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The Arf6 Activator Efa6/PSD3 Confers Regional Specificity and Modulates Ethanol Consumption in *Drosophila* and Humans

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AUTHOR CONTRIBUTIONS

D.A.G., J.H.P., S.A.O., S.F.A., and A.R. conceived, performed, and analyzed the *Drosophila* experiments. S.D., T.B., C.B., A.L.W.B., P.J.C., H.F., B.I., M.L., J.M., T.P., M.S., G.S., and the IMAGEN consortium acquired the human data. T.J., B.X., and G.S. analyzed the human data. D.A.G., T.J., J.H.P., J.L.H., A.R.R., G.S., and A.R. wrote the paper.

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CONFLICT OF INTEREST

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SUMMARY

Ubiquitously expressed genes have been implicated in a variety of specific behaviors, including responses to ethanol. However, the mechanisms that confer this behavioral specificity have remained elusive. Previously, we showed that the ubiquitously expressed small GTPase Arf6 is required for normal ethanol-induced sedation in adult *Drosophila*. Here, we show that this behavioral response also requires Efa6, one of (at least) three *Drosophila* Arf6 guanine exchange factors. Ethanol-naïve *Arf6* and *Efa6* mutants were sensitive to ethanol-induced sedation and lacked rapid tolerance upon re-exposure to ethanol, when compared to wild-type flies. In contrast to wild type-flies, both *Arf6* and *Efa6* mutants preferred alcohol-containing food without prior ethanol experience. An analysis of the human ortholog of *Arf6* and orthologs of *Efa6* (*PSD1-4*) revealed that the minor G-allele of SNP *rs13265422* in *PSD3*, as well as a haplotype containing *rs13265422*, were associated with increased frequency of drinking and binge drinking episodes in adolescents. The same haplotype was also associated with increased alcohol dependence in an independent European cohort. Unlike the ubiquitously expressed human Arf6 GTPase, *PSD3* localization is restricted to the brain, particularly the prefrontal cortex (PFC). Functional magnetic resonance imaging (fMRI) revealed that the same *PSD3* haplotype was also associated with a differential fMRI signal in the PFC during a Go/No-Go task, which engages PFC-mediated executive control. Our translational analysis therefore suggests that *PSD3* confers regional specificity to ubiquitous *Arf6* in the PFC to modulate human alcohol-drinking behaviors.

INTRODUCTION

Alcohol is one of the most used and abused drugs in the world.[1,2] Excessive alcohol consumption can lead to alcohol use disorders (AUD) and addiction,[1] and the behavioral changes that associate with these include tolerance and increased consumption and preference.[3–5] Genetic predisposition has been estimated to contribute 40–60% to the development of AUDs,[6–10] but the molecular mechanisms involved in this process remain poorly understood.

Numerous model organisms have been established to investigate genes and mechanisms that underlie the development of AUDs. Upon acute alcohol exposure, the vinegar fly,

Drosophila melanogaster, exhibits behaviors similar to mammals,[11,12] such as disinhibition[13] and locomotor hyperactivity,[14] followed by sedation.[14] Repeat exposures can lead to rapid or chronic tolerance, i.e. reduced sensitivity to ethanol-induced sedation upon re-exposure.[15] Flies also develop preference for alcohol consumption in a two choice paradigm, both within a day of being able to choose,[16] or after a defined ethanol pre-exposure.[17]

Mechanistically, there is striking conservation, from flies to humans, of genes that regulate ethanol-responses,[18,19] including the Rho-family of small GTPases. Rho GTPases are ubiquitously expressed signaling molecules that regulate actin dynamics and affect many neuronal processes, including addiction-related behaviors, like cocaine-induced place preference and sensitivity to alcohol.[20] The Rho-family GTPase Rac1 acts in conjunction with the ubiquitously expressed GTPase Arf6 to regulate ethanol-induced behaviors.[19,21] Arf6 regulates receptor trafficking and actin dynamics at the plasma membrane[22–24] and acts in a signaling pathway that includes the insulin receptor, mTor, and S6 kinase.[25] This pathway is activated by alcohol self-administration in rodents,[26] and inhibition of the direct S6k activator mTor reduces drinking relapse.[27] One potential problem for therapeutic drugs targeting members in this Arf6 pathway is toxicity, caused by the fact that the proteins involved are expressed in nearly every tissue in the body.[28,29]

Here, we use *Drosophila* to isolate conserved genes associated with human alcohol behaviors. We show that *Drosophila Efa6* (also known as *dPSD*), a guanine exchange factor (GEF) and activator of *Arf6,* is required for normal alcohol-induced behaviors. We show that a single nucleotide polymorphism (SNP) in one of the four human orthologs, *PSD3*, and one haplotype phase containing that SNP are associated with the frequency of drinking in adolescents, and the same haplotype phase is also associated with alcohol dependence in an independent sample. *PSD3* is specifically expressed in the brain, especially the prefrontal cortex (PFC).[29] Functional brain imaging revealed an association of the same haplotype phase with differential activation in the right inferior frontal gyrus of adolescents during a Go/No-Go executive control task. The specific expression of *PSD3* in the PFC, coupled with its association with specific phenotypes in prefrontal activation and in alcohol consumption suggests a general mechanism by which a ubiquitous signaling pathway can be controlled by a spatially restricted regulator. It also suggests a strategy for the development of specific therapeutic intervention with fewer side effects.

MATERIALS AND METHODS

Fly Methods

Flies were kept on standard cornmeal/molasses food at 25°C on a 12 hr light : 12 hr dark cycle at constant humidity (76 %) and were grown to regular density (~200–400 F1 flies per bottle). All fly lines were outcrossed (for at least 5 generations) to w^{-} *Berlin* prior to behavioral experiments. Fly strains not described in[21] were obtained from the Bloomington *Drosophila* Stock Center ($Efa6^{PB}$, Stock #10314), and Dr. Yang Hong ($Efa6^{KO}$, a knock-out line generated by homologous recombination).[30] Adult males (unless otherwise noted) were collected 1–5 days after eclosion and used for experiments after at least one day of recovery within the next one to three days. Sedation and tolerance were determined by observing the

