu1 does not contain a potential Rac-inactivating, GTPase-activating (GAP) domain, we hypothesize that Rsu1 prevents Rac1 from interacting with its relevant activators and/or effectors, possibly by sequestering them or occluding binding sites within Rac1. We show that normal ethanol-induced behaviors, including sedation sensitivity and consumption preference, require proper Rsu1 and Rac1 function in the adult nervous system. Together, our findings suggest that integrin signals to Rac1 through Rsu1 to regulate actin dynamics, which is known to be required for proper synaptic function (43) as well as behavioral responses to drugs of abuse (13). It also establishes integrin/Rsu1 as an important functional input into the regulation of actin dynamics with behaviorally manifest consequences.

Involvement of Rsu1 in Higher Behaviors. Our further characterization revealed different behavioral roles for Rsu1 in anatomically distinct neuronal circuits. For normal naïve responses to ethanol, Rsu1 functions in the nervous system outside of the MB. Absence of Rsu1 from these non-MB neurons resulted in reduced sensitivity to ethanol-induced sedation as well as naïve preference for ethanol in a choice assay. In contrast, loss of Rsu1 in MB led to normal naïve ethanol sedation sensitivity and consumption preference but caused a failure to acquire ethanol preference, suggesting that activated Rac1 in the MB prevents this behavioral plasticity. Indeed, when we overexpressed $Rac1^{CA}$ in adult MB, the flies failed to acquire ethanol preference. Conversely, flies lacking Rsu1 throughout the brain showed high naïve ethanol preference, suggesting that activation of Rac1 outside the MB promotes naïve preference. Thus, Rsu1 has opposite effects on ethanol preference depending on the affected circuits. Such opposite effects are reminiscent of mouse findings, where suppression of Rac1 in the nucleus accumbens promoted conditioned place preference (CPP) for cocaine (44), whereas global Kalirin7 KO (a Rac1 activator) led to reduced cocaine CPP (45). Our data expand on these findings by showing that (*i*) similar to mammals, gene function in distinct circuits can differentially affect drug preference in *Drosophila*, (*ii*) in addition to Kalirin7-mediated activation, integrin/Rsu1-regulated suppression is an important input into Rac1 regulation, and (*iii*) we extend the mouse Rac1 findings from effects on cocaine-mediated reinforcement (in CPP) to voluntary drug/alcohol consumption in both *Drosophila* and humans.

Previous studies have shown a remarkable conservation of genetic determinants of alcohol and substance use behavior across both species (5). We investigated whether *RSU1* was involved in human reward processing and alcohol-drinking behaviors, including addiction, by analyzing several datasets, including the IMAGEN adolescent imaging genetics cohort (46), the SAGE alcohol dependence dataset (40), and the NFBC1966 (41). Like most other large genetic datasets, these samples have been analyzed in various different projects. Repeat analysis raises the question of a potentially greater false-positive rate, because correction for multiple testing is usually confined to the number of tests within one project. Although an increased risk for false-positives is a real possibility, we have mitigated it by (*i*) testing a very specific hypothesis, which has been experimentally supported in the *Drosophila* studies presented, and (*ii*) validating our results across different independent datasets.

Because we were interested in investigating the genetic basis of mechanisms that convey increased risk for alcohol-drinking behavior, we first analyzed the population-based IMAGEN sample of 14-y-old adolescents who did not meet criteria for AUDs. In this sample, a generic reward stimulus as presented in the MID task might be more salient and a more reliable activator of the reward system than alcohol-specific cues. Using this approach, we first carried out single-SNP analyses to identify a marker for the strongest genetic signal for VS activation during reward anticipation in the RSU1 gene. This analysis resulted in the detection of the association of VS activation during reward anticipation but not frequency of lifetime drinking with SNP rs7078011 localized in intron 7 of *RSU1*.

We hypothesized that rs7078011 might be a marker of potentially causative rare genetic variants. Indeed, analyzing whole-genome sequencing data, we detected several rare variants in the genomic locus delineated by 22 SNPs in strong linkage disequilibrium with rs7078011 that probably impair protein function of Rsu1. Although our datasets were underpowered to carry out a genetic association analysis of the rare variants detected, we were able to carry out a kernel-based association analysis with these 22 SNPs. Using the kernel method, we confirmed the association of *RSU1* with VS activation during reward anticipation in the IMAGEN dataset, and we also found an association of the *RSU1* kernel with frequency of lifetime drinking in the same sample. The fact that the association of rs7078011 with the investigated phenotypes was less stable than the association of the kernel is in keeping with the possibility of rare variants underlying the observed associations. When rare causal variants are present, their linkage disequilibrium with noncausal SNPs with higher frequencies might vary from sample to sample. This variability can be caused by recurrent rare mutations or admixture of populations with different genetic backgrounds. It is, thus, possible that the same rare variant can be linked with different alleles in different samples. Such differential linkage could lead to false-negative findings if only the same SNP was analyzed. Alternatively, different rare variants within the observed gene locus might associate with different phenotypes under study. Using a kernel analysis allowed us to overcome these problems.