flies' loss-of-righting reflex (LORR).[31] To do so, flies were exposed to a mixture of two air flows at predetermined ratios, one water-saturated (air), and the other saturated with ethanol (EtOH). The combined (EtOH/air) flowrate was kept at 150 units. Flies were visually inspected every 5 min for LORR upon gentle tapping during the exposure. The time when 50% of the flies sedated (ST-50) was determined for each tube of 20 flies, for an $n = 1$. For rapid tolerance, flies were exposed to ethanol for 30 min, and then re-exposed 4 hr later. Preference assays were done in an abbreviated, 16 hr, "2-bottle choice" capillary feeder (CAFÉ) assay,[16] following a 20 min mock, or preference-inducing ethanol pre-exposure 24 hr prior.[17] Sample sizes of at least 6 for sedation and tolerance and 12 groups of flies for CAFÉ preference were chosen. Genotypes were randomly assigned to the different exposure tubes/chambers, numerically coded, and the experimenter was blind to genotype during the assay. All groups of flies were included in the analyses. Data were analyzed using Prism (GraphPad 6 Software, La Jolla, CA). Chi-square test was used for the discontinuous data in Figure 1a, and ANOVAS for the rest of Figures 1–3 where values were normally distributed (D'Agostino & Pearson normality test with Bonferroni correction). No differences in variances were detected (Brown-Forsythe test). Only in Figure 2D was one variable not normally distributed (due to one "outlier"), but as all data points were included, a non-parametric test was performed in that case (Kruskal-Wallis, with Dunn's post-hoc test).

Lethality Screen

To determine viability of the *whir*^{3} allele, we crossed *whir*^{3} virgins to wild type males. Because the *whir* gene is on the X-chromosone, this resulted in F1 progeny consisting of *whir*^{3} mutant males, and *whir*^{3}/+ heterozygous, phenotypically normal females. At 25°C, only ~25% as many males survived, compared to females, hence the designation of *whir*^{3} as semi-lethal (note that individual flies were only scored as alive when reaching the adult stage, and the designation "semi-lethal" applied to the genotype, not individual flies of that genotype). At 28°C, only ~1% of *whir*^{3} males survived, and the suppressor screen was performed at that temperature. Unbiased mutations on the third chromosome (*mut(III);* limited to chromosome 3 only for the crossing scheme that follows) were crossed to *UAS-hid* virgins (to get rid of 3rd chromosome balancer chromosomes), resulting in *mut(III)/UAS-hid* progeny. Virgins of *whir*^{3} were then crossed to *mut(III)/UAS-hid* males. The *whir*^{3} mutant also contains a Gal4-driver that causes all *whir*^{3}*;+/UAS-hid* males and *whir*^{3}*/+;+/UAS-hid* females to die (data not shown), therefore the surviving flies were all of the desired *whir*^{3}*;mut(III)/+* and *whir*^{3}*/+;mut(III)/+* genotype. Vials with >5 surviving males were scored as putative suppressors, and the cross was repeated with the same 3rd chromosome *mut(III)*, to confirm the suppression. All mutations had been mapped, and the affected genes determined (Exelixis, San Francisco, CA). We confirmed the insertion sites by PCR (see Supplementary Figure 1).

Biochemical Assays

Arf6.GTP levels were determined using a specific GG3A-PBD (protein binding domain) conjugated to GST, which binds to activated Arf6 only. Arf6.GTP was then pulled-down using glutathione-agarose beads and compared to total levels of Arf6 in 3% lysate in two independent experiments (Active Arf6 Pull-Down and Detection Kit, Thermo Scientific).

Western blots were performed using anti-Arf6 antibody (1:1000, Sigma-Aldrich, #A5230) and visualized using enhanced chemiluminescence (Amersham).

Human Cohort and Analysis

The IMAGEN Study was approved by local ethics research committees at each research site: King's College London, University of Nottingham, Trinity College Dublin, University of Heidelberg, Technische Universität Dresden, Commissariat à l'Energie Atomique et aux Energies Alternatives, and Universitätsklinikum Hamburg Eppendorf. Informed consent was sought from all participants and a parent/guardian of each participant. 1363 IMAGEN adolescent individuals (712 females) were included in the association analyses between frequency of drinking and binge drinking in the last 30 days (age mean = 16.46, range =13.81–18.80, s.d. = 0.51) and SNPs/haplotypes of *PSD* genes. Alcohol usage behaviors were assessed with the European School Survey Project on Alcohol and Other Drugs (ESPAD) questionnaire,[32] and substantial alcohol use was observed in these 16-year old IMAGEN participants, of which 1004 (74%) individuals had experience of drinking in the last 30 days, while 528 (39%) individuals had experience of binge drinking, which is in line with previous findings.[33] For the functional MRI experiments, 1771 IMAGEN individuals (914 females) were assessed for their blood oxygen-level dependent (BOLD) response contrast between 'stop success vs. go success' during the Stop Signal Task (Go/No-Go) at 14-years old (mean age: 14.43; range: 12.56–18.67; s.d = 0.42). See[34] for description of the IMAGEN cohort and analysis, and the Supplementary Materials and Methods for additional detail. *The Study of Addiction:* Genetics and Environment (SAGE) dataset has been described by Bierut and colleagues[35] which integrates alcohol dependent individuals and controls from different sub-datasets. Specifically, 2544 subjects of European descent were included in our analyses to match the genetic background of IMAGEN sample. Descriptive statistics for both IMAGEN and SAGE drinking behaviors are summarized in Supplementary Table 1 and 2.

Genetic and Haplotype Analyses

For SNP data, the linear regression/partial correlation was adopted to conduct univariate analysis between SNP and phenotypes. Given a sequence of SNPs with high linkage disequilibrium, e.g. D′, a haplotype analysis could help to investigate the underlying hidden haplotype structure through estimating most probable haplotype phases for each individual. Hence established haplotype phases represent allele combinations of a chromosomal region (i.e. determined haplotype blocks) that are inheritable without recombination and could help to capture information from non-genotyped SNPs.[36,37] In the IMAGEN sample, the LD/haplotype blocks of the *PSD3* gene were generated and illustrated through the Haploview software.[38] The exact haplotype phases of both IMAGEN and SAGE samples were estimated with the PLINK software.[39] To investigate sexual dimorphism, the gender-specific correlations were compared by using Fisher r-to-Z transformation. See Supplementary Materials and Methods for more details. The IMAGEN data are available from a dedicated database: https://imagen2.cea.fr.

RESULTS

Efa6 Genetically Interacts with Mutations of *RhoGAP18B*

The *Drosophila* Rho-family GTPase activating protein RhoGap18B acts on the ubiquitous small GTPase *Rac1*,[40] which is downstream of the integrin cell adhesion molecule,[19] and upstream of the actin-severing protein cofilin.[40] Activity of this pathway in neurons modulates sensitivity to ethanol-induced sedation,[19] and loss-of-function mutations in the *white rabbit* (*whir*) gene, encoding RhoGAP18B, cause resistance to ethanol-induced sedation.[31] The two RhoGAP18B alleles $whir^{1}$ and $whir^{3}$ (caused by a transposable element inserted in two different locations in the gene) show similar extents of ethanol-induced resistance, but the stronger $whir^{3}$ allele also causes reduced viability.[31] In order to isolate additional genes contributing to the signaling cascade that includes RhoGAP18B, we performed a genetic modifier screen. We hypothesized that mutations that reduce the lethality of the $whir^{3}$ allele would also reduce the ethanol-resistance of $whir^{1}$ and would therefore be in a genetic network with RhoGAP18B regulating ethanol-induced behaviors. We performed a screen of 300 transposable elements on the third chromosome and isolated two suppressors of $whir^{3}$ semi-lethality, *Arfip* and *Efa6* (Figure 1a). Mutation in *Arfaptin* ($Arfip^{x12}$) suppressed $whir^{3}$ semi-lethality, and we have previously shown that the Arf6 and Rac1 GTPase-binding protein Arfaptin is involved in regulating ethanol-induced behaviors in the adult nervous system.[21] The other suppressor we isolated was a mutation in *Efa6* (Figure 1a), an activator of *Arf6*.[41] Given that both Arfip and Efa6 are associated with Arf6, we tested if a mutation in *Arf6* would also affect $whir^{3}$ semi-lethality. As hypothesized, $Arf6^{G4}$ loss of function mutants[21] also suppressed $whir^{3}$ semi-lethality (Figure 1a).

Mutations in *Arf6* and *Arfip* suppressed the ethanol-resistance of $whir^{1}$ flies.[21] Because Efa6 is an activator of Arf6, we next tested whether mutation in *Efa6* would also suppress $whir^{1}$-mediated ethanol-resistance. Unlike experiments with the $whir^{3}$ allele, this $whir^{1}$ genetic interaction experiment was not confounded by developmental semi-lethality. Double mutant $whir^{1}$;$Efa6^{PB}/+$ flies showed a significant suppression of the $whir^{1}$ ethanol-resistance phenotype (Figure 1b), while in a wild-type background, $Efa6^{PB}/+$ heterozygotes showed no phenotype (Figure 1c). These data suggest that *Efa6* acts in concert with RhoGAP18B to regulate ethanol-induced behaviors, as do *Arfip* and *Arf6*.[21]

Efa6 Activates Arf6 and Regulates Ethanol-Induced Sedation

We then tested whether *Efa6* mutants alone would have an ethanol-induced sedation phenotype. The $Efa6^{PB}$ allele we isolated is a transposable element insertion in the open reading frame of *Efa6*, disrupting the Sec7 GEF domain essential for GTP loading (Supplementary Figure 1). Homozygous $Efa6^{PB}$ mutants were viable, but male sterile. This is the same phenotype that was previously reported for an $Efa6^{KO}$ knock out allele generated by targeted, homologous recombination at the *Efa6* locus.[30] Homozygous $Efa6^{PB}$ males showed significantly enhanced sensitivity to ethanol-induced sedation, as did $Efa6^{PB}/Efa6^{KO}$ trans-heterozygotes (Figure 1c). *Efa6* is therefore required to counteract ethanol-induced sedation.

Loss of either *Efa6* or *Arf6* caused enhanced sensitivity to ethanol sedation (Figure 1c).[21] Therefore, we hypothesized that Efa6 acts as an activator of Arf6 in the specific physiological context of ethanol-induced sedation. We first tested this hypothesis genetically by generating *Efa6;Arf6* double mutants. If loss of *Efa6* function causes loss of *Arf6* activity, then introducing a loss-of-function *Arf6* mutation should not enhance the *Efa6* phenotype any further. As predicted, *Arf6*, *Efa6*, and the *Arf6;Efa6* double mutants all had indistinguishable ethanol-sensitivity phenotypes, and the introduction of the *Arf6* loss-of-function mutation did not make the *Efa6* phenotype any more severe (Figure 1d). This lack of enhanced sensitivity was not due to a floor effect, because we used a very low dose of ethanol during exposure, which was unable to sedate wild-type flies, and we have previously shown that the *Arf6* phenotype can be enhanced with an unrelated, ethanol-sensitive *Amn* mutation.[21]

If Efa6 acts as an activator of Arf6, we next hypothesized that $Efa6^{PB}$ mutants should show reduced Arf6 activation as measured by GTP loading. We tested this prediction by specifically pulling down activated Arf6.GTP from head extracts in our set of mutants ($Efa6^{PB}$, $Efa6^{KO}$ and *Arf6*). Western blots revealed a reduction of activated Arf6 in the mutants compared to wild-type controls (Figure 1e). Together, these data show that Efa6 acts upstream of Arf6 to regulate Arf6 activation and together, they counteract ethanol-induced sedation.

Efa6 and *Arf6* Regulate Ethanol-Induced Tolerance and Consumption Preference

One hallmark of addiction is altered behavioral responses after prior alcohol exposure, including reduced sedation, increased tolerance, and heightened preference and consumption. We therefore tested if *Efa6* and *Arf6* regulate ethanol-induced behavioral plasticity in addition to naïve ethanol-induced sedation. Flies develop rapid functional tolerance after being exposed to a sedating dose of ethanol, a behavioral change that can be measured upon a second exposure.[15] For example, it takes wild-type flies twice as long to sedate during the second exposure, indicating that they developed 100% tolerance relative to the sedation time during the first exposure (Figure 2a,b). *Arf6* mutant flies were as sensitive to sedation during the second exposure as they were during the first exposure, indicating that they did not develop tolerance (Figure 2a,b). We were able to rescue that phenotype when expressing *Arf6-cDNA* driven by the endogenous *Arf6* promoter (using the $Arf6^{G4}$ mutant allele which includes a Gal4-driver expressed from endogenous Arf6 promoter/enhancers). Because a tolerance phenotype might be confounded by the initial sedation-sensitivity of *Arf6* mutants, we also tested the ethanol-sensitive Amn^{c651} mutant for their tolerance. We found that while these mutants showed initial sensitivity to sedation (as expected),[42] they developed tolerance similar to that of the wild type (Figure 2a,b). These results indicate that initial sensitivity to alcohol and rapid tolerance phenotypes can be separated, as found by Devineni and colleagues.[43] Similar to *Arf6* mutants, *Efa6* mutants also failed to develop tolerance to repeat ethanol exposures (Figure 2c, d), further strengthening the functional connection between the two genes.