Our kernel analyses in additional independent datasets revealed an association of *RSU1* with adult alcohol dependence but not with adult drinking behavior in a general population sample. Because early substance use in adolescents is a risk factor for adult alcohol dependence (47), these results might indicate that the effect of Rsu1 on reward processing influences a risk drinking trajectory at very early stages of exposure to alcohol. However, one limitation of our study is that it is not possible to unambiguously rule out an association of adult alcohol drinking in the population with *RSU1*. The markedly different LD structure of *RSU1* in the NFBC1966 cohort might have masked an association of the kernel. The observed nominal association of the *RSU1* haplotype 4 with the amount of drinking might, indeed, indicate a weak signal in this locus.

The haplotypes included in the kernel are distributed around exon 8, which encodes one of seven leucine-rich repeats found in the Rsu1 protein that are crucial for its interaction with PINCH1 (48). In human glioma cells, an alternative splicing site has been described, which gives rise to an exon 8-deleted splice variant of *RSU1* translating into a less stable protein with reduced function (49). It is possible that the rare variants detected might result in an impaired interaction of *Rsu1* protein with PINCH and/or decreased protein stability. Such decreases might disrupt Rsu1 function in a way analogous to the knockdown of Rsu1 in *Drosophila*, causing the alcohol preference phenotype. However, additional investigations are required to analyze the effect of these variants on Rsu1 function and test their association with alcohol drinking in large metaanalyses.

Together, our data show that Rsu1 regulates reward-related behaviors, such as ethanol consumption, in flies and humans. We found no structural abnormalities associated with Rsu1 variants in either flies or humans but show that Rsu1 is required after development in adult flies for normal ethanol-induced behaviors. Our data from both species are, therefore, highly concordant. We hypothesize that the physiological process underlying these phenotypes is synaptic plasticity. In the integrin/Rsu1/Rac1 signaling cascade, both integrin (50) and Rac1 (13) are known to affect synaptic structure and plasticity. Our findings, thus, underscore the use of model organisms. For one, they are useful in elucidating the molecular mechanisms of genes mediating addiction-like behaviors. Also, they show remarkable predictive power with unbiased forward genetic screens in generating testable hypotheses that can be translated to human phenotypes.

Materials and Methods

Detailed methods can be found in *SI Appendix, SI Materials and Methods*.

***Drosophila* Experiments.** Flies were kept and assayed as described (51) with the CAFÉ modified from ref. 12. Standard cell culture and biochemistry approaches were used.

Human Data. This project was approved by the ethics committee at King's College London as well as the local ethics committees at each recruitment

site. Informed consent was obtained from each participant and at least one parent. A detailed description of recruitment and assessment procedures, as well as in/exclusion criteria, has previously been published (46). The IMAGEN cohort and assays are described in ref. 46, and the frequency of drinking phenotype was defined using an adapted version of the 2007 ESPAD (European School Survey Project on Alcohol and Other Drugs) questionnaire (www.espad.org), which assesses the number of times alcoholic drinks were consumed until 14 y of age. Individuals were ranked into seven categories from 0 (nondrinker) to 6 (over 50 times; mean = 2.0, SD = 1.8, median = 2.0 in males; mean = 2.1, SD = 1.7, median = 2.0 in females). The NFBC is described in ref. 41, and the quantity of drinking phenotype was determined using an adapted version of the 2007 ESPAD questionnaire (www.espad.org), which assesses "the quantity of alcohol consumed on a TYPICAL DAY when you are drinking." In this cohort, the mean alcohol intake was 9.1 g alcohol per day (mean = 13.8, SD = 19.7, median = 7.8 in males; mean = 4.8, SD = 7.9, median = 2.2 in females). In our analysis, these original values were analyzed in a quantitative way without any presumed threshold for case–control split. The SAGE dataset is described in ref. 40, which integrates different case–control studies for alcohol dependence. Particularly, 2,509 Caucasian cohorts (case = 688, control = 404 in males; case = 461, control = 956 in females) were chosen in our study because of similar genetic background to the IMAGEN dataset. The descriptive statistics of all three datasets are summarized in *SI Appendix*, Table S2. We used kernel-generalized variance (38) to quantify the dependency between the functional BOLD and behavioral responses with *RSU1* in three cohorts.

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