We then tested our *Arf6* and *Efa6* mutant flies for their ethanol preference in an assay similar to a two-bottle choice paradigm. We used the capillary feeder (CAFÉ) assay, where

flies choose between two capillaries, one containing liquid food only, and the other liquid food with 15% ethanol. Wild-type flies showed initial indifference to ethanol on the first day and then acquire ethanol preference over the course of three to four days.[16,44,45] Pre-exposing naïve flies to ethanol vapor, 24 hr before the choice assay, also results in acquisition of preference.[17] In an abbreviated 16-hr CAFÉ, naïve flies showed acute aversion to alcohol (as found in),[17] and a pre-exposure to vaporized ethanol (20 min, 80/70 ethanol/air mix, 24 hr prior to test) induced alcohol preference (Figure 3). *Arf6* mutants showed high initial preference, which did not change upon pre-exposure, and this phenotype was rescued by expressing *Arf6-cDNA* (driven by the $Arf6^{Gal4}$ mutant/driver; Figure 3a). Similarly, *Efa6* mutants also showed high initial, and unchanging preference for ethanol (Figure 3b). *Arf6* and *Efa6* are therefore required for normal alcohol-induced tolerance and preference.

Because variants in the human ortholog of *Efa6* showed a trend towards gender-specific phenotypes (albeit non-significant; see below), and the above experiments were performed in male flies (see also legend to Figure 1), we also tested *Drosophila Arf6* and *Ef6* mutant females. We found that the mutations equally affected acute aversion to alcohol in naïve males and females (Supplementary Figure 3), but that some of the tolerance and sedation phenotypes were more pronounced in males, compared to females (Supplementary Figure 2). Overall, however, the sexual dimorphism of these *Drosophila* alcohol phenotypes was subtle, and just as in human adolescents (see below), not pervasive.

Variants in Human *PSD3* Associate with Frequency of Alcohol Consumption and with PFC Activation

Previous genes identified in *Drosophila* that function in alcohol-induced behaviors have orthologs associated with human alcohol behaviors.[19] We therefore decided to examined the human orthologs of *Efa6* and *Arf6*. The human genome encodes for one *Arf6* ortholog, and four *Efa6* orthologs, *PSD1-4* (for Pleckstrin and Sec7 GEF domain-containing proteins – the same domains as found in *Drosophila* EFA6; see Supplementary Figure 4 for gene structures). We first studied association of *PSD1-4* and human *Arf6* with drinking behavior in adolescents, including frequency of drinking and binge drinking (Supplementary Table 1 and 2). In the IMAGEN sample of 16-year old European adolescents genotype information was available for 252 SNPs in *PSD3*, as well as one SNP in *PSD1*, nine SNPS in *PSD2*, two SNPs in *PSD4*, and two SNPs in the *Arf6* gene region (Supplementary Table 3). In *PSD1*, *PSD2*, *PSD4*, and *Arf6*, there was no significant association (Supplementary Table 3). However, in *PSD3* we found overall significant association of the minor G allele of *rs13265422* (Figure 4, Supplementary Figure 5; minor allele frequency = 0.3) with frequency of drinking in the last 30 days ($r = 0.11$, $t = 3.97$, $P = 7.3\times10^{-5}$; P corrected for 266 SNPs of all five genes, as well as two phenotypes, based on 100,000 permutations, $P_{corrected} = 0.031$). While this result was mainly observed in girls ($r = 0.14$, $t = 3.63$, $P = 2.8\times10^{-4}$), but less so in boys ($r = 0.08$, $t = 1.99$, $P = 0.047$), the directionality of association was similar in both genders, and there was no significant difference in the effect between boys and girls ($z = 1.06$, $P = 0.29$). We also found a nominally significant association of *rs13265422* with frequency of binge drinking in the last 30 days, a measure of alcohol abuse ($r = 0.06$, $t = 2.06$, $P = 0.040$; girls: $r = 0.09$, $t = 2.48$, $P = 0.013$; boys: $r = 0.02$, $t = 0.52$, $P =$

0.60), and again there was no significant difference between boys and girls ($z = 1.33$, $P = 0.18$).

The SNP *rs13265422* is localized 40k nucleotides downstream of the stop codon (Supplementary Figure 5) and shows strong/moderate linkage disequilibrium with SNPs within the 3′ UTR of *PSD3*, i.e. *rs3739398* ($r^2 = 0.50$), *rs3739396* ($r^2 = 0.47$), *rs901933* ($r^2 = 0.52$), *rs901934* ($r^2 = 0.35$); *rs13265422* might therefore be a marker of functional SNPs within the 3′ UTR that can potentially affect the stability and translation of mRNA.[46]

To further investigate the genetic structure of *PSD3* we carried out a haplotype analysis of this gene (see Supplementary Materials and Methods), and detected 29 haplotype blocks (Supplementary Figure 6a). The haplotype block that contains SNP *rs13265422* (Supplementary Figure 6b) was significantly associated with frequency of drinking ($\eta^2_{\text{partial}} = 0.022$, $F_{(9,1345)} = 3.37$, $P = 4.3\times10^{-4}$, omnibus test; girls: $\eta^2_{\text{partial}} = 0.042$, $F_{(9,695)} = 3.40$, $P = 4.2\times10^{-4}$; boys: $\eta^2_{\text{partial}} = 0.039$, $F_{(9,634)} = 2.85$, $P = 0.0026$). This haplotype block contains 12 SNPs that give rise to ten individual haplotype phases with frequency > 1% (Supplementary Table 4). Within this block, haplotype phase 7 (Figure 4, Supplementary Table 5; frequency 8%), containing the G allele of *rs13265422*, showed the strongest association with frequency of drinking ($r = 0.11$, $t = 3.98$, $P = 6.9\times10^{-5}$; girls: $r = 0.074$, $t = 1.96$, $P = 0.050$; boys: $r = 0.14$, $t = 3.55$, $P = 3.8\times10^{-4}$), as well as frequency of binge drinking ($r = 0.095$, $t = 3.50$, $P = 4.7\times10^{-4}$; girls: $r = 0.09$, $t = 2.27$, $P = 0.023$; boys: $r = 0.10$, $t = 2.57$, $P = 0.010$); there was no statistically significant gender difference ($z = -1.21$, $P = 0.23$ for frequency of drinking; $z = -0.29$, $P = 0.77$ for frequency of binge drinking). We then investigated *rs13265422* and the haplotype block containing *rs13265422* in the European sample of the Study of Addiction: Genetics and Environment (SAGE) cohort ($n = 2544$, Supplementary Table 1), an independent adult alcohol-dependence sample. We did not find significant association with *rs13265422* (OR = 1.09, $\chi^2_{\text{df=1}} = 1.76$, $P = 0.18$) (Figure 4), but we observed a very similar haplotype structure of ten individual haplotype phases (Supplementary Table 4). While there was no omnibus significance, we again found significant association of haplotype phase 7 (Figure 4, Supplementary Table 5) with increased alcohol dependence (OR = 1.28, $\chi^2_{\text{df=1}} = 4.80$, $P_{\text{one-tailed}} = 0.014$; girls: OR = 1.36, $\chi^2_{\text{df=1}} = 3.99$, $P_{\text{one-tailed}} = 0.023$, $n = 1433$; boys: OR = 1.22, $\chi^2_{\text{df=1}} = 1.48$, $P_{\text{one-tailed}} = 0.11$, $n = 1111$), and again no significant gender difference was observed ($z = 0.41$, $P = 0.69$).

Drosophila Arf6 is a ubiquitous protein[23,47] involved in plasma membrane trafficking of numerous cell surface receptors,[48] and *Drosophila Efa6* is also expressed in all tissues.[47] Human *Arf6* is also expressed ubiquitously.[29,49] In contrast, *PSD3* is specifically expressed in the human brain, especially the PFC (Supplementary Figure 7).[29,49] The PFC is required for behaviors involving executive control,[50] including alcohol use disorders.[51,52] We therefore analyzed activation of the PFC using functional MRI during a Stop Signal Task, which engages executive control in the PFC. In this task, probands are asked to press one of two buttons most of the time (Go), and not press either button (No-Go) one out of six times (on average). We selected a region of interest, right inferior frontal gyrus (rIFG; see Supplementary Materials and Methods), in the PFC and measured the association of 'No-Go success vs. Go success' during this task with the *PSD3* SNP *rs13265422* and haplotype

phase 7. There was a trend towards association of the BOLD response of rIFG with *rs13265422* ($r = 0.04$, $t = 1.71$, $P = 0.086$; girls $r = 0.08$, $t = 2.56$, $P = 0.010$; boys $r = -0.00$, $t = -0.00$, $P = 1.00$), and a significant association with haplotype phase 7 ($r = 0.06$, $t = 2.38$, $P = 0.017$; girls: $r = 0.08$, $t = 2.44$, $P = 0.015$; boys: $r = 0.03$, $t = 0.88$, $P = 0.38$; Figure 4) – indicating that in carriers of the risk genotype/haplotype a greater effort was required to carry out a successful inhibition. This might lead to a greater risk for increased frequency of alcohol drinking/binge drinking, which was also observed with this genotype/haplotype. While the differences were again driven by girls, the two genders were not significantly different (SNP: $z = 1.78$, $P = 0.075$; haplotype phase 7: $z = 1.06$, $P = 0.29$). In a whole brain survey (without region of interest hypothesis) of the same functional contrast we found no significant association after correction for multiple testing, neither for *rs13265422* nor for *PSD3* haplotype phase 7, however, both SNP and haplotype phase had their highest peak level significance in the right inferior frontal gyrus region (Supplementary Figure 8). Overall, our data suggest a modulating role for *Efa6*/*PSD3* in brain activity during behavioral inhibition, and in the regulation of alcohol drinking.

DISCUSSION

We have previously shown that Arf6 acts in the adult nervous system to regulate ethanol-induced sedation in flies.[21] Here, we expand on this genetic network regulating alcohol-induced behaviors in *Drosophila* (see Figure 5) by showing that one of its activators, Efa6, is also required for normal behavioral sensitivity to ethanol. In addition, both Arf6 and Efa6 are required for ethanol-induced tolerance, a behavioral change upon repeat alcohol exposure that is thought to be a precursor to alcohol addiction.[53] Indeed, flies lacking either of these genes showed abnormal alcohol consumption preference. In humans, the minor G allele of SNP *rs13265422* and a haplotype phase containing this SNP of *PSD3*, one of the four human *Efa6* orthologs, were associated with increased drinking frequency and binge drinking frequency in adolescents. The same haplotype phase was also associated with alcohol dependence in the independent SAGE sample. *Arf6* and *PSD3* are involved in neurite and dendrite outgrowth[54–56] and might therefore also be involved in neuronal plasticity underlying the behavioral changes seen during addiction. Arf6 is a ubiquitously-expressed protein in flies[57,58] and humans.[23,29,49] *Drosophila* Efa6 is also found in most tissues,[57,58] with an enrichment in the nervous system.[30] Human *PSD3*, on the other hand, is highly specific for the human brain and shows a particular enrichment in the PFC,[29,49] a region known to be involved in addiction[51,52] and critical for executive control.[50] We show that in a Go/No-Go task, which engages prefrontal cortical areas, the same *PSD3* haplotype phase also associated with differential fMRI activation, specifically in the rIFG. This *PSD3* haplotype phase thus affected both alcohol-drinking behavior, as well as prefrontal activity during an executive control task. Insufficient executive control is well known to be a risk factor for externalizing disorders, including alcohol abuse and addiction.[59]

Mutations in the Efa6 activator of ubiquitous Arf6 GTPase were identified in our genetic interaction screen with mutants for *whir*, encoding the RhoGAP18B inactivator of ubiquitous Rac1 GTPase. In this expanding genetic network regulating *Drosophila* behavioral ethanol responses (Figure 5), neuronal Arf6 acts in the signaling pathway linking the insulin receptor to S6 kinase to regulate ethanol-induced sedation in *Drosophila*.[25] In

rodents, inhibition of the mTor kinase in the homologous pathway can reduce alcohol drinking[26] and relapse behavior.[27] The mTor inhibitor rapamycin is a clinically used, FDA-approved drug for human use, but it causes a large number of side effects, likely due to the fact that the pathway (insulin receptor/Arf6/mTor/S6 kinase) is found in most every cell and tissue and controls cell growth and survival. Furthermore, both Arf6 and the Rho-family of small GTPases are ubiquitous proteins that are involved in many cellular and neuronal processes.[20,47] In contrast, our study suggests a mechanism by which the restricted expression of a regulator, in this case PSD3, can confer highly specific regulation of this ubiquitous pathway: by activating Arf6 in specific anatomical regions and thereby also achieving regulation of specific behaviors. This has implications for the targeted treatment of specific behavioral disorders, in this case alcohol use disorders. By targeting the restricted PSD3, as opposed to the ubiquitous insulin receptor/Arf6/mTor/S6 kinase pathway, one might improve on therapeutic efficacy while decreasing side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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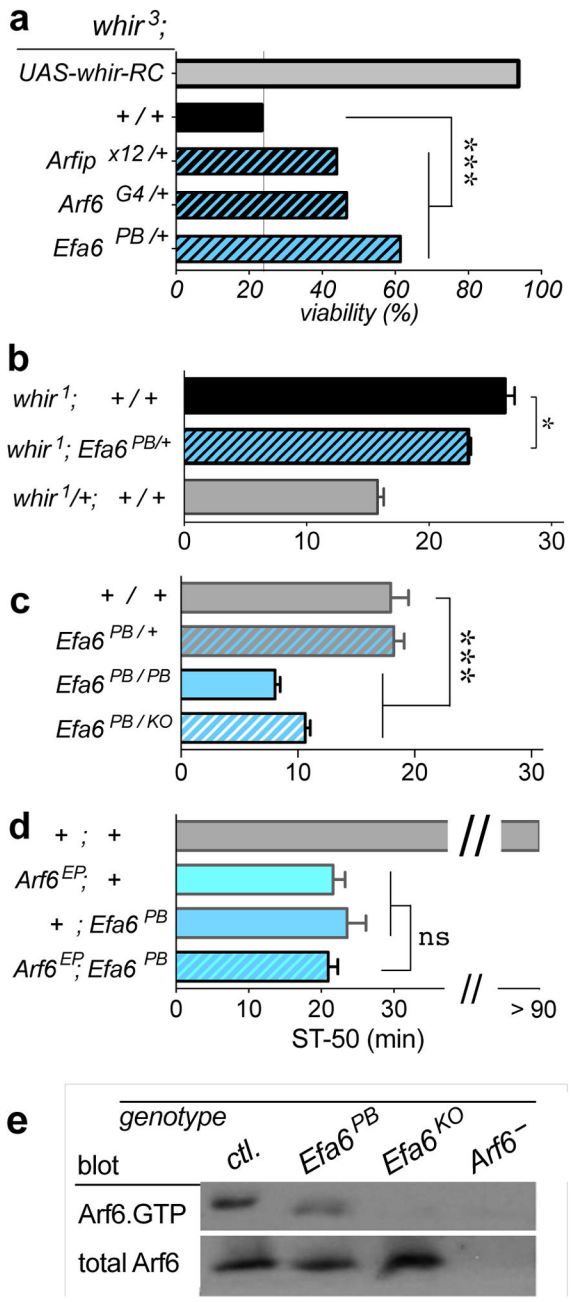

Figure 1.

Efa6 mutant phenotypes. **(a)** Viability phenotypes of various *whir*^{3} genotypes. Displayed are the ratios of surviving *whir*^{3}*/Y;genotype* males to *whir*^{3}*/+;genotype* females. All the genotypes are in the context of the *whir*^{3} mutation, indicated by the *whir*^{3}; atop. The *whir*^{3} allele causes semi-lethality (*whir*^{3}*;+/+*, black bar), which can be rescued by re-introducing the RhoGAP18B-PC isoform (*whir*^{3}*;UAS-whir-RC*, where *RC* is the RNA transcript encoding the PC protein isoform, grey bar) driven by the Gal4-driver inserted in the *whir*^{3} mutant. (Chi-square test with Bonferroni correction, $z = 12.4$, $df = 153.6,1$, $P < 0.001$, $n =$ 1776 flies). This isoform also rescues the *whir*^{3}-mediated ethanol resistance.[31] Heterozygous

Arfip/+, *Arf6/+*, and *Efa6/+* mutations partially suppress the $whir^{3}$ semi-lethality phenotype (*df* = 113.3,3, ***$P < 0.001$, $n > 329$ flies per genotype). **(b)** Heterozygous *Efa6* mutation partially suppresses the ethanol-resistance phenotype of $whir^{1}$ mutants (One-way ANOVA with Dunnett's multiple comparison vs. $whir^{1}$, $F(2,16) = 70.3$, *$P = 0.011$, $n = 8,5,6$; top to bottom). In all of the Figures in this manuscript, error bars depicted are the standard error of the mean. Here, and in the following Figures, flies were exposed to 130/20 Ethanol/Air vapor (unless otherwise noted). Male flies were used in all behavioral experiments with the exception of $whir^{1}$/+ females here (as the *whir* gene is on the X chromosome) and the females indicated in Supplementary Figures 2 and 3. **(c)** Homozygous $Efa6^{PB}$ mutants show significantly enhanced sensitivity to ethanol-induced sedation, as do $Efa6^{PB/KO}$ trans-heterozygous mutants (One-way ANOVA, with Dunnett's multiple comparison vs. $+/+\ w^{-}$ *Berlin*, $F(3,33) = 48.0$, ***$P < 0.001$, $n = 6,7,11,13$; top to bottom). The $Efa6^{KO}$ allele is a molecularly-targeted knock out, previously described.[30] **(d)** *Arf6;Efa6* double mutants are no more sensitive than either single mutant alone ($F(2,33) = 4.6$, *ns* $P > 0.54$, $n = 12$ per genotype). Flies were exposed to 30/120 Ethanol/Air, a low dose that did not sedate wild-type flies after 90 min. (**e**) Activated Arf6.GTP pull-down from head extracts, followed by anti-Arf6 Western blot. Both $Efa6^{PB}$, and $Efa6^{KO}$ mutants show reduced *Arf6* activation (and GTP-loading) compared to w^{-} *Berlin* control (*ctl.*). Lysates from $Arf6^{-}$ flies contain undetectable levels of *Arf6*. A representative blot from 2 replicates is shown.

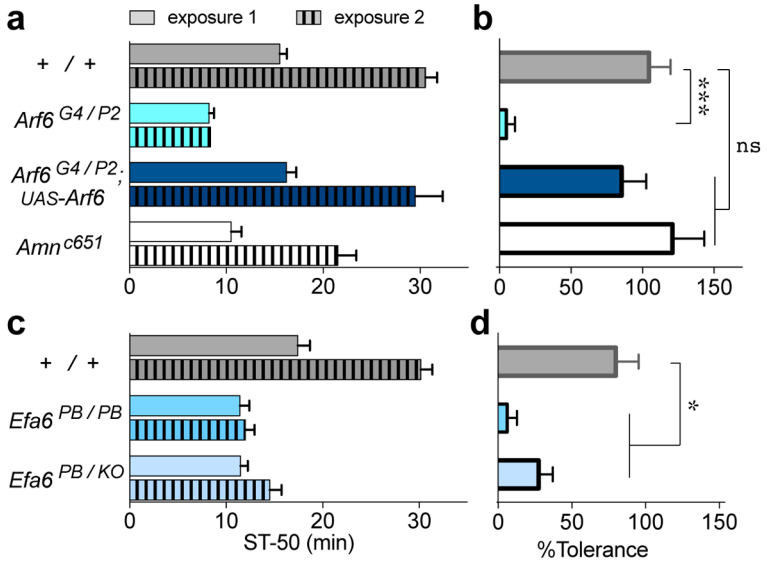

Figure 2.

Efa6 and *Arf6* are required for rapid ethanol tolerance. **(a)** Flies were exposed for 30 min to 130/20 Ethanol/Air (exposure 1, plain bars), and after a 4 hr recovery they were re-exposed (exposure 2, striped). Two-way ANOVA reveals significant effects for exposure ($F_{(1,96)}$ = 89), genotype ($F_{(3,96)}$ = 42), and interaction ($F_{(3,96)}$ = 9, n = 14,11,13,14; top to bottom). Both *Arf6* mutants (light blue, $P < 0.01$, Dunnett's post-hoc test) and *Amn* (white, $P < 0.05$) are sensitive to ethanol-induced sedation during the first exposure when compared to the +/+ control, as previously reported.[21,42] **(b)** Wild-type and *Amn* mutant flies develop tolerance (expressed as the % increase in ST-50 from first to second exposure), but *Arf6* mutants do not (One-way ANOVA with Dunnett's post-hoc test, ***$P < 0.001$). Note that expression of *UAS-Arf6-cDNA* driven by the endogenous Arf6 promoter (Gal4-expressing $Arf6^{G4}$ allele) rescues both $Arf6^{-}$ sedation **(a)** and tolerance **(b)** phenotypes. **(c)** Mutants of *Efa6* are sensitive, compared to +/+ control, to ethanol-induced sedation during the first exposure ($P <$ 0.01, n = 8,6,9; top to bottom) and also develop significantly less tolerance **(d)** than wild-type flies. This is true for $Efa6^{PB}$ homozygous, and $Efa6^{PB/KO}$ trans-heterozygous flies (*P < 0.05, Kruskal-Wallis non-parametric test with Dunn's post-hoc test. Non-normality of the data was due to one +/+ "outlier", which was nevertheless included in the analysis). All +/+ controls in these experiments are w^{-} *Berlin* flies.

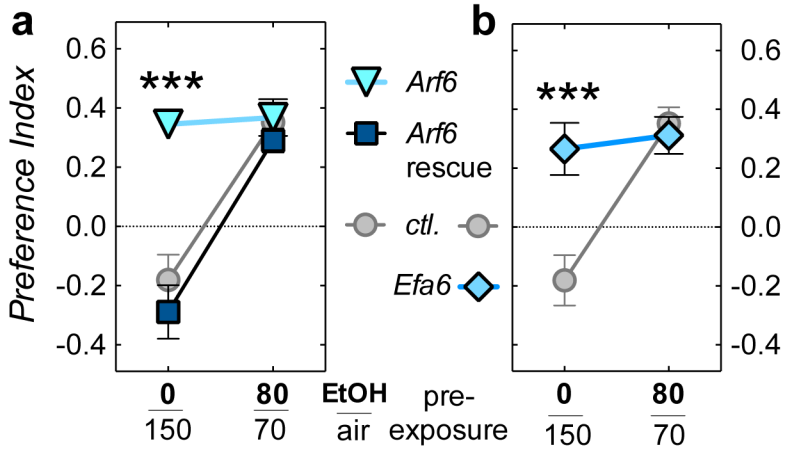

Figure 3.

Arf6 and *Efa6* mutants show increased alcohol consumption preference. **(a)** Flies were offered a choice between liquid food, and liquid food containing 15% ethanol. Naïve wild-type flies (mock exposed to 0/150 Ethanol/Air) avoid ethanol (Preference Index < 0; w^{-} *Berlin ctl.* flies). This changes to preference (PI > 0) after a 20 min 80/70 Ethanol/Air pre-exposure the day before (Two-way ANOVA, $F(1,80)_{exposure} = 44$, $P < 0.001$, n =13,18,12,17,12,14, left to right, top to bottom). *Arf6* mutants show high, naïve ethanol preference, independent of a pre-exposure (Dunnett's post-hoc test, ***$P < 0.001$). This phenotype is rescued by *UAS-Arf6-cDNA* expression (Arf6 rescue, genotypes as in Figure 3A). **(b)** $Efa6^{PB}$ homozygotes display the same naïve preference phenotype (***$P < 0.001$, n = 13,18,12,12, left to right, top to bottom).

		Frequency of Drinking in Last 30 Days at 16 years old (IMAGEN)	Alcohol Dependence (SAGE Caucasian)	Inferior Frontal Gyrus BOLD response at 14 years old (IMAGEN)
SNP *rs13265422* (G allele freq =0.33)	Full Sample	T=3.97, df=1353, P=7.3x10^{-5}***	$\chi^2_{df=1}$=1.76, df=2537, $P_{one\text{-}tailed}$ =0.092	T=1.71, df=1761, P=0.086
	Boys	T=1.99, df=642, P=0.047*	$\chi^2_{df=1}$=0.79, df=1105, $P_{one\text{-}tailed}$ =0.21	T=-0.00, df=848, P=1.0
	Girls	T=3.63, df=703, P=2.8x10^{-4}***	$\chi^2_{df=1}$=0.37, df=1427, $P_{one\text{-}tailed}$ =0.35	T=2.56, df=905, P=0.010*
Haplotype Phase 7 (Freq = 0.08)	Full Sample	T=3.98, df=1353, P=6.9x10^{-5}***	$\chi^2_{df=1}$=4.80, df=2537, $P_{one\text{-}tailed}$ =0.014*	T=2.38, df=1761, P=0.017*
	Boys	T=3.55, df=642, P=3.8x10^{-4}***	$\chi^2_{df=1}$=1.48, df=1105, $P_{one\text{-}tailed}$ =0.11	T=0.88, df=848, P=0.38
	Girls	T=1.96, df=703, P=0.050*	$\chi^2_{df=1}$=3.99, df=1427, $P_{one\text{-}tailed}$ =0.023*	T=2.44, df=905, P=0.015*

significant at level 0.05 *, 0.01**, 0.001***

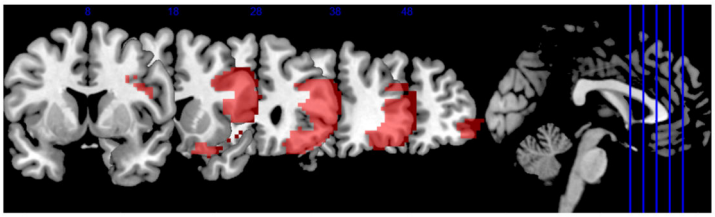

Figure 4.

Human *PSD3* phenotypes. (Top) Associations of *PSD3* with frequency of drinking (IMAGEN), alcohol dependence (SAGE Caucasian) and BOLD response during Go/No-Go task (IMAGEN), a measure of executive control. The minor G allele of SNP *rs13265422* is associated with increased frequency of drinking in the last 30 days in the IMAGEN cohort of 16-year old European adolescents (GG-genotype average score = 1.77, n = 146; GT-genotype average score = 1.49 n = 620; TT-genotype average score = 1.34, n = 597). The haplotype phase 7 shows association not only with increased frequency of drinking in the last 30 days (IMAGEN), but also shows association with increased alcohol dependence in an independent sample of Caucasian adults (SAGE), as well as increased activation contrast between 'No-Go success vs. Go success' in the right inferior frontal gyrus (IMAGEN). Gender and sites were controlled for all analyses where applicable, and handedness was controlled for fMRI phenotypes. (Bottom) Illustration of right inferior frontal gyrus (rIFG) activation as red area in coronal view. The slides were acquired based on MNI sagittal coordinates 8, 18, 28, 38, 48 (from left to right).

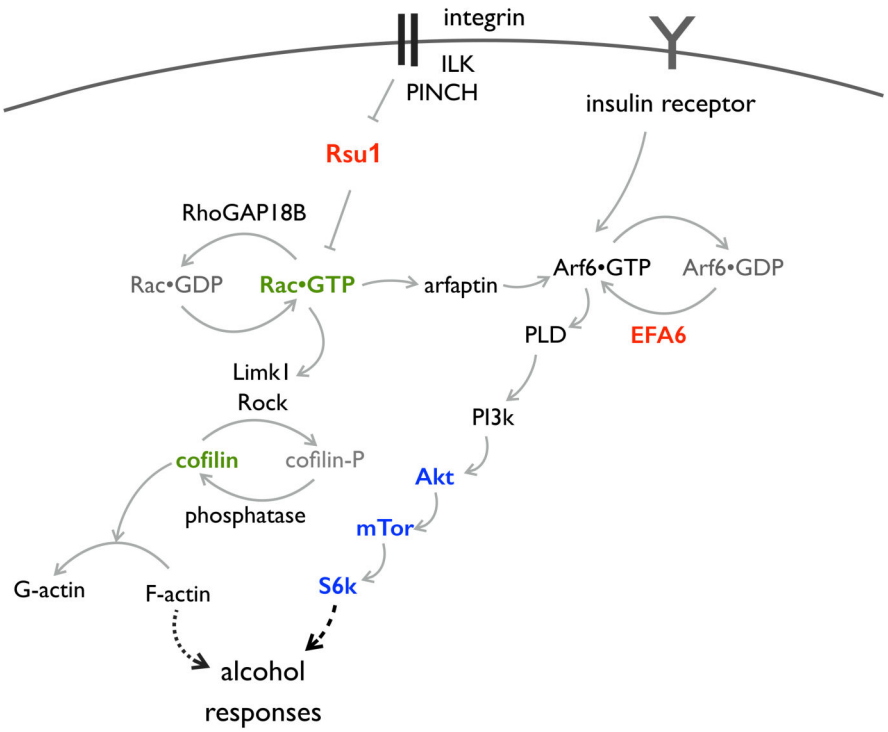

Figure 5.

Model of molecular mechanisms involved in *Drosophila* alcohol responses. Interactions (arrows) are based on our genetic and biochemical data[19,21,25,31,40] (as well as other published data cited therein). Of particular relevance to this report: RhoGAP18B (encoded by the *whir* gene) binds to, and acts on Rac1.[11,40] Rac1 is linked to Arf6 via Arfaptin, which binds to either activated GTPase.[21] Here, we show that Efa6 is required for Arf6 activation and behavioral ethanol responses, and together, these biochemical data support our initial finding of a genetic interaction between *whir* and *Efa6*, placing them in the same network. Note that all molecules depicted here have mammalian orthologs, with the exception of RhoGAP18B, which contains a GTPase activating GAP domain and long stretches without any other characterized domains. Proteins whose genes are associated with human alcohol drinking are depicted in red (Rsu1,[40] Efa6, this report), ones involved in rodent alcohol drinking in blue,[27,60] and ones linked to rodent cocaine-induced behaviors in green (Rac1,cofilin).[61,62] Abbreviations: ILK: integrin-linked kinase, PINCH: particularly interesting Cys-His rich protein, Rsu1: Ras-suppressor 1, Rac: Ras-related C3 botulinum toxin substrate, Limk1: LIM domain kinase 1, Rock: Rho-associated kinase, Arf6: ADP-ribosylation factor 6, PLD: phospholipase D, Akt: Thymona-associated kinase from the Ak strain, mTor: mechanistic target of rapamycin, S6k: S6 kinase